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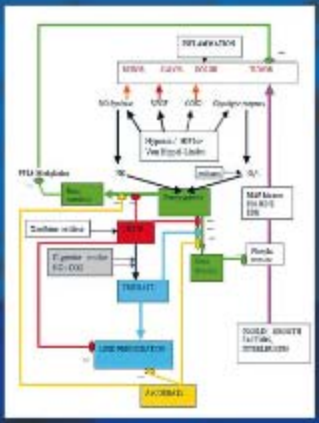

Cancer Microenvironment and Therapeutic Implications

Tumor Pathophysiology Mechanisms and Therapeutic Strategies

Springer

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The book is dedicated to Pietro M Gullino,
an uncommon friend and teacher

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List of Abbreviations

BDMCs	Bone marrow derived myeloid cells
CCL22	Chemokine ligand 22
CD	Cluster of differentiation
DCs	Dendritic cells
G-CSF	Granulocyte colony stimulating factor
GF-β	Trasforming growth factor beta
GM-CSF	Granulocyte macrophage colony stimulating factor
GAL-1	Galectin 1
HIF-1	Hypoxia inducible factor 1
HSP	Heat shock protein
IFN-γ	Interferon gamma
IDO	Indoleamine 2,3-dioxygenase immunoregulatory cells
IL-1	Interleukin1
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin6
IL-10	Interleukin 10
IL-12	Interleukin 12
IF	Interstitial fluid
IFP	Interstitial fluid pressure
LAK	Lymphokine-activated killer cell
MDSC	Immature myeloid-derived suppressor cells
MHC	Major histocompatibility complex
NF-kB	Nuclear factor kappa B
NO	Nitric oxide
PDGF	Platelet derived growth factor
pH_e	Low extracellular pH
ROS	Reactive oxygen species
SDF-1	Stromal cell derived growth factor1
TAA	Tumor associated antigens
TNF-α	Tumor necrosis factor alpha
TLR	Toll like receptors
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor

Introduction

Revolutionary changes have swept through cancer diagnosis and treatment over the past century. In the early 1900s the struggle was to diagnose. Then came the struggle to cut it out. Then the struggle to keep it away.

In the late 1900s scientists and physicians turned inward towards cancer cell mechanics and that is when the explosion of oncologic sciences erupted. A fountain of genes, transcription factors, signaling pathways, degradation fates, apoptosis chains, receptor interactions and cell-cell contact points provide a wellspring of opportunity to interfere with runaway cell growth and metastasis.

In the post-genomic era, cancer is a genetic disease.

But somewhere in the rush of genetic reductionism, the cancer research community has increasingly neglected the importance of the tumor microenvironment and the interplay between cancer cells, dysplastic cells and normal cells.

It is in this realm that this book is written.

The genesis of cancer is not as simple as a gene abnormality or even a set of gene abnormalties. A permissive environment must allow unchecked cell division. For cancer to initiate, the immune system must look the other way, nutrients and oxygen must be served at the right time, temperature and concentration, and a hospitable bed of stroma and extracellular matrices must support burgeoning growth and spread. Cancer is a chance co-op with a panoply of factors traded between normal and malignant.

Chief interactions in this co-op are exposed herein and implications for anti-cancer therapies are discussed. This book is not intended to serve as a simple review; rather, to spark new ideas and provocative questions so that better anti-neoplastic therapies are conceived and promulgated.

In this book we assembled a team of contributors who share amongst them serious experiences at the laboratory bench and in the clinic. These physician-scientists have dedicated themselves to the tension between the urgency for breakthroughs and the technical challenges of discovery. Their thoughts and perspectives on the state of cancer biology, ecology and implications for treatment are gathered herein. We intend for this book to roadmap outstanding questions and potential answers for the eradication of cancers.

Part I
Tumor Physiopathology and Microenvironment Genesis

Chapter 1
Inflammation and Carcinogenesis: A Change in the Metabolic Process

L. Schwartz, M. Israël and Icard Philippe

Abstract Since the seminal work it is known that inflammation is a major risk factor for cancer. Inflammation can be caused by agents as diverse as heat, cold, foreign body or chemicals. In every case, there is a protein leak from the damaged capillaries. This results in increased oncotic pressure which is in turn responsible for the methylation of the PP2A phosphatase. The activation of PP2A results in the translocation of NF κB and the activation of several metabolic pathways.

Keywords Inflammation · Chronic inflammation · Carcinogenesis · Methylation · PP2A phosphatase · NF-κB

Cancer is frequently associated with pre-existing inflammation and fibrosis. Between 60% and 90% of hepatocellular carcinoma occurs in patients with hepatic macronodular cirrhosis (De Vita et al. 1993, Podolsky and Isselbacher 1994). Chronic liver disease of any type is a risk factor for liver cancer. The cancer may be caused by hepatitis B or C, alcoholic liver disease, antitrypsin deficiency,hemochromatosis, and tyrosinemia. Its features result from hepatocyte necrosis, extensivefibrosis, connective tissue deposition, vascular distortion and nodular regeneration of the remaining tissue parenchyma (Podolsky and Isselbacher 1994). Evidence for a cause-effect link between cirrhosis and hepatocarcinoma is lacking. The relation may often be one of chance alone, since not all cirrhotics develop cancer. Nonetheless, diseases that cause cirrhosis also increase the risk of hepatocarcinoma (Podolsky and Isselbacher 1994). Furthermore, the more disorganized the liver becomes, the higher the risk of hepatocarcinoma (Podolsky and Isselbacher 1994, Baffis et al. 1999).

Similarly, lung cancer is most common among patients suffering from any form of chronic lung disease (Ernster 1996, Maitre et al. 1996). History of chronic bronchitis, emphysema, primary lung fibrosis, chronic lung infection and even lung

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irradiation are associated with increased risk (Ernster 1996, Maitre et al. 1996). There is no evidence for a relation of bronchitis or emphysema with lung cancer that could not be explained by independent links to exposure to tobacco smoke or other noxious agents. Nevertheless, the risk of lung cancer increases with the extent of disruption of normal lung architecture. For example, the risk of lung cancer is higher among patients suffering from chronic bronchitis and severe impairment of the lungs'carbon monoxide Breast cancer genesis also seems to be linked to architectural changes. A woman's reproductive history is one of the most important determinants of breast cancer risk. This is not a new notion. Ramaziani in 1700 first showed that breast cancer risk was higher among nuns (Haagensen 1986). Early in the 1900s, investigations noted that nulliparity and a history of having never breast-fed an infant were risk factors. Modern epidemiological cohorts have confirmed the increased risk for breast cancer after early puberty, late menarche, hormonal stimulation (Haagensen 1986).

In 1977, Ing reported a disproportionate increase of post menopausal breast cancer in the left breast of Tanka women of Hong-Kong thought to have nursed only with the right breast (Ing et al. 1997). All these risk factors and others, like radiation to the developing breast, are related (causally or not) to change in architecture of the breast.

Breast cancer is rare among young women. It is before the menopause, when the architecture of the mammary gland starts to undergo fatty tissue involution, that the incidence of cancer rises (Haagensen 1986). With the completion of menopause, the breast changes, it becomes somewhat smaller and less dense. There is a decrease in the number and size of the ducts. These atrophic lobules are seen lying in a dense fibrous matrix. Increase in the connective tissue is a prominent feature of this aging process.

The rare cases of breast cancer in young women are often due to hereditary anomalies. The most studied gene is BRCA-1. BRCA-1 is a nuclear phosphoprotein expressed in a broad spectrum of tissues during cell division. The inheritance of a mutant BRCA1 allele dramatically increases a woman's lifetime risk for developing both breast and ovarian cancers. This increased risk may be secondary to architectural changes. Analysis of a prophylactic subcutaneous mastectomy after genetic counseling for either carrying the BRCA-1 gene or belonging to a pedigree with familial breast cancer shows a different architectural pattern. The BRCA-1 or related genes may have a functional role in the branching pattern of the breast during lobular development, mainly in epithelial-stroma interaction (Russo et al. 2001). BRCA-1 deficient mice display multiple malformations (Cressman et al.1999).

Cancers occurring during childhood (nephroblastoma, medulloblastoma, retinoblastoma or Li-Fraumeni syndrome) are also associated with tissue disorganization from embryonic remnants (De Vita et al. 1993, Pivnick et al. 1998, Boyle et al. 2000). Patients suffering from genetically-encoded hereditary tumors like Li-Fraumeni syndrome and retinoblastoma have both mutated epithelial cells and fibroblasts with impaired growth, resulting in concomitant malformations (Boyle et al. 2000, Le Couter et al. 1998).

Experimental Evidence that Chronic Inflammation Induces Fibrosis and Cancer

Chemical Carcinogenesis

Exposure to chemical carcinogens is considered to cause most human cancer (De Vita et al. 1993). In animal carcinogenesis experiments, a first chemical (initiator) is responsible of an intense inflammatory reaction. A second chemical (promotor) is genotoxic. The word "genotoxic" has been created to replace the older previous term: toxic. The effect of genotoxic compounds is not confined to the epithelial cell; they kill epithelial and stromal cells. Genotoxicity causes tissue disruption through cell killing and replacement. For example, hepatocyte necrosis induced by genotoxic compounds, which precedes hepatic carcinoma, is associated with substantial damage to surviving hepatocytes, as well as extensive mesenchymal changes and loss of normal liver architecture (Ames and Gold 1990).

In vitro, however, carcinogens have not always successfully transformed normal human cells in culture (Mc Cormick et al. 1990). For these normal cells to be transformed, they often need to be immortalized by transfection with a cancer-associated virus prior to exposure to a carcinogen (Dipaolo et al. 1986).

Radiation-Induced Cancer

Ionizing radiation induces cancer in humans and animals (Rhim et al. 1993, Barcellos-Hoff and Ravani 2000). In vitro, the vast majority of attempts to achieve transformation of normal human cells into cancer cells have been unsuccessful (Barcellos-Hoff and Ravani 2000). In fact radiation induced carcinogenesis appears to be a consequence of inflammation and fibrosis.

The female mammary gland is unique among all glands in that the epithelium develops after the birth from a rudiment that can be easily removed at about three weeks of age. Barcellos-Hoff irradiated the whole mammary gland of nurturing mice. After the irradiation, the epithelial cells are surgically removed and replaced with transplanted normal mammary cells. The cancer arises from these normal non-irradiated epithelial cells. Tumor growth appears as a consequence of the changes in the irradiated stroma (Barcellos-Hoff and Ravani2000).

Physical Carcinogenesis

Chemicals and radiation induce inflammation and fibrosis. The question is whether these architectural changes and the fact that tissue disruption is a risk factor for neoplasia may be explained by chronically increased epithelial cell proliferation resulting in an increased rate of mutation (Moore and Tsuda 1998). The answer lies in the old literature on physical carcinogenesis. It has been documented that some

foreign bodies induce cancer (Sonnenschein and Soto 1999, Brand 1982, Stanton and Wrench 1972, IARC 1999, Lipkin 1980). The carcinogenicity of foreign bodies is linked to their shape. Cellulose membrane filters of specific shape, texture or size generate sarcoma. Intense inflammation and proliferative fibrosis precede tumor formation. The combination of shape and size (about the width of a human cell) may also be critical (Lipkin 1980). The carcinogenicity of this chemically inert molecule is also linked to particle shape and size.

The International Agency for Research on Cancer evaluated the carcinogenic effect of surgical implants and other foreign bodies in humans (IARC 1999). The evaluation resulted in a group 2B classification (possibly carcinogenic for humans) for polymeric implants prepared as thin smooth films, and implanted foreign bodies consisting of metallic cobalt, or nickel, and a particular alloy powder consisting of 66–67% nickel, 13–16% chromium and 7% iron. The evaluation also resulted in a group 3 classification (not classifiable as to their carcinogenicity to humans) for organic polymeric materials as a group, orthopaedic implants of complex composition, cardiac pacemakers, silicone breast implants, dental materials and ceramic implants.

Physical carcinogenesis may also be a "transforming" factor. In nude mice, the implantation of both colon adenoma cells and of a plastic plate are necessary for tumorigenic growth. Again, locally, there is intense inflammation (Okada et al. 2000).

Inflammation Is a Metabolic Disease

Of the cardinal symptoms of inflammation: rubor, calor, dolor and tumor, we shall particularly consider a cellular aspect covered by the word tumor. Indeed, inflammation, like cancer, triggers the mitosis of a variety of cells, and common mechanisms may have to be controlled in order to remain within physiological limits. We shall first recall a few basic facts.

Inflammation is a natural response of the organism to an aggression, which can be traumatic, bacterial or viral. The defense involves first non-specific (innate) mechanisms, and second specific immunological mechanisms. The innate mechanisms cover the complement cascade, which leads to the activation of proteases able to destroy cells, the complement found in the serum, responds to an antigen-antibody complex, or might be directly activated by bacterial antigens. The innate defense is also associated to the release by the liver, of a variety of proteins the Hageman factor for example. The latter, induces a variety of events, first the coagulation cascade (thrombine-fibrinogen-fibrine); then the induction of the fibrinolytic-plasmine cascade; finally, the activation of the so called kallikrein-kinin system, which will have major actions on vascular permeability, leading to infiltration of tissues and pain, and to the release of lipid mediators of inflammation, eicosanoids-leucotrienes. The liver releases many other proteins (C reactive protein) the latter, binds phospholipids from bacteria, controlling the recruitment of macrophages, it also promotes the activation of the complement. The increase of proteins in the serum

increases the sedimentation velocity of red blood cells, measuring the intensity of inflammation.

An essential player of the innate line of defense is the mast cell; it will release histamine, causing vasodilatation, and TNF α (tumor necrosis factor); it will produce and release Interleukins (IL1) and generate lipid mediators from arachidonic acid: leukotrienes, prostaglandins. The mast cell also releases a platelet activation factor (PAF), which promotes the release of serotonin from platelets. The activation of the mast cell may result from an injury, or from an allergen-IgE recognition. Endothelial cells neutrophils and macrophages, which are attracted on the site by chemotactic factors (e.g., RANTES), form this first line of defense. There is a vasodilatation, the site is red and hot (histamine-serotonin), the vascular permeability increases (prostaglandin), swelling gives pain since nerves are compressed, and bradykinine is involved. As for IL1 and TNF α, they act on the hypothalamic center controlling fever. They cytokines also trigger the release of prostaglandin (E2), which activates cAMP dependent processes and catabolism, providing substrates to the site of inflammation. The patient experiences cachexia, anorexia, fatigue, and fever. This innate line of defense already explains three of the major symptoms of inflammation: rubor, calor, and dolor. But what about cell multiplication and "tumor"? We shall see that both lines of defense trigger the increased mitosis leading to the accumulation of neutrophils and macrophages.

The mitogenic effect involves signaling processes mediated by tyrosine phosphorylations, that have common features with tyrosine kinase receptors such as the insulin receptor. If we consider for example the Toll/ILl-1 receptors, or the TLR4 receptors that respond respectively to interleukin 1 (IL1) or to bacterial lipopolysaccharides, they both have a tyrosine kinase, intracellular domain. Downstream of the tyrosine kinase signal, and with the help of an adapter protein (MyD88), the IL-1R kinase (IRAK) gets activated, which leads after several steps, to a phosphorylation and to proteolysis of Iκb. This dissociates Iκb from its partener NF-κb. The latter can then move to the nucleus and transcribes proteins controlling cell mitosis. Nitric oxide released by macrophages may nitrosylate Iκb and dissociate it from NF-κb.

We shall now discuss mitogenic effects associated to the specific defense line. When a virus infects a cell for example, it presents on its surface the antigen, viral proteins for example, associated to the major histocompatibility complex of class 1 (MHC1). The complex will be recognized by a T cell lymphocyte of the cytotoxic type. The T cell receptor recognizes not only MHC1 as a self-identification device, but also motifes of the antigen. The cytotoxic T cell receptor will signal via its CD3 components, and via an essential protein (CD8) that a correct recognition of both the antigen and MHC1 took place, which activates again a tyrosine kinase (P56lck). In the case of cytotoxic T cells, the tyrosine kinase signal induces synthesis and secretion of enzymes perforating the contaminated cell, which is eliminated. The situation is different for helper T cells, they recognize antigens presented by the major histocompatibility complex of class 2 (MHC2) that appear on the surface of macrophages or B lymphocytes. The helper T cell receptor recognizes not only self-MHC2, but also the presented antigen. The helper T cell receptor will signal via

its CD3 component, but also via an essential protein of helper T cells (CD4), that the recognition of the antigen-MHC2 complex took place, which activates again the P56lck kinase. A variable menu of lymphokines are consequently produced, triggering a massive multiplication of immunologically competent cells. In all cases interleukin 2 (IL-2) autoactivates the multiplication of the helper T cell. In the case of an antigen presented by a macrophage, the helper T cell and the macrophage secrete a cocktail of lymphokines: Il-6, TNFα, INFα, and colony stimulating factors for granulocyte and macrophages (G-CSF and GM-CSF). In the case of an antigen presented to helpers T cells by a B lymphocyte, which binds circulating antigens, the helper T cell secretes IL-4 and IL-6, which induce proliferation of the antigen presenting B lymphocyte. The selected population will proliferate and convert into a plasmocyte secreting antibodies in the plasma, which neutralize circulating antigens. For macrophage presentation, a cocktail of the same lymphokines induces their proliferation.

After this brief survey of immunological defense mechanisms, the point we want to make in relation to cancer is that these lymphokines will act on receptors that activate cellular proliferation via similar MAP kinase signaling pathways. In spite of many common features, there are different modalities to consider. In response to growth factors, the receptor is autophosphorylated on tyrosines, which bind the SH2 domains of adapter proteins. Then, RAS-GTP steps activate the Raf serine-threonine kinase, follows the mixed MEK kinase, which finally activates ERK. The nuclear translocation of ERK will allow the phosphorylation of transcription factors, regulating the expression of genes involved in mitosis. Two other MAP kinase pathways have been identified, they are particularly activated in response to inflammatory cytokines; in these pathways, JNK or P38 replaces ERK. At the receptor level, there are also differences, particularly when the receptor itself has no kinase activity. In this case it recruits a tyrosine kinase JAK, or src. In the case of JAK, there is a short cut, which activates STAT, the latter being translocated to the nucleus.

An interesting modality is related to the CD45 phosphatase that serves as receptor in lymphocytes, it will then modulate a downstream tyrosine kinase step, promoting mitosis. It is probable that scaffold proteins are involved for selecting among the different MAP kinase pathways.

After having recalled these basic features of inflammation, we shall discuss some possible links with cancer.

The proliferation of immunologically competent cells, the multiplication of embryonic tissues, or the proliferation of tumor cells seems to require the activation of a signaling mechanism, which indeed, depend on MAP kinases. Their activation first depends on a tyrosine kinase reaction, mediated by specific enzymes, or result from auto-phosphorylations, which can be induced by the binding of a ligand, or an antigen, to the tyrosine kinase receptor.

In cancer formation, oncogenes often results in a perturbation of normal signaling pathways. Similar perturbations can also occur in inflammation, when proliferating cells involved in immunologic defense escape from homeostatic controls. Indeed, if factors that trigger the proliferation of cells related to inflammation,

interleukins for example, are continually secreted because inflammation persists, the enhanced tyrosine kinase signals may result in uncontrolled proliferation of tissues and then oncogenic transformation. We know that tyrosine kinase receptor signals are not only triggers for the MAP kinase cascade, but that they also activate PI3 kinase, this is the case for the insulin receptor for example. Receptors coupled to G proteins, activated by transmitters released by inflammatory cells, may also activate PI3 kinase, leading in both cases to the conversion of phosphatidyl inositol 4,5, bisphosphate (PIP2) into phosphatidyl inositol 3,4,5 (PIP3). We know from the work of Hokin and Hokin (Hokin and Hokin 1953) that transmitters such as acetylcholine (ACh) acting on muscarinic receptors, elicit hydrolysis of phosphoinositides. Other transmitters released during inflammation (serotonin or histamine) may also activate such "Gq coupled" receptors, increasing phosphoinositides via the stimulation of a phospholipase C. If PI3kinase is activated as well, inositol 1,3,4,5 (IP4) is formed, if not, inositol 1,4,5 (IP3) is released. In general, Gq coupled receptors activate phospholipase Cβ while tyrosine kinase receptors activate phospholipase Cγ. The released inositides mobilize calcium, thereby helping the exocytotic incorporation of the glucose transporter. Another action of phospholipids, like diacyl glycerol (DAG), activates protein kinase C, which mimics the tumoral action of phorbol esters, probably via a direct activation of ERK. The inflammatory transmitters also activate a phospholipase A2, releasing arachidonic acid. The latter, lead via cyclooxygenases (COX1, COX2) to prostaglandins and thromboxanes or to leukotrienes via lipoxygenases. The preventive effect of non-steroidal anti-inflammatory drugs in colon cancers likely depend on the inhibition of COX2, decreasing inflammation. But above all, we should recall that PTEN phosphatase hydrolyses phosphoinositides, decreasing their effects. Hence, if this phosphatase is down regulated, as it is observed in cancer, the PI3 kinase pathway and related lipidic mediators of inflammation will exaggerate their effects. If PI3 kinase signaling is upregulated, PTEN control is lost, resulting in increased frequency of mitosis. This may not necessarily lead to cancer, unless some other perturbation takes place at the level of the MAP kinase pathway, which controls mitosis. The synthesis of cell cycle proteins or tumor suppressor proteins is partly controlled by this pathway through transcription factors activated by ERK, JUNE, or P38, after their translocation in the nucleus.

So what might perturb the MAP kinase pathway in inflammation, and why should this favor the transformation of cells? Fig. 1.1 answers in part the questions raised. The tyrosine phosphorylation step is essential. In order to limit the mitogenic effects of the MAP kinase pathway, we have a natural inhibitory system performing tyrosine nitrosylation of proteins. This inhibitory system competes with phosphorylation of these tyrosines when the pathway is activated. Normally, nitrosylation is operated by peroxynitrite, formed when NO meets superoxides, which are generated by NADH oxidase, or if respiration does not generate enough electrons to fully reduce oxygen. If an aggression takes place, hypoxia induces via HIF1/Von Hippel-Lindau factor: NO synthase, glycolytic enzymes, carbonic anhydrase, VEGF, COX2, leading to inflammation; except that cell mitosis is still limited by the tyrosine nitrosylation mechanism. Then, purine catabolism gets activated,

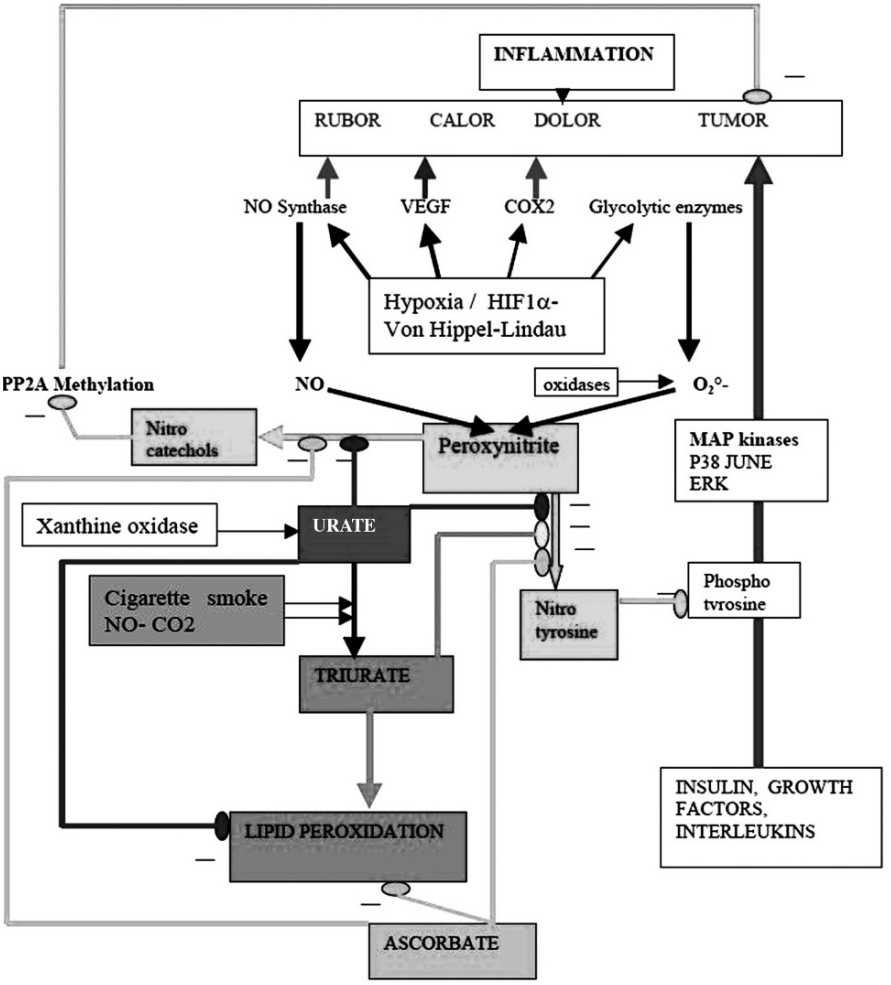

Fig. 1.1 Inflammation. Metabolic stressors of tissues, such as hypoxia, induce via HIF1α and Von Hippel-Lindau factors: NOsynthase, VEGF, COX2-prostaglandins, which explains three of the cardinal signs of inflammation (rubor, calor, dolor). Chemokines secreted at the site attract leukocytes and macrophages. Glycolytic enzymes are also induced in the inflamed tissue. The other sign of inflammation (tumor), involves mitosis of cells ensuring immunological defense

xanthine oxidase becomes not only a source of superoxide, but also a source of an essential product: uric acid. The latter, is the natural inhibitor for tyrosine nitrosylations, which increases tyrosine phosphorylation and triggers the MAP kinase cascade. Mitosis occurs resulting in tumor formation, a cardinal sign of inflammation. In primates, who lack urate oxidase (Oda et al. 2002, Spitsin et al. 2002), uric acid becomes the natural activator of the MAP kinase pathway since it removes the nitrosylation brake on the MAP kinase pathway (Teng 2002). But uric acid also

inhibits formation of nitrocatechols and nitroindols, which normally inhibit methylases (Perez and Avila 1999, Huotari et al. 2001). Hence, uric acid helps methylation of PP2A and its activation, thereby counteracting the MAP kinase trigger of mitosis and limits its tumorigenic effects.

In other works we have discussed the role of nitrocatechols and nitroindols in relation to neurologic diseases, and compared them to endogenous neuroleptics (Israël 2004). The levels of these molecules decrease when uric acid increases. In normal conditions, uric acid controls this defense mechanism in primates; it is still operational when an excess of peroxynitrite or peroxycarbonate is formed, such as after inhaling NO or CO2 with cigarette smoke. But in this case, peroxynitrite converts urate into triurate, which inhibits tyrosine nitration, and thus pushes the MAP kinase pathway. Triurate has deleterious actions on lipid peroxidation (Robinson et al. 2004) and cannot inhibit formation of nitrocatechols as well as urate. Nitrocatechols increase, and the methylase is more inhibited, leading to PP2A becoming poorly methylated and inactive towards given substrates. We have seen that this situation favors mitosis. In sum, toxin exposure generates triurates which lead to poorly activated PP2A, which then fails to counteract the MAP kinase activation of mitosis.

A protection against the effects of triurate on lipid peroxidation and on PP2A could come from ascorbate (Frei 1991) or vitamine E, but primates do not synthesize these vitamins. Other mammals, such as rodents, can synthesize ascorbate. Rodents use ascorbate, as a single protection mechanism against lipid peroxidation and tyrosine nitrosylation. Probably, this explains why cigarette smoke interferes with urate defenses of primates, resulting in lung cancer, while rodents with different defense mechanisms, based on ascorbate, rather than urate, are more resistant to developing lung cancer after toxin exposure.

In this discussion we highlight major players in inflammation such as PP2A and PTEN. Specifically, PTEN is down regulated in cancer because the gene is hypermethylated, while PP2A is inactivated because of a cytosolic hypomethylation.

Cancer as a Metabolic Disease: Back to Otto Warburg

To understand regulation of human cell division, a detour to the world of microbiology is necessary, beginning with the work of Louis Pasteur himself. Pasteur (1822–1895) showed that the conversion from sugar to ethanol required living organisms, rather than a chemical catalyst, demonstrating that by decreasing the oxygen content in a yeast broth, the yeast cells could be made to divide, multiply, and ferment vigorously (the “Pasteur Effect”). In modern terms, this effect could be described as an activation of anaerobic glycolysis to meet cellular ATP needs.

Otto Warburg developed the work of Pasteur on fermentation. During his lifetime Warburg was generally regarded as the greatest biochemist of the 20th century. Warburg, Krebs and Meyerhoff showed that cancer cells were anaerobic in nature, more akin to fungus or bacteria than to normal mammalian cells.

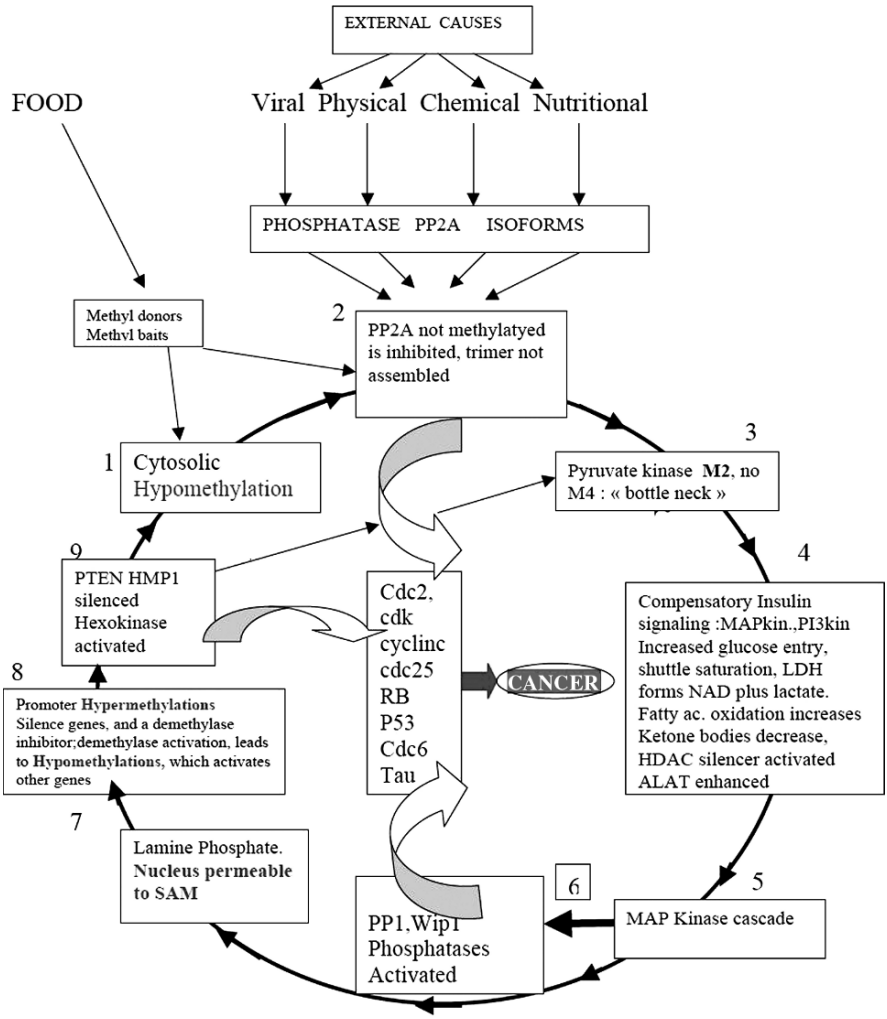

Fig. 1.2 A vicious circle leading to cancer 1. There are conditions related to food: methyl sources /methyl baits, vitamins, or genetic susceptibilities, that lead to a methylation deficit in the cytosol. **2**-This affects the activity of PP2A phosphatase, this enzyme has hundreds of variable isoforms which may be affected by many different external agents: viral, physical or chemicals that after the different isoforms. The resulting effect is a hypomethylation in affected cells. The PP2A subunits do not assemble. **3**-The phosphatase looses its specificity for given protein targets, leading to effects on proteins controlling mitosis, and also to a glycolytic metabolic "bottle neck", because Pyruvate kinase remains in its inactive M2 phosphorylated form, rather than switching to the active M4 tetramer. **4**-there is a compensatory insulin signaling process, entering glucose, saturating mitochondria shuttles above the neck, Lactate dehydrogenase forms NAD+ and lactate (Warburg effect). Below the neck, Alanine transaminase (ALAT), the Malic enzyme, form pyruvate, but the lactate sink is deep. Hence, fatty acid catabolism has to form the necessary acetyl-coA, not provided by pyruvate dehydrogenase, oxaloacetate comes via an abnormal carboxylation of phosphoenolpyruvate by mitochondrial PEP carboxy kinase. **5**-The activation of insulin–tyrosine kinase–MAP kinase signaling, activates PP1 phosphatase; **6**-PP1 acts on the cell cycle

As early as 1920, Warburg knew how to inject tumoral suspensions into the peritoneum of mice, and how to measure their gas concentration. He understood that cancer is a disease of cellular breathing and that cancers are often hypoxic.

He also understood that all cancer-producing substances (arsenic, tars, and cyanide) decrease cellular respiration. Cancer cells ferment even in presence of oxygen. Either oxygen cannot reach the cell, or it cannot be utilized. In the 1920's, Warburg had identified these two phases: first, hypoxia alters cell metabolism; second, if the cell survives these anomalies, the latter will produce cancer. Despite the Nobel prizes discerned to Otto Warburg, Krebs and Meyerhoff their work has largely been forgotten. The main reason for this ignorance of the work done at the beginning of the past century lies in the multiple dramas of that period.

And what could be easier, when you understand cellular respiration than to choke it? From the start of the First World War, German scientists working on cellular respiration lent their knowledge to the German war effort, with the resulting devastation caused by yperite and other combat gases. Yet another poison was synthesized: Zyklon B, used so "successfully" in the death camps of the Second World War. Needless to say, this research on cellular respiration mechanisms – whatever it's original merits – suffered greatly from the ensuing madness. Furthermore, Warburg was an eccentric genius who had many enemies. He proposed a theory of cancer dependent on glycolysis that was initially greeted enthusiastically but was later widely ridiculed in academic circles (Nachmansohn 1979, Guillemin and Krasnow 1997). Although Warburg's data were impeccable, other scientists later claimed to find exceptions to Warburg's rule. In time, Warburg's theory became not just old-fashioned but anathema to a scientific establishment that was increasingly focused on viruses and aberrant genes as the source of cancer.

Since then, the Positron Emitting Tomography (PET) scan has revived Warburg's work.

For long, the prodigious expense and ponderous size of the cyclotron (housed in its own building and managed via a network of intricate controls) and the cumbersome requirements of staff prohibited its clinical use. Over the years, refinements made it more practical, more manageable, and less complicated. The cyclotron was downsized. It could fit into a hospital.

←

Fig. 1.2 (continued) synergistically with the PP2A deficit, leading to the permanent activation of mitosis. **7**-MAP kinases affect lamine phosphorylation, the nuclear membrane becomes permeable to methyl donors such as S-adenosyl methionine (SAM), it is retained in the nucleus, cytosolic methylase become less active, while the nuclear methylases are boosted. **8**-There is an increase of nuclear methylations, the hypermethylation of promoters will silence genes (PTEN), but also demethylase inhibitors; thus, other parts of the genome get hypomethylated, activating other genes (hexokinase gene). **9**-Consequently PTEN and HPM1 phosphatases are silenced, increasing insulin – PI3 kinase – MAP kinase effects, while hexokinase and glucose influx increase, aggravating the effect of the PP2A failure, on the cell cycle. In sum there is a cytosolic hypomethylation and a nuclear hypermethylationhypomethylation process, which upsets the phosphatases and leads to catastrophic changes of metabolism and mitosis, generating cancer

Using a radioactive analog of glucose, PET scan examination has revived Warburg's work. Modern imaging confirms the increased uptake by cancer of large quantities of glucose.

Fermentation provides energy, though less efficiently than respiration (Guillemin and Krasnow 1997, Schwartz 2004) for, in the presence of sufficient oxygen, glucose is completely degraded into water and carbonic gas, hence the efficiency of respiratory metabolism. At lower concentrations of oxygen, by contrast, the glucose is incompletely degraded and waste is produced, a portion of which is released into the extracellular component. Some wastes such as ethanol produced by glycolysis are highly valuable.

Other "waste products" of anaerobic glycolysis remain within cells, causing cell mass to increase, and reacting to form aminoacids, lipids, glucids and nucleic acids (Schwartz 2004, Palmer 1985, Wang et al. 1995, Gerasimovskaya et al. 2002, Wada et al. 2002). These aminoacids, in turn, form proteins, the lipids are transformed into hormones; and the nucleic acid provides DNA and RNA. In this manner, anaerobic glycolysis provides cells with all the requirements for mitosis, both physical (sufficient cell mass) and biochemical (suitable molecular species).

Modern biology has been hindered by the discoveries of hundreds of what have been identified and named or, rather, misnamed, "growth factors". Originally, the concept of "growth factors" was limited to polypeptides, but it now extends to include sugars and fats such as triglycerides. All such molecules have one thing in common: they deliver energy to the cell. There are, for example, no polypeptidic "growth factors" which cannot be transformed into energy. These so-called growth factors can be utilized on the spot or exported as secretions to be used by distant cells, as in the case of steroid hormones (derivatives of cholesterol, that is, of fat). Some growth factors like insulin or insulin like growth factor (IGF1 and 2) further increase the glucose uptake and metabolism.

Pharmacologists modify "growth factors" and synthesize false nutrients that cannot be transformed into available energy for the cell. As a result, cell metabolism is reduced and the cell may ultimately starve to death.

Once cancerous, the metabolism of a cancer cell remains glycolytic even in the presence of oxygen (the Warburg Effect), with concurrent hypoxia responsible for an elevated incidence of mutations whose pattern is similar to that observed in tumors (Reynolds et al. 2002). For example, an oncogene originally activated by hypoxia will result in the accumulation of p53 and an increased concentration of the Hypoxia Inducible Factor (HIF), thus mimicking a situation of reduced oxygen availability (Chan et al. 2002). Hypoxia also results in the secretion of proteases. These may destroy the basement membrane and enable the cancer cells to invade the surrounding normal tissue and spread to distant organs.

Most if not all of the properties of cancer can be explained by hypoxia and the resulting anaerobic glycolysis: carcinogenesis, cancer fractal growth, cell proliferation, loss of cell differentiation, loss of cell polarity, metastasis, and resistance to conventional cancer

Inflammation and Cancer Are Dysmethylation Syndroms

Recent work from Abdolhassani (Abolhassani et al. 2008) and his colleagues demonstrates that inflammation is a consequence of a dysmethylation syndrome. In short, they show that in every model of inflammation studied, the inflammatory cascade is a direct consequence of the methylation of the catalytic subunit of PP2A.

Similarly work by Israel (Israel and Schwartz 2006) and then Guenin (Guenin et al. 2008) demonstrates that tumor response is controlled by methylation of PP2A adding further insight into the epigenetic regulation of cancer.

The cholinergic deficit and the increase of homocysteine are often found in Alzheimer's disease. Another essential methylation is also deficient in this disease: the methylation of PP2A phosphatase. As previously stated, this phosphatase controls key enzymes of glycolysis, pyruvate kinase, but also the phosphorylation of other proteins, Tau protein, controlling in this way, tubulin polymerization. The PP2A deficit leads to hyperphosphorylated Tau and tangles. Homocysteine also reacts with serine, giving cysteine, a step requiring vitamin B6. Cysteines like glutathion control the folding of proteins, their proteolysis and are in this way involved in the formation of plaques.

The same effect could take place in inflammation and tumors, and the PP2A block towards given proteins, presumably shuts down pyruvate kinase, leading to a "bottle neck" in glycolysis, and to the Warburg effect (Israel and Schwartz 2006). The PP2A methylation deficit also alters tubulin polymerization and perturbs the spindle. But in cancer, the possible SAM decrease, which impaired the cytosolic methylation of PP2A, seems to be associated to an excess of nuclear DNA methylations, leading to the silencing of genes such as PTEN, and to changes activating demethylases, which favor the expression of other genes such as hexokinase. The metabolic result, is a facilitation of PI3kinase signals linked to the insulin-tyrosine kinase pathway, which also gets enhanced. Consequently MAP kinases are activated, and mitosis is triggered.

The activation of nuclear methylases, could be a consequence of inflammation or hypoxia acting via arachidonic inflammatory derivatives on these methylases, the nuclear membrane becomes permeable to SAM, while the cytosolic methylases are blocked in a non-specific configuration. We have abundantly discussed the different external triggers leading to this metabolic situation in which cytosolic hypomethylations are associated to nuclear hypermethylations-hypomethylation shifts. In comparison and schematically, Alzheimer's disease displays only the cytosolic methylation deficit, while tumors alter as well their nuclear methylation programs.

Tumors also secrete proteases that disrupt the controls of differentiation, which are also linked to the proteolysis of contact proteins. Mitosis and the development of the cancer mass is certainly most impressive, but the catastrophic metabolic situation in which the organism burns proteins and lipids for burning glucose is really terrible. Clinically, this can present as cancer cachexia.

Furthermore, the cytosolic methylation deficit of PP2A might be associated to a hypermethylation of DNA, silencing genes such as PTEN and activating other such as hexokinase. Methylation shifts take place, and change the expression of many genes linked to glycolysis, oxidative metabolism, inflammation or angiogenesis mentioned in the metabolic analysis. A new picture of carbohydrate metabolism characterizes the situation, in which fundamental cellular mechanisms: mitosis, differentiation, apoptosis are no longer controlled, leading to cancer. It is a terrible fate to endure: Alzheimer or cancer. In the case of cancer, it may be useful to methylate PP2A with methyl donors, in order to correct, via PP2A, the pyruvate kinase block. It might also be necessary to inhibit phosphoenolpyruvate carboxykinase, carbonic anhydrase, lactate dehydrogenase, and citrate synthase to control the entry of glucose, amino acids and vitamins. Clinically, it is essential not to aggravate the situation. Methyl donors will have to be associated with histone deacetylase (HDAC) inhibitors or with histone acetylase agonists, in order to cancel the effect of methyl donors on DNA methylation. The treatment must then take into account each particular situation, in order to decide if it is more useful to remethylate genes, or to avoid their hypermethylation via the HDAC inhibitors or HAT agonists. Other phosphatases such as PP1 must on the contrary be inhibited. We have also discussed the impact of nitrosylations on tyrosine kinases, etc.

What we present, is that cancer might have epigenetic causes, affecting genes that are also oncogenes targets. This perspective is more frequently considered (Li et al. 2001). Dysmethylation syndromes changes cellular expression of genes leading to uncontrolled mitosis and immortality-cancer. If one attacked with multiple compounds the different metabolic branches that are affected, closing those that are activated, and helping those that fail, one may then hope to reverse or attenuate the process, or to eliminate tumor cells. This is certainly a complex task. Development of systems biology roadmaps will reduce this complexity. One may at least try to understand the mechanisms leading to this autophagy, and hope that we shall be able to block the triggers that transform normal cells, into a hungry beast.

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Chapter 2
Tumor Microenvironment: Aspects of Stromal-Parenchymal Interaction

Attila Zalatnai

Abstract Solid tumors are composed of malignant parenchymal cells embedded in nonneoplastic stroma. A large body of evidence emphasizes that the actual biological behavior of the given tumor is determined by continuous and mutual molecular interactions between the stromal cells and matrix, as well as the malignant cells. Cancer cells create their own microenvironment, which promotes or suppresses the growth, invasion and spread of the malignant cells. The scenario is highly complex, because there are numerous interactions not jut between the parenchyma and the stroma, but also between extracellular matrix and stromal elements. These microenvironmental changes usually serve as a selection advantage for the malignant growths, therefore, recently stroma-targeted novel therapeutic strategies are being investigated.

Keywords Tumor stroma · Tumor-associated fibroblasts · Tumor-associated immune cells · Extracellular matrix

Composition of the Malignant Tumors

Solid malignant tumors are basically composed of neoplastic cells, collectively called *parenchyma*, and non-tumorous elements, the *stroma*. Without stroma the tumor cells are unable to survive and grow, because they need appropriate blood, oxygen and nutrient supply. The stroma, however, is not merely a supportive framework of the cancer, instead, it is newly established and modified by the parenchymal cells, and there are reciprocal and continuous interactions between the components of the neoplasm. The stroma is highly complex, composed of mesenchymal stem cells, fibroblasts/myofibroblasts, different immune cells including tumor-infiltrating

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CD4+/CD8+ T-lymphocytes (TIL), small number of B-lymphocytes and plasma cells, rare natural killer (NK) cells, antigen-presenting dendritic cells, eosinophils, mast cells, macrophages, polymorphonuclear leukocytes (PMNs), immature myeloid cells, vascular and lymphatic channels, perivascular mesenchymal cells (pericytes), adipocytes – all embedded into the extracellular matrix (ECM). This creates a unique microenvironment, in which tumor growth can be modified.

Stromal Elements of Malignant Tumors: Unchanged or Modified?

When malignant tumors infiltrate underlying stroma, their cells modify the original components and significant alterations develop both in the stromal cells and in the matrix. This process is called by some authors as stromatogenesis (Sivridis et al. 2004). It involves formation of new, specific, tumor-associated stroma as an integral part of tumor invasion and governed primarily by the neoplastic cells at the beginning of the process. This newly established stroma differs from the original one, and it usually enables a growth advantage for the tumor. For example, it is rich in collagen III, but a depleted amount of collagen I and fibronectin is observable (Sivridis et al. 2004) Galectin -1 (a member of β-galactosidase-binding galectin family) which is not present in normal tissues, is frequently accumulated in cancer stroma (Van den Brule et al. 2001). The extra domain B of fibronectin is also expressed in the majority of the solid tumor stroma, but not in normal stroma (Birchler et al. 2003).

The distribution of ECM proteins also differs from that of the nontumorous mesenchyma (Linder et al. 2001). Basement membranes show discontinuous/absent staining with collagen type IV, laminin, and vitronectin antibodies (Shimoyama et al. 1995). Fibronectin is mainly seen pericellularly in the tumor-surrounding stroma, and its fragmentation is an early sign of malignant transformation (Labat-Robert 2002). The matrix proteoglycans similarly exhibit quantitative and qualitative changes: decorin was shown to be markedly elevated in the stroma of colonic or laryngeal cancer (Iozzo and Cohen 1993, Skandalis et al. 2004), but there was a loss of aggrecan (Skandalis et al. 2004). There is also a shift in the proportion of 6-sulfated and 4-sulfated delta disaccharides (Skandalis et al. 2004). In many cancerous tissues there is a high level of matrix metalloproteinase (MMP) activity, usually at the tumor-stroma interface (Lynch and Matrisian 2002). The stromal syndecan-1 expression is also increased in head and neck squamous cell carcinomas, (Mukunyadzi et al. 2003), and the loss of the normal architecture of different integrins is frequently observed (Charalabopoulos et al. 2005).

The cellular composition of malignancy associated stroma also significantly differs from that of the nontumorous counterparts. For example, a consistent lack of CD34+ stromal cells is a characteristic feature in invasive breast, colonic, lung, or pancreatic cancers (Barth et al. 2002b, Kuroda et al. 2005, Kuroda, Nakayama

et al. 2005, Nakayama et al. 2003, Barth et al. 2002a), microvessel density is changed (Hasan et al. 2002), and fibroblasts are converted into specialized myofibroblasts (Desmoulière et al. 2004). The vessels in the tumor stroma show many morphological and functional abnormalities. In some tumors the process of neovascularization is defective, which is partly responsible for local hypoxia that further induces significant alterations in the tumor. These, and other data clearly demonstrate that the tumor stroma is actively formed, peculiar, and during neoplastic progression, numerous profound alterations develop.

Malignant Cells Alter Their Microenvironment

It is generally accepted when that neoplastic cells locally proliferate and infiltrate the surrounding stroma they trigger significant changes in the non-tumorous elements by either releasing soluble factors or by cell-to-cell contact.

In the process of the metastatic cascade tumor cells secrete a number of factors facilitating their invasiveness. Among them, various proteases are of pivotal importance (Koblinski et al. 2000). Most details are available about the function of the cathepsins, especially cathepsin B. In many different carcinomas an increased expression and activity of cathepsins were detected, making possible for cancer cells to contact and interact directly with the stromal elements. Experimental studies clearly revealed a strong correlation between cathepsin B activity and metastatic potential of human cancer when the cells were injected intrasplenically in nude mice (Tzanakakis et al. 2003). Clinical reports also show that high expression of this cysteine protease was associated with a poorer prognosis in various cancers (Kayser et al. 2003, Niedergethmann et al. 2004, Scorilas et al. 2002). The role of other cathepsins (cathepsins F, H, K, S, X) is not exactly established, and it seems to be different in various cancer types (Zalatnai 2006).

The matrix metalloproteinases (MMPs) have been extensively studied and now it is clear that their role is much more complex than it was thought earlier: they are not just involved in the degradation of extracellular matrix (ECM) for tumor invasion and metastasis, but are also involved in the early stages of tumorigenesis, migration, growth, angiogenesis, and selection of apoptosis-resistant subpopulations (Lynch and Matrisian 2002). This complex effect is often reflected by their striking accumulation at the tumor/stroma interface. In addition to the parenchymal cells, however, MMPs are also produced by stromal elements after being triggered by cancer cells. In this regulated process a large number of cytokines, growth factors, cell-matrix or cell-to-cell interactions participate (Gabison et al. 2005). One of the inducing factors is the extracellular matrix metalloproteinases inducer (EMMPRIN; CD147) which is highly expressed on the surface of tumor cells and stimulates surrounding fibroblasts (or the carcinoma cells by autocrine mechanism) to produce MMPs and activate T-lymphocytes (Nabeshima et al. 2006). EMMPRIN, however, is not a universal MMP-inducer. Various cells respond differently. For example, EMMPRIN

regulates MMP-1, MMP-2, and MMP-3 expressions, but has no effect on MMP-9 or the tissue inhibitors of MMPs (TIMP-1, TIMP-2) (Caudroy et al. 2002). Moreover, EMMPRIN-levels are strongly influenced by hormones, various growth factors, glycosylation and membrane shedding (Gabison et al. 2005, Yan et al. 2005).

Recent data indicate that the urokinase plasminogen activator (uPA) system is also involved in the progression of the malignant tumors by remodeling the ECM, enhancing migration and proliferation of tumor cells or modulating cell adhesion (Duffy 2004). Its receptor (uPAR) which is located on the cell surface was found to be overexpressed in many cancer types and recently it has become one of the targets of anticancer therapy (de Bock 2004).

Other mechanisms and soluble factors should also be taken into consideration. In vitro studies have shown that the adhesion of cancer cells to endothelial cells also seems to be important in activating signaling pathways to induce MMP production, although these pathways for different MMPs are somewhat different (Hasebe et al. 2007). The influence of hormonal effects has also been demonstrated by using endometrial tumor models: administration of estrogens upregulated the mRNA levels of MMP-13 in vitro, and downregulation was observed upon antiestrogen treatment. In transplanted tumors, however, just the opposite effects were noted (Tushaus et al. 2003). Cancer cell-derived tumor necrosis factor α (TNF-α) or transforming growth factor β (TGF-β) also induced MMP-9 expression in vitro (Stuelten et al. 2005).

The best example of the profound modifying influence of cancer cells on the stroma and vice versa is the formation of bone metastasis. Some malignant tumors (breast, thyroid, lung, kidney, prostate) have a high affinity to bones and their secreted factors trigger osteolytic or osteoblastic metastasis (Virk and Lieberman 2007). Osteolysis is initiated by activation of osteoclasts through a number of soluble factors: parathormon-related protein (PTHrP), soluble receptor activator of nuclear factor κ-B ligand (RANKL), IL-1, IL-6, IL-8, IL-11, TNF-α, macrophage colony stimulating factor (M-CSF), TGF-β, prostaglandins, VEGF, and various MMPs. During osteolysis, further growth factors are released (TGF-β, IGF-1, bFGF) resulting in a facilitation of the tumor growth. More tumor cells mean more pro-osteolytic factors – and a vicious circle ensues. Osteoblastic metastasis is also triggered by carcinoma cells, but the cytokines, growth factors, transcription factors are different: TGF-β, endothelin-1 (ET-1), bone morphogenetic proteins (BMPs), IGF-1, IGF-2, IL-6, wingless int (Wnt), urokinase, FGF-1. FGF-2, FGF-8, and PDGF-BB. These compounds lead to proliferation, differentiation, and maturation of osteoblasts. Moreover, they secrete VEGF that in turn, potentiates tumor growth and survival.

The plasminogen activator inhibitor-1 (PAI-1) is regularly expressed at high levels in extracts of malignant tumors and it promotes tumor growth and spread. Although PAI-1 is mainly expressed in stromal fibroblasts and endothelial cells, in some malignant tumors the predominant PAI-1 expression is from cancer cells. In addition to its activity of inhibiting plasminogen activator, it binds the ECM protein vitronectin and endocytosis receptors of the low density lipoprotein receptor family (Andreasen 2007). Nevertheless, its exact function in tumor biology is still unclear,

although it was shown that high levels of PAI-1 is correlated with poor prognosis in human cancers (Durand et al. 2004).

Impact of Stromal Elements on Cancer Cells

Tumor-Associated Fibroblasts/Myofibroblasts

In most cases, fibroblasts and myofibroblasts cells account for the majority of stromal elements in malignant tumors (especially in carcinomas with desmoplastic reaction) and they can further modify the biological behavior of the tumor. But their specific contribution to tumor growth is yet to be fully elucidated. Morphologically, stroma elements appear similar, but functionally they are heterogeneous cell populations, with different biosynthetic and differentiation potentials. Some stroma may produce more growth factors, cytokines, chemokines, matrix degrading enzymes, while others may deposit matrix and are capable of influencing the local immune response.

Tumor-associated fibroblasts (TAFs) differ from their normal counterpart in many ways. Microarray studies revealed that in these cells 170 to 22,000 genes were upregulated including those encoding various growth factors, Cox-2, and adhesion molecules (Nakagawa et al. 2004). These fibroblasts generally have advantageous effects on cancer cells: although coculture experiments did not result in a malignant proliferation, in vivo studies revealed an accelerated growth and shorter latency period when epithelial cells and transformed fibroblasts were inoculated into nude mice (Camps et al 1990). Fibroblast activation protein (FAP), a 97-kDa surface glycoprotein, is overexpressed in a majority of cancer-related fibroblasts and it was shown to promote tumor growth in animal model systems. It may also have clinical impact, since stromal FAP was found to negatively correlate with tumor stage and tumor size in colonic cancer xenografts, and its greater level in patients with metastases was associated with a shorter survival (Henry et al. 2007). The cells of the immune system are also influenced by tumor-associated fibroblasts. Although they are phenotypically and functionally heterogeneous, in human lung cancer they may enhance or suppress tumor-associated T-lymphocyte functions (Nazareth et al. 2007). In vitro data show evidence that these stromal cells recruited blood monocytes into tumors by producing monocyte chemotactic protein-1 (MCP-1) which was regulated by endogenous IL-6 (Silzle et al. 2003).

Experimental data clearly indicate that stromal fibroblasts and collagens generate a supportive microenvironment for malignant cells. Coculture of squamous carcinoma cells and fibroblasts results in enhancement of the induction of an active form of MMP-9, cell motility, and activation of NF-kB in tumor cells (Ikebe et al. 2004). Similarly, it was demonstrated in vitro that fibroblasts are essential for melanoma cell invasion into a collagen I matrix after interaction with soluble factors (e.g. bFGF) (Wandel et al. 2002). Induction of syndecan-1 expression in stromal fibroblasts also promotes proliferation of human breast cancer cells (Maeda et al. 2004.). The role of PAI-1 was mentioned above.

Myofibroblasts ("activated fibroblasts") represent a subset of tumor-associated fibroblasts combining the characteristics of fibroblasts and smooth muscle cells. They are a major source of extracellular matrix components. These cells produce a large amount of stroma cell derived factor-1 (SDF1) promoting tumor growth and metastatic spread through CXCR4 receptors. In addition, this chemokine can activate endothelial cells leading to increased angiogenesis (Burger and Kipps 2006, Orimo and Weinberg 2006). High expression levels of this receptor were found to be associated with more aggressive phenotype of prostatic and breast cancer in experimental systems (Darash-Yahana et al. 2004), In contrast to fibroblasts, myofibroblasts secrete insulin-like growth factor (IGF-1), hepatocyte growth factor, HGF, VEGF, IL-6 – all these compounds result in a significant increase of the invasive capacity of tumor cells (Cat et al. 2006). HGF production is a nice example of the mutual interactions between the carcinoma and the stromal elements: tumor cells release IL-1, bFGF, PDGF which induce HGF-secretion from myofibroblasts; whereas HGF, in turn, potentiates the invasiveness of carcinoma cells (Nakamura et al. 1997, Matsumoto and Nakamura 2006). The significance of IGF-1, and its receptor in malignancies is less understood: in malignant neuroendocrine tumors, for example, IGF-1/IGF-1R is associated with more aggressive biological behavior, (Furukawa et al. 2005); however, the importance of the IGF-system in some other tumors, for example in advanced breast cancer is not clear (Helle 2004).

The origin of tumor-associated fibroblasts/myofibroblasts is still an unsettled issue. Various growth factors secreted by cancer cells (TGF-α, TGF-β, IGF-I, IGF-II, PDGF) transform stromal cells (Ronnov-Jessin and Petersen 1993, Ellis et al. 1994, Bronzert et al. 1987), but *Zeisberg* and coworkers reported that endothelial to mesenchymal transition could be a novel mechanism for the accumulation of TAFs (Zeisberg et al. 2007).

Cellular Elements of the Immune System

The stroma of most solid malignant tumors is usually infiltrated by macrophages, lymphoid cells, dendritic cells, mast cells, eosinophils, PMNs. But the cellular composition varies from case to case. These cells are recruited either by parenchymal cells or stromal fibroblasts.

Tumor-Associated Macrophages (TAMs)

The stroma of malignant tumors is very frequently infiltrated by a large number of recruited, modified macrophages. But their role in the neoplastic process is intriguing and somewhat contradictory. Both neoplastic and stromal cells are able to recruit blood monocytes. After migrating into the tumor and contacting with carcinoma cells these mononuclear cells acquire new properties, both phenotypically and genotypically. Gene expression of macrophages isolated from malignant tumors significantly differs from that of seen in wound macrophages or resting peritoneal macrophages. For example there is an increase in proliferation-associated

genes (Duff et al. 2007). The differentiation marker carboxypeptidase M was shown to be suppressed, while other markers (HLA-DR, CD14, CD16) were upregulated (Gottfried et al. 2003). Other studies have revealed that over 50 genes were differently expressed from those of control macrophages including IL-6, IL-8, MMP-9 (Chen et al. 2005). Tumor-associated macrophages express high levels of hypoxia-inducible factor-2α (HIF-2α) and this is an independent prognostic factor for poor outcome, while normal tissue macrophages contain negligible levels (Knowles et al. 2004). They produce much less nitric oxide than the normal monocytes and demonstrate impaired phagocytic activity (Baskic et al. 2001). They release toxic compounds (TNFα, NO, H_2O_2), proteases, immunosuppressive materials, various growth factors and cytokines (EGF, PGDF, IGF-1, bFGF, VEGF, M-CSF, IL-1, IL-8), and the net result is increased mitotic activity, promoted invasiveness and enhanced neoangiogenesis (Mantovani et al. 1992). Coculturing with carcinoma cell lines, TAMs increase their invasiveness, by upregulated EMMPRIN expression (Hagemann et al. 2005). The clinical significance of these data is intriguing. Indeed, macrophage density in the tumor is generally negatively correlated with relapse-free and overall survival, but their localizations also seem to be important. The association between TAM-density and survival of cancer patients depends on the number of intratumoral or peritumoral macrophages counted (Welsh et al. 2005). One cannot generalize about role of these cells in malignant tumors. In a tumor microenvironment there are areas where TAMs enhance tumor proliferation, namely in the perivascular areas where they promote metastatic capacity of cancer cells, while around the necrotic areas where the tissues are hypoxic, they stimulate angiogenesis favoring tumor spread (Lewis and Pollard 2006).

Tumor-Infiltrating Lymphocytes (TILs)

It has long been recognized that the stroma of some malignant tumors is heavily infiltrated by lymphoid cells, but their prognostic (and therapeutic) significance has been a topic of debate. In melanoma, ovarian cancer, colonic cancer the presence of TILs is frequently observed, and in these patients some reports claimed favorable clinical outcomes (Liakou et al. 2007), but other investigations could not reinforce these data. In renal cell cancer the presence and number of intratumoral lymphocytes or macrophages had no prognostic value (Usubütün et al. 1998); on the contrary the abundant infiltration of the tumor with CD4+ and CD8+ lymphocytes was associated with shorter survival (Nakano et al. 2001). Originally, it was thought that these cells represent a defense against tumor cells, but the scene is much more complex, because they represent a heterogeneous cell population. While CD8+ (suppressor) T cells and non-regulatory CD4+ helper cells have a beneficial effect, the accumulated CD4+/CD25+ regulatory T cells suppress tumor-specific host response (Yu and Fu 2006). Apart from direct cytotoxic effects, the tumor infiltrating lymphocytes may alter the stroma of the malignant neoplasms by secreting T-cell cytokines (TNF-α, IL-4, IL-10). The inhibition of stroma formation and angiogenesis may contribute to tumor rejection (Blankenstein 2005).

Tumor-Infiltrating Dendritic Cells

Dendritic cells, the most effective antigen presenting cells, frequently infiltrate tumor stroma together with other inflammatory cells. They also represent a heterogeneous cell population, especially with regard to their maturation. Early studies have found that the dendritic cell maturation process could be inhibited by soluble factors released by the tumor cells (mainly VEGF, TGF-β), but that mature dendritic cells were not affected. The authors concluded that suppressed dendritic cells contribute to mechanisms by which malignant cells escape from the host immune system (Gabrilovich et al. 1996). Some carcinomas (liver, colorectal, pancreatic) produce IL-8, which attracts monocyte-derived dendritic cells, but most of them remain inside the tumor where their migration toward lymphoid tissues is inhibited (Feijoó et al. 2005). CD4+ lymphocytes are also regulated by them. It was found that when the ratio of dendritic cells to T-cells was 1 : 10, an enhanced lymphocyte proliferation was seen; however, the ratio of 1 : 2 resulted in a proliferation arrest in naive CD4+ helper cells (Höpken et al. 2005). All these effects seem to favor local invasion of tumor cells. Breast cancer cells have also been reported to facilitate their own proliferation by exploiting dendritic cells. The tumor triggered these cells to induce IL-13 release from T-helper lymphocytes, which in turn promoted the growth of the carcinoma (Aspord et al. 2007). Bone marrow-derived dendritic cells, however, may display a powerful tumoricidal effect upon cell-cell contact. In addition to their retained phagocytic activity, mature dendritic cells produce large amounts of IL-6, IL-12, TNF-α, and NO that are mainly responsible for tumor cell death (Nicolas et al. 2007). Tumor-infiltrating dendritic cells may be either suppressive or protective of the immune response, because a subset of them expresses high levels of interferon-γ and IL-6, whereas other cells secrete a substantial amount of TGF-β (Liu et al. 2005). Thus, tumor-infiltrating dendritic cells also have an ambient effect on the malignant parenchyma.

Other Inflammatory Cells

B-lymphocytes and NK-cells are rarely found in the tumor stroma, and their exact role is unknown. NK and dendritic cells, however, can bidirectionally activate each other's signals. Activated NK cells induce maturation of myeloid dendritic cells, enhance their ability to produce pro-inflammatory cytokines, while dendritic cells promote the production of cytokines and cytotoxicity of the natural killer cells, enhancing NK cell tumoricidal activity (Kalinski et al. 2005). Recently, the clinical efficacy of combined NK-dendritic cell therapy is being investigated in malignant melanoma.

In some malignant tumors (skin, breast) mast cells are frequently accumulated in the stroma due to secretion of chemoattractans from the tumor cells, and also contribute to tumor growth and progression. They release diverse factors: in addition to heparin, histamine, other molecules (bFGF-2, IL-8) provide direct mutagenic effects for fibroblasts or epithelial cells, while various proteases are involved in degradation and remodeling of ECM. Mast cells are also a major source of VEGF,

inducing endothelial cell proliferation and leakage of the vessel walls (Ch'ng et al. 2006, Conti et al. 2007, Coussens and Werb 2001). All these effects can benefit the malignant cells.

Blood and Lymphatic Vessels

When the diameter of malignant tumors exceed 1–2 mm, they require neovascularization for their survival. The process of angiogenesis is one of the "hot spots" of oncological research. To date in the literature more than 20, 000 papers are available reporting its significance, mechanism and potential therapeutic applications. In many malignant tumors intratumoral microvessel density (MVD) has been implicated as a prognostic factor. In resected pancreatic carcinoma cases, for example, high MVD was correlated with shorter survival (Khan et al. 2002, Niedergethmann et al. 2000), but in a subset of patients with endocrine pancreatic neoplasms unfavorable outcome was associated with a low number of stromal vessels (Marion-Audibert et al. 2003). Although microvessel density and total microvascular area are of clinical impact in malignant tumors, one cannot generalize about them: they usually have predictive value in tumors that induce significant neoangiogenesis (e.g. breast, prostate, hematological malignancies), but in others (e.g. lung cancer, urinary bladder carcinoma) no such an association is seen (Sharma et al. 2005).

The proliferation of endothel cells is mainly triggered by vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). These factors are released by tumor cells themselves, but also by stromal fibroblasts or tumor-associated macrophages. Cyclooxygenase-2 (Cox-2) also seems to play role in this process: various neoplasms display an immunohistochemical colocalization of Cox-2, VEGF and TGF-β, and their strong expression is correlated with high MVD. Similarly, new vessels proliferate in areas where the tumor also expresses Cox-2 (Fosslien 2001). This enzyme is highly upregulated in tumor cells and in angiogenic endothelial cells during neoplastic progression, and it promotes the αVβ3-mediated endothelial cell adhesion, spreading, or migration (Rüegg et al. 2004). Recent data show that neural cell adhesion molecule (NCAM) and pericytes also play roles in the maintenance and integrity of vessels. NCAM results in stabilization of the vessel walls by maintaining normal deposition and synthesis of ECM molecules around them, and formation of perivascular matrix (including basement membrane) facilitates pericyte integration (Xian et al. 2006). According to these authors this whole process may limit tumor cell dissemination.

Lymphatic spread is an important step in the progression of malignant tumors. Recently, it has been recognized that infiltrating tumors do not simply exploit preexisting lymphatic channels, but they actively participate in lymphangiogenesis. Mainly tumor-derived VEGF-C, D and A trigger formation of new lymphatic vessels, but some other signaling molecules (HGF, FGF, PDGF, IGF, angiopoetin) have also been identified to promote this process (Achen and Stacker 2006, Jiang et al. 2005, Tobler and Detmar 2006). On the other hand both interferon-α and

interferon-γ cause a growth inhibition of lymphatic endothelial cells by inducing apoptosis (Shao and Liu 2006).

Lymphatic vessel density (LVD) and its significance may vary from tumor to tumor. In melanoma, intratumoral LVD was significantly higher than that in benign melanocytic nevi (Giorgadze et al. 2004) in pancreatic endocrine tumors LVD and VEGF-C expression correlated with malignant behavior (Rubbia-Brandt et al. 2004). The tumor-secreted VEGF-C in prostatic carcinoma proved to be an important inducer for intratumoral lymphangiogenesis (Wong et al. 2005). However, in breast cancer, lymphangiogenesis was not evident (Agarwal et al. 2005). In colorectal cancer there was no relationship between LVD and the clinicopathological features (Duff et al. 2007), although in this tumor the distribution of lymphatic channels was uneven: they were mainly found in peritumoral localization, while inside the tumor their number was sparse.

In some tumors, for example in non small cell lung cancer, Cox-2 also promotes lymphangiogenesis. Immunohistochemically, Cox-2 levels correlate with LVD, VEGF-C and clinicopathological parameters. In cell lines Cox-2-mediated VEGF-C upregulation was commonly observed and this activation was mediated by HER-2 tyrosine kinase receptor (Su et al. 2004).

It should be mentioned that apart from these factors, in the process of lymphangiogenesis, the extracellular matrix also plays an important role: for the growth, migration, survival of lymphatic endothelial cells several ECM molecules participate as well, among them MMPs, hyaluronan, integrins, reelin, IL-7, etc. (Ji 2006).

Extracellular Matrix (ECM)

The role of ECM is not limited to being a supportive scaffold and barrier against tumor invasion, but it also serves as a reservoir of various growth factors and cell binding proteins all affecting the biological behavior of tumor parenchyma. Proteases released by tumor cells and stroma elements significantly modify the ECM, leading to altered cell-to-cell and cell-to-matrix interactions.

The elements of the extracellular matrix (collagens, proteoglycans, elastin, fibronectin, laminin, vitronectin, tenascin, entactin, osteonectin, osteopontin, thrombospondin) and degrading enzymes (MMPs, heparinase) are produced by the parenchymal or stromal cells, and their quantity and quality are important for tumor growth. Because of wide diversity of these molecules, the crosstalk among ECM components and tumor cells are extremely complicated.

In some malignant tumors (pancreas, biliary tract, breast, left colon, stomach) the presence of abundant connective tissue is a characteristic feature. In the desmoplastic stroma of pancreatic carcinoma the mean collagen content (collagen I, III, V) is about 3-fold higher than in normal pancreatic tissue (Imamura et al. 1995), and in this process several factors (TGF-beta1, connective tissue growth factor, and platelet-derived growth factor, etc.) participate (Lohr et al. 2001). In bile duct carcinoma, deposited collagen type IV was found to stimulate tumor cell proliferation, adhesion and migration in a dose-dependent fashion (Chen et al. 2001), but other

effects similarly may favor the progression: the malignant cells stimulate expression of collagen XIII, and this collagen may contribute to tumor progression by modulating the cell-matrix interaction (Väisänen et al. 2005). Moreover, the increased amount of stromal collagen serves as a barrier for CD8+ T-cells, so the tumor cells might escape from the host immune reaction (Ohno et al. 2002).

It has long been recognized that in the stroma of solid tumors fibrinogen/fibrin are also deposited, although their amount is variable from case to case. Fibrinogen/fibrin play important roles not just in clotting blood, but also in several cellular and matrix interactions. Binding to other compounds, they promote fibroblast proliferation, endothel cell spreading, angiogenesis, and all these activities are enhanced by binding to FGF-2, VEGF, IL-1 (Mosesson 2005). The deposition process resembles wound healing in many respects, but it differs fundamentally in that invading tumor cells render the newly formed vessels hyperpermeable resulting in an accumulation of these molecules. Platelets have no role in this process (Nagy et al. 1989). Apart from exudation, there are experimental data available that some tumors (breast cancer, for example) also synthesize and secrete fibrinogen polypeptides (Simpson-Haidaris and Rybarczyk 2001). The deposited fibrinogen/fibrin seem to play an important role in cancer growth and dissemination, but the data are somewhat contradictory. In genetically fibrinogen-deficient mice, development of spontaneous lung metastases was found to be strongly diminished without effecting the primary lung tumor or angiogenesis (Palumbo et al. 2002). On the other hand, experimental data showed that fibrinogen exerted an induction of apoptosis on endothelial cells, blocked their tube-formation, and human tumor cells that secreted COOH-terminal globular domain of fibrinogen displayed a markedly suppressed tumor growth and a reduced number of intratumoral vessels (Akakura et al. 2006).

Among adhesion molecules, *integrins* have received great attention during the past decade. These heterodimer cell surface molecules on one side link to actin cytoskeleton to the cell membrane, and on the other side, mediate bidirectional matrix-cell interactions. This structure enables transmission of signals from the extracellular space to the cell. In this process various molecules are involved (focal adhesion kinase [FAK], integrin-linked kinase [ILK], particularly interesting new cystidin-histidine rich protein [PINCH], non-catalytic region of tyrosine kinase adaptor protein [Nck2]). They also act synergistically with several pathways, including the Rho-effector pathway, the growth factor pathway, and the Ras-MAP kinase pathways (Wu et al. 2007). Through these signaling cascades they regulate cell proliferation, differentiation, migration, but in many malignant tumors these pathways are partly altered (Hehlgans et al. 2007). In addition, integrins may be involved in the oncogenic transformation of normal cells (Eble and Haier 2006). They are highly expressed in neovascular endothelial cells (especially $\alpha V\beta 3$, $\alpha 5\beta 1$) (Rüegg et al. 2004) and may contribute to extracellular matrix-mediated multidrug resistance, too (Elliott and Sethi 2002).

Parenchymal-stromal interactions are also involved in production of ECM components as it was shown by *in vitro* studies. Coculturing colon cancer and non-tumorous stromal cells resulted in an increased tenascin-C, chondroitin sulphate, chondroitin-6-sulphate, or versican expression, and this effect was mediated by

TGF-β and PDGF (Mukaratirwa et al. 2005). In pancreatic cancer a marked overexpression of pro-metastatic versican, or antitumor lumican and decorin was found by RT-PCR and by immunohistochemistry. Abundance of these proteoglycans was present in the ECM, but not inside cancer cells, because the main source of these proteins is the pancreatic stellate cells, but the TGF-β secreted by the carcinomatous elements actively influenced their expression (Köninger et al. 2004).

Galectin-1, a soluble lectin is secreted by many different cells (normal, tumorous and stromal elements) into the peritumoral ECM, and is able to regulate proliferation, differentiation, migration, and tumor cell death (He and Baum 2006). Elimination of immunoreactive T-lymphocytes can also be initiated from this molecule. It was shown that it could induce an apoptotic death of the tumor-infiltrating T-cells. This process may or may not require cell-cell contact: the majority of galectin-1 exerts its lymphocyte killing effect on bound T cells, but its soluble form is similarly active. As a sum result, the decreased number of T cells in the tumor stroma favors again the invasive capacity (He and Baum 2004).

Cancer cells can also specifically interact with other ECM proteins, like elastin, which is mediated by two elastin-binding proteins and galectin-3. The expression of these elastin-binding proteins was found to be closely associated with the invasive potential of several tumor types. Certain malignant tumor cells can synthesize elastin and express lysil oxidase and in these carcinomas (breast, stomach) elastic tissue is frequently demonstrable in the stroma. This elastic tissue may have some role in the angiogenesis, since this ECM protein can store angiostatic molecules (Lapis and Timár 2002).

Conclusions

The interaction between parenchymal and stromal elements in malignant tumors is a highly complex, multidirectional phenomenon involving the active participation of neoplastic cells and the fibroblasts, myofibroblasts, blood and lymphatic vessels, various cells of the immune system, and the extracellular matrix. All these components influence each other and they can mutually induce genotypic/phenotypic changes either by cell-cell contact or by soluble factors (Fig. 2.1). The tumor stroma differs from normal tissues, both quantitatively and qualitatively, and these alterations are primarily governed by the neoplastic cells. The modified stromal cells in this newly formed and reorganized microenvironment release numerous cytokines, growth factors and in turn, facilitate or suppress proliferation of the malignant elements. Generally, stromal changes serve as advantageous alterations for cancer, promoting its survival, providing new vessels and lymphatic channels, secreting growth factors for its progression, and inducing drug-resistance. However, the altered stroma has an ambient effect on the tumor, because in some experiments the invasiveness of the cancer was decreased by the stroma. Hence, one cannot generalize about the role of the microenvironment, partly because different tumors may display different stromal composition, and partly because the interactions between the parenchymal and stromal elements are dynamically changing during the tumor

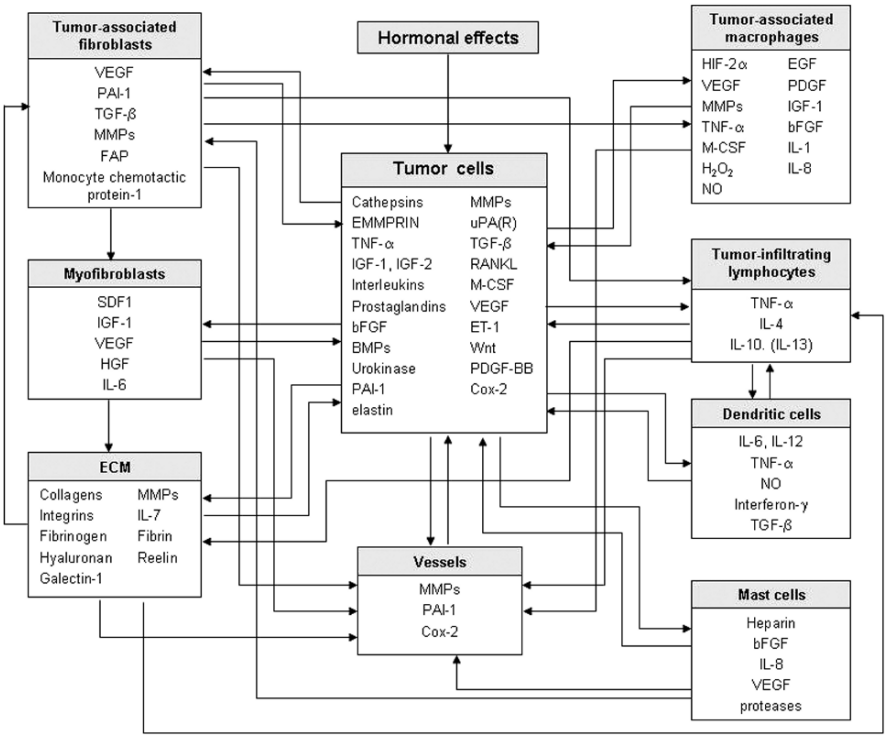

Fig. 2.1 Among the malignant cells and the microenvironment there is a highly complex, multidirectional relationship, they can mutually modify each other's effect. bFGF: basic fibroblast growth factor; BMPs: bone morphogenetic proteins; Cox-2: cyclooxygenase-2; ECM: extracellular matrix; EGF: epidermal growth factor; EMMPRIN: extracellular matrix metalloproteinases inducer; ET-1: endothelin-1; FAP: fibroblast activation protein; HGF: hepatocyte growth factor; IL: interleukin; HIF-2α: hypoxia inducible factor-2α; IGF-1: insulin-like growth factor-1; M-CSF: macrophage colony stimulating factor; MMPs: matrix metalloproteinases; NO: nitric oxide; PAI-1: plasminogen activator inhibitor-1; PDGF-BB: platelet derived growth factor BB; RANKL: receptor activator of nuclear factor κ-B ligand; SDF1: stromal cell derived factor-1; TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor-α; uPA(R): urokinase plasminogen activator (receptor); VEGF: vascular endothelial growth factor; Wnt: wingless int

development. Nevertheless, the recognition that the stromal microenvironment plays a significant role in the progression of malignancies, novel therapeutic strategies targeted the stromal components are established and are being investigated.

Abbreviations

bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
Cox-2	cyclooxygenase-2
ECM	extracellular matrix

EGF	epidermal growth factor
EMMPRIN	extracellular matrix metalloproteinases inducer
ET-1	endothelin-1
FAP	fibroblast activation protein
FGF	fibroblast growth factor
HGF	hepatocyte growth factor
HIF-2α	hypoxia inducible factor-2α
IGF-1	insulin-like growth factor 1
IL	interleukin
IGF-1R	insulin-like growth factor 1 receptor
LVD	lymphatic vessel density
M-CSF	macrophage colony stimulating factor
MMP	matrix metalloproteinase
MVD	microvessel density
NCAM	neural cell adhesion molecule
NF-kB	nuclear factor kappa B
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet derived growth factor
PMN	polymorphonuclear leukocyte
PTHrP	parathormon related protein
RANKL	receptor activator of nuclear factor κ-B ligand
SDF1	stromal cell derived factor-1
TAF	tumor-associated fibroblast
TAM	tumor-associated macrophage
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinases
TNF-α	tumor necrosis factor-α
uPA(R)	urokinase plasminogen activator (receptor)
VEGF	vascular endothelial growth factor
Wnt	wingless int

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Chapter 3
Significance of Tumor Microenvironment on the Genesis of: Interstitial Fluid, Angiogenesis, Haemostatic/Haemorheologic Abnormalities. Pathogenesis and Therapeutic Aspects

Gianfranco Baronzio, Isabel Freitas and Kwan Hau

Abstract In order for tumors to grow more than a few millimetres in size, new vasculature must be established. However, the new vascular support is generally inefficient, poorly organized and cannot keep up with the pace of the tumor proliferation. This creates undernourished and hypoxic regions in a new environment, with neo-vasculature having a perturbed structure and function. Two abnormalities are peculiarly important. The first is an excessive leakiness of the endothelium due to a malfunction of Starling's law that regulates liquid distribution across the endothelium, resulting in increased accumulation of liquid in the tumor interstitium. The second is the enhanced procoagulant activity of the endothelium, as shown by numerous clinical and experimental findings on the interaction between the tumor and the coagulation and fibrinolytic systems. Through these interactions, tumor growth and dissemination are enhanced while the tumor can generate a matrix supporting neoangiogenesis. In the unique tumor microenvironment, both endothelial cells and tumor cells express a wide variety of factors, including procoagulants, cell adhesion molecules, vasomotor substances and cell survival signals. The up-regulation by tumor microenvironment of MET oncogenes and other hemostatic oncogenes further contribute to alterations of the hemostatic balance. Platelets (PLTs), leukocytes (LKs), and red blood cells (RBCs) behave differently in the tumor capillary lumen, than in normally capillary lumen, such as being more hemoconcentrated. These alterations create the conditions for an anomalous hemorheology and leukocyte endothelium interaction. PLTs, LKs, and RBCs may bind to the fibrin that envelopes tumor cells in the capillary lumen, and form a cocoon-like structure that protect them from colliding with the endothelium, and from immune surveillance, and thus not eliminated by the body's defensive

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system. This feature contributes partially to tumor progression and metastasization. We believe that these findings may have important therapeutic implications. In this overview, an attempt will be made to describe how tumor cells abrogate normal physiologic mechanisms to suit the environment for their own purposes and review the common pathways between coagulation, angiogenesis and the unique tumor patho-physiology.

Keywords Tumor microenvironment · Hypoxia · Tumor interstitial fluid · Coagulation abnormalities · Hemostasis · Thrombosis · Neoangiogenesis · Trousseau syndrome · Hemorheology

Introduction: Generation of the Unique Tumor Microenvironment and Angiogenesis

Tumors are not merely an accumulation of neoplastic cells, but rather a complex tissue with blood vessels, stromal cells, infiltrating immune-competent cells, and a differentiated extracellular matrix. All these cell types interact each other to build a unique tumor microenvironment (Van Kempen et al. 2003). This heterogeneous mass grows until it reaches a volume of $2\,mm^3$, beyond which the diffusion of nutrients and oxygen cannot take place and areas of hypoxia and acidosis develop. At this stage the development of a new vasculature (neoangiogenesis) is necessary for permitting tumor rapid growth and for compensating the state of hypoxia and anoxia. Neoangiogenesis results from the incorporation of host vessels, sprouting and growth of new blood vessels from preexisting ones or recruitment of bone marrow precursors, and is initiated by a series of hypoxia-induced growth factors and cytokines (Raughunand). A major stimulus for this process is cellular hypoxia, a condition that is present in almost all solid cancers (Vaupel and Mayer 2007). Hypoxia induces transcriptional activation of genes that alter cellular metabolism and promote neoangiogenesis. The first to be activated when oxygenation is low ($O_2 \leq 1\%$) is hypoxia inducible factor 1 (HIF-1), shown to be induced in a multitude of tumor types, especially in hypoxic areas (Vaupel and Mayer 2007). HIF-1, is a heterodimeric protein consisting of α and β subunits. Under normoxic conditions, the α-subunit is a target of an oxygen-dependent prolyl-hydroxylase and quickly degraded in proteasomes, but when there is hypoxia the complex translocates to the nucleus and modulate the expression of genes essential for cell survival under hypoxic conditions, such as those of inducible nitric oxide synthase, vascular endothelial growth factor (VEGF), heme oxygenase-1 and enzymes involved in the intermediate reactions of glycolysis. converting tumor metabolism from aerobic to anaerobic (Kim and Dang 2006). Among the factors induced by HIF-1, VEGF is considered the key angiogenesis factor (Fukumura and Jain 2007,Vaupel and Mayer 2007). Four subfamilies (A,B,C,D) of VEGF are known to bind specific tyrosine kinase receptors (VEGFRs) on endothelial and on lymphatic cells. VEGF-A binds to VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). VEGFR-2 appears

to mediate almost all of the known cellular responses to VEGF. VEGFA binds to all VEGFRs, and can increase the motility, the mitosis and the migration of endothelial cells. VEGFB is implicated in embryonic angiogenesis and VEGFC in lymphangiogenesis. The action of VEGFD is currently unknown (Goh et al. 2007). Other growth factors involved in angiogenesis are platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β) and basic fibroblast growth factor (bFGF). Both tumor and stroma cells produce these factors in an attempt to carry nutrients to the deprived environment in solid tumors. Despite these efforts, hypoxia persists as oxygen demand from rapid tumor growth outstrips the blood supply. The conditions thus do not allow a complete resolution of hypoxia and anoxia in the tumor microenvironment resulting in a persistence of nonproliferating hypoxic cells (Baronzio et al. 2003). As previously reported, even though neovascularization exerts a profound effect on tumor growth and metastasis, the neovascularization has an abnormal anatomic architecture compared with its normal counterpart (Baluk et al. 2005). These abnormalities favor the formation of excessive interstitial fluid (TIF), a stagnant and irregular tumor microcirculatory flow with increased viscosity predisposing to thrombosis, and a persistence of hypoxia (Baronzio et al. 2003). Initially hypoxia was thought to be due exclusively to anomalous tumor physiology but now it is becoming evident that tumor exploits and builds this microenvironment for its own purposes. In fact, cancer cells can subvert the normal tissue architecture and reprogram the activity of stromal cells to their advantage. An example is the peritumoural inflammatory reaction. This reaction is normally an attempt of the complex homeostatic mechanism of the body to hamper tumor development (Albini et al. 2005), however when inflammatory cells, such as macrophages arrive in the tumor environment they polarize from one phenotype (TAM1) to another (TAM2). TAMs1 have antitumoural activity, whereas TAMs2 have tumor-prone activity (Colombo and Mantovani 2005, Crowther et al. 2001, Lamagna et al. 2006). TAMs accumulate in large numbers in tumor hypoxic zones, being recruited by several cytokines/chemokines (IL-6; Il-8, IL-10, human monocyte chemoattractant protein-1 (MCP-1/CCL2)) and growth factors (VEGF, SDF-1) induced by tumor hypoxia (Murdoch et al. 2005, Lewis and Murdoch 2005). Fibroblast and other stromal cells such as neutrophils and mast cells along with tumor cells in hypoxic conditions form a distinctive microenvironment that favors tumor progression (Baronzio and Freitas 2008). Furthermore, tumor hypoxia not only induce the expression of hypoxia inducible factor 1 (HIF-1) but over-express oncogenes such as MET, with a role in tumor hemostasis (Boccaccio and Comoglio 2005). The recruitment of macrophages and lymphocytes, neutrophils and fibroblasts leads to an inflamed and injured environment. This microenvironment is characterized by low levels of oxygen and glucose and high levels of inflammatory cytokines, reactive oxygen, nitrogen species and metabolites. Associated with the low oxygen concentration in inflamed tissue there is an up-regulation of HIF-1 by the inflamed tissue (Frede et al. 2007). Additionally, inflamed tumor stroma are rich in proinflammatory cytokines including IL-1, IL-6, IL-8, IL-10. These cytokines along with tissue factor (TF) and VEGF strongly influence the vascular structure (Wojtukiewicz et al. 2001). Two important involvements of vasculature will be reviewed. The first is

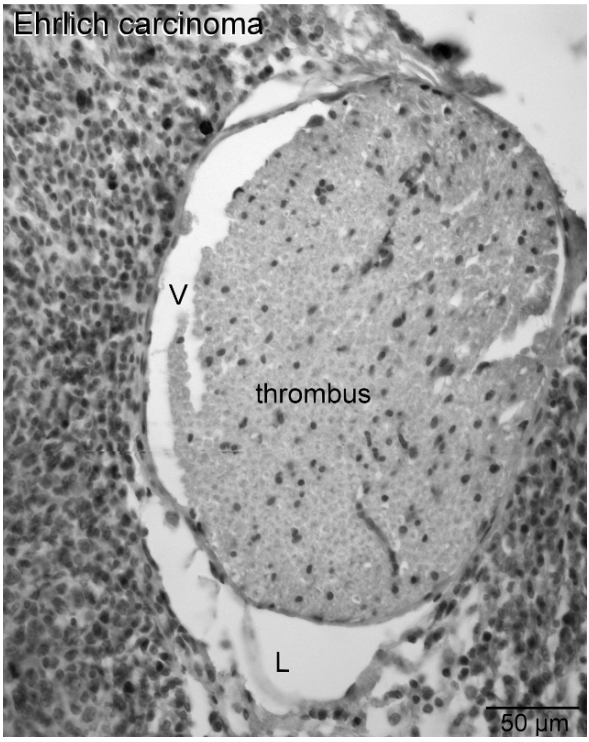

Fig. 3.1 Large heterotypic thrombus in the lumen of a vein at the periphery of an Ehrlich carcinoma, containing erythrocytes, leukocytes, tumor cells and fibrin. Immunoperoxidase staining for Hypoxia Inducible Factor-1 alpha (HIF-α); counterstaining with Hematoxylin. V: vein; L: lymphatic vessel

the altered fluid distribution between the tumor vasculature and the host interstitium. The second regards the altered blood flow in the vessel lumen with disruption of the Virchow triad and contribution to venous thrombosis (see Fig. 3.1).

Altered Blood-Tumor Interstitial Fluid Exchange

Fluid Exchange Between Blood and Normal Tissues

Normally, cell vitality is maintained by a suitable transport medium that enables the exchange of oxygen, nutrients and waste products. This medium or space located between the capillary walls and the cells is called interstitium. As reviewed by Aukland and Reed, the basic structure of the interstitium is similar in all tissues: collagens build the fiber framework that contains a gel phase composed of glycosaminoglycans (GAGs), a salt solution, and proteins derived from plasma. 70% of this medium is fluid and principally water (Guyton and Hall 2006).

Structure of Normal Capillary

The normal capillary wall has three basic structural constituents: the endothelium, the basal lamina and pericytes. The inner diameter is between 5 to 10 μm, their length is 20 to 100 mm and their density reflects the magnitude of metabolic rates. As revealed by electron microscopy, the endothelium, that is a sheet of thin squamous epithelium can be continuous, fenestrated or discontinuous, depending on the presence or absence of small transcellular openings (fenestrae) in its wall (Simionescu and Simionescu 1998). Continuous capillaries are present in skeletal, smooth, cardiac muscle, lung and central nervous system. Fenestrated capillaries are present in renal glomeruli, endocrine glands and gastrointestinal system. Discontinuous capillaries are found in spleen, bone marrow and liver. Normal endothelium rests on a basal lamina (basement membrane) that varies in thickness and continuity (Simionescu and Simionescu 1998). The exchange of substances required for cellular metabolism when capillaries are not discontinuous or fenestrated, takes place through two type of small passageways connecting the capillary with the exterior: the vesicular channels and the intercellular clefts. Careful measurements in laboratory animals have proved that vesicular forms of transport are quantitatively of little importance, whereas fluid percolates mainly through intercellular clefts. Intercellular clefts are thin slit curved channels with a uniform spacing of about 6–7 nm (60–70 Å) slightly smaller than the diameter of albumin (Guyton and Hall 2006). Pericytes, mesenchymal-like cells, play several roles in regulating vascular formation, stabilization, remodeling, and function. Pericytes also have a role in exchange, by breaking the barrier formed by the basement membrane and by regulating blood flow at the capillary level (Shepro and Morel 1993).

Starling Equation

Under normal conditions water movement across capillaries is the result of a balance between filtration and reabsorption following changes in capillary and tissue hydrostatic and oncotic pressure. In 1896, the British physiologist Ernest Starling was the first to define the interrelationship between these two forces, and since then the relation is named Starling law (Starling 1896).

The Starling law is expressed in quantitative terms by the following equation (eq. 3.1) (see Fig. 3.2).:

$$Jv = K_F A[(Pc - Pi) - \sigma(\pi c - \pi i)] \tag{3.1}$$

J_v is the amount of fluid filtered or reabsorbed per unit time. The reflection coefficient (σ) is a measure of the ease of solute penetration through the capillary wall and its value ranges between 0 and 1, where 0 indicates no restriction for the solute and 1 indicates that capillary is impermeable to solute. K_F, the filtration constant, is determined by the physical properties of the barrier (i.e., size and number of pores

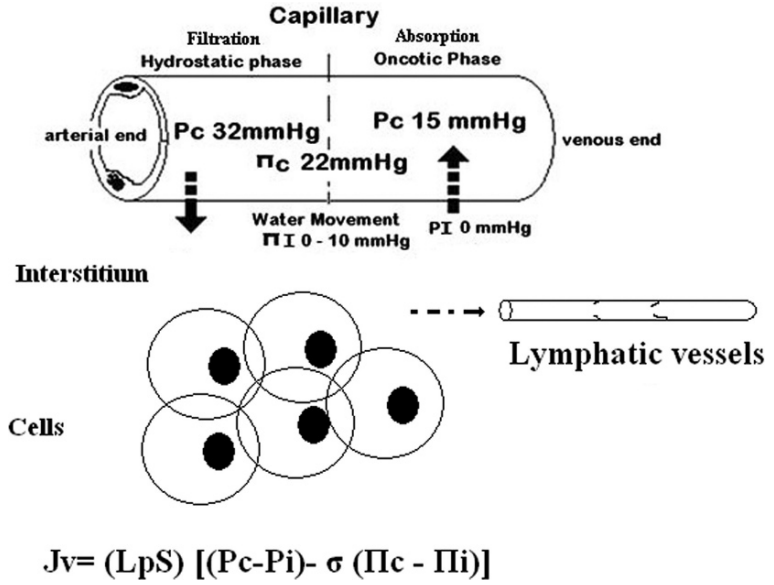

Fig. 3.2 The Starling law with the various forces acting on and across capillary wall are illustrated. Pc: capillary pressure. Pt: interstitial pressure; πc: Capillary osmotic pressure; π i; interstitial osmotic pressure

and the thickness of the barrier). P_C is the capillary hydrostatic pressure, Pi the tissue interstitial hydrostatic pressure, Π_C the capillary plasma oncotic pressure, Πi the tissue interstitial oncotic pressure and A the area of exchange. The expression in brackets $(P_C - Pi) - \sigma(\Pi_C - \Pi i)$ represents the net driving force for moving fluid into and out of circulatory system. 70% of the osmotic pressure within the capillary is generated by albumin (Guyton and Hall 2006) and remains constant along the capillary length (see Fig. 3.2). Thus under normal conditions, one can distinguish two phases operating in the control of water exchange: the hydrostatic phase and the oncotic phase (Fig. 3.2). The hydrostatic phase predominates at the arterial side of the capillary where water is lost since the P_C is $> \Pi_C$; by contrast, the oncotic phase predominates on the venous end of capillary where water is reabsorbed because Π_C is $> P_C > Pi$.

Recently, it has become evident that an additional factor contributes to the control of fluid quantity in the interstitium, i.e., the lymphatic flow. Lymphatic drainage proceeds at constant rate and is generally quite low (Levick 1995, Aukland and Reed 1993). The presence of lymphatic drainage, modify eq. 3.1 in:

$$Jv = KFA[(Pc - Pi) - \sigma(\pi c - \pi i)] - L \tag{3.2}$$

L stand for lymphatic drainage and is negative because it removes liquid from the interstitium. Another important aspect to take in consideration is that capillary pressure may drop along the length of capillary lumen. The differences between the

arterial and venous end can be of 15–30 mmHg, and the P_C at the arterial end is more influenced by P_C at the venous end that by the systemic pressure. Interstitial fluid pressure Pi is generally negative or near zero (Guyton and Hall 2006) and increases only in encased tissues like brain, kidney or when there is a lymphatic blockade or under certain pathologic conditions that we will describe below.

Fluid Exchange Between Blood and Tumor Tissue

Clinical studies and animal studies have demonstrated an accumulation of fluid in the tumor interstitium (Gullino 1966, Jain 1988, Milosevic et al. 2001). Several factors contribute to this accumulation and are dependent on: (a) capillary structure and physiology; (b) hydrostatic and osmotic pressure differences; (c) lack of lymphatic drainage. The differences will be reviewed in greater detail in the following.

Tumor Capillary Structure and Physiology

The tumor endothelium presents several differences compared to normal counterpart. First, many endothelial cells originating from tumour vessels do not form a normal monolayer but are irregularly shaped and disorganized with some overlapping one another (McDonald and Foss 2000). They have loose interconnections and focal intercellular openings. The size of the opening determined by electron microscope, is generally less than 2 μm in diameter (McDonald and Baluk 2002). Pericytes that play an important role in the regulation of vascular formation, stabilization, remodelling and function (Armulik et al. 2005), have a different role in tumour vessels (Morikawa et al. 2002). They show various alterations, such as increased perivascular deposition of extracellular matrix (ECM) components, different expression of cytoskeleton proteins, loose association with endothelial cells and extension of their cytoplasmatic processes deep in the tumour tissue; they also play a role in vessel sprout growth and metastasis (Morikawa et al. 2002, Xian et al. 2006). Furthermore, the tumor itself produces not only proangiogenic factors such as Vascular Endothelial Growth Factor (VEGF) but also vasoactive factors such as bradykinin, nitric oxide (NO) and peroxynitrite ($ONOO^-$) (Dvorak 2002, Ferrara and Davis-Smyth 1997, Maeda et al. 2000) that make tumor endothelial cells more permeable and leaky (McDonald and Foss 2000). Tumor vessels leakiness may enhance the efficiency of fluid exchange between the vascular and the interstitial space. This unbalance can increase the tumor interstitial space that becomes very large; for instance in the hepatoma it may be twice that of host liver (Gullino et al. 1965). This enlargement in interstitial space has been demonstrated to be present in several animal and human tumors (for a review see Jain 1987).

Tumor vessels are tortuous, and lack the normal hierarchical arrangement of arterioles, capillaries and venules. Furthermore, tumor endothelial cells show abnormalities in gene expression, require growth factors for survival and have defective barrier function to plasma proteins. Pericytes on tumor vessels are also abnormal. Aberrant endothelial cells and pericytes generate defective basement membrane. (Baluk et al. 2005).

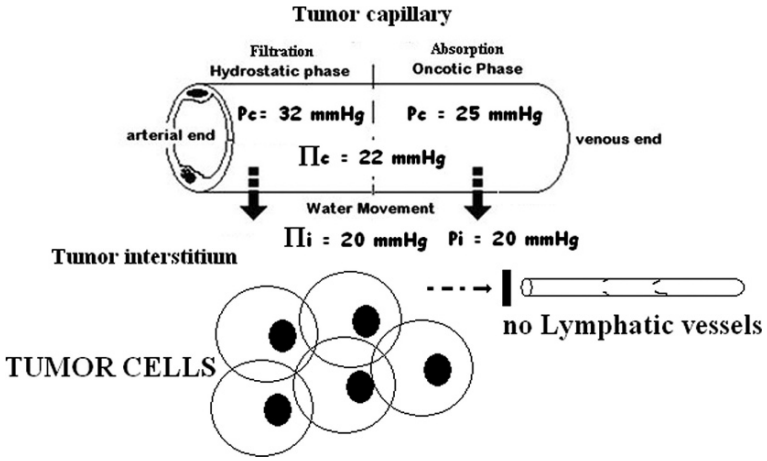

Fig. 3.3 The non obedience of Starling Law in tumor is outlined as the various forces acting across the tumor vascular wall. Pc: capillary pressure. Pi: interstitial pressure; πc: Capillary osmotic pressure; πi: interstitial osmotic pressure

Hydrostatic Pressure and Osmotic Forces Differences

P_C is the main driving force in regulating fluid filtration and, as seen in Fig. 3.2, is generally between 28–35 mm Hg and has an axial decreases of 15–20 mm Hg. This drop is called arterial venous pressure difference (a-v Δp). This difference in tumor capillary is less than in normal capillary, and P_C at the arterial end approaches that at the venous end with a median drop of 8–10 mmHg (see Fig. 3.3) (Heldin et al. 2004, Jain 1987). This permits to develop a gradient at the venous end for Πi compared to Pi. The reasons for this behavior are not completely understood and can be ascribed to a higher number of arteriovenous anastomoses, to an increased tortuosity of tumor neovasculature and to a stagnant blood flow (Jain 1988). The decreased reabsorption together with tumor vessel leakiness, cause a flow of macromolecules toward the interstitium, as compared to physiologic conditions (McDonald and Foss 2000, Jain 1987). Albumin, that generates 70% of the oncotic pressure, is lost in greater quantity leading to an increased oncotic pressure (Πi) in the interstitium, as demonstrated in animal studies (Stohrer et al. 2000, Butler et al. 1975,Gullino 1966). This factor contributes further to develop a gradient at the venous end for Πi compared to Pi and to decrease the water reabsorption at the venous end (see Fig. 3.3).

Lack of Lymphatic Drainage

Lymphatics are present at the tumor periphery but not within the tumor mass (see Fig. 3.3) (Gullino 1966, Heldin et al. 2004, Jain 1987). This does not permit fluid reabsorption, and along with the above factors, create a progressive increase in tumor interstitial fluid pressure (TIFP) from the periphery to the center of the tumor (Freitas et al. 1997, Gullino 1966, Jain 1987, 1988).

Brief View of Normal Hemostasis

Hemostasis is an intricate, highly regulated and dynamic process able to seal focal injury and to maintain blood fluidity (Riddel et al. 2007). When the integrity of blood vessel wall is disrupted, the haemostatic process is an active process with the initiation of a cascade of biochemical reactions to minimize blood loss, restoring endothelium continuity. The biochemical cascade involves both intracellular and extracellular components, and is sequential. Its first aim is that of forming a clot, followed immediately by the dissolution of this clot. The hemostatic process can be divided in two stages namely: (a) primary hemostasis and (b) secondary hemostasis (Handin et al. 2005, Hoffman and Monroe 2007). Process (a) involves the formation of a platelet plug at the site of injury; process (b) refers to the cascade of reactions of plasma coagulation system that result in fibrin formation. As reported by Handin, the normal hemostatic system limits blood loss by regulating the interactions between three different components and namely vessel wall, blood platelets and plasma proteins. The endothelium normally produces and releases procoagulant and anti-coagulant factors. Usually, anticoagulant activity predominates, and is attributable to the presence of heparin-like molecules that activate antithrombin, the production of thrombomodulin (a co-factor of protein C), the competitive inhibition of Factor V activation, and the endogenous synthesis of prostacyclin and nitric oxid. Further anticoagulant property is present in the layer of glycosaminoglycans on endothelial surface. These along with heparin-like molecules have negative surface charges, repelling platelets and anticoagulant factors in the vessel lumen (Colvin 2004, Van Hinsbergh 2001). When injury to the endothelium occurs, the first phase of primary hemostasis requires the interaction of platelet adhesion, aggregation and plasma constituents such as thrombin. They will be described separately.

Primary Hemostasis: Platelet Adhesion and Aggregation

Activated platelets aggregate to each other to form a plaque that seals the loss of integrity of endothelium. Within few seconds after injury, platelets adhere to the collagen fibrils present in the vascular sub endothelium. The adhesion to endothelium is facilitated by the interaction of platelets with two collagen receptors: glycoprotein (Gp) Gp Ia/IIa and Gp VI. This adhesion to the endothelium is stabilized by von Willebrand Factor (VWF) an adhesive Gp (Handin et al. 2005, Born et al. 1981). The aggregation is promoted by agonists secreted from platelets themselves and by fibrinogen that binds to the platelet surface via GPII/ IIIa. As, the primary hemostatic plug is formed plasma coagulation proteins are activated to initiate secondary hemostasis.

Secondary Hemostasis

Tissue factor (TF) is the major physiological initiator of blood coagulation (Lwaleed et al. 2007). It is a transmembrane protein and a member of class II cytokines

and hemapoietic growth factor receptor family. When TF, that is normally kept separated from circulating blood cells by the endothelial barrier, comes in contact and binds both zymogen and activated form of Factor VII (FVIIa), it forms a complex that activates factor IX and X. Factors IXa and Xa together with factor VIIa and Va, respectively, form the tenase and prothrombinase complexes that activate FX and prothrombin. Tenase complex is constituted by FVIIIa, Calcium and phospholipids that can activate FX to FXa and converts inactive prothrombin to α-thrombin, a serine protease. Thrombin formation occurs via several steps and once formed it can convert fibrinogen to fibrin that in turn can activate platelets (Norris 2003). The formation of fibrin is the final stage of the coagulation process. In conclusion, soluble fibrinogen is converted into insoluble fibrin polymer that is converted by thrombin in soluble fibrin monomers (Fibrinopeptide A and Fibrinopeptide B). These monomers interact each other and form strands that cover as a mesh the plug of platelets that have sealed the injured area (Norris 2003).

Fibrinolysis, the Safety Catch

When the hemostatic function of the fibrin clot is no longer needed, it is removed by fibrinolysis. This process is achieved by the enzyme *plasmin*, a potent proteolytic enzyme with a broad spectrum of activity. Plasmin is derived from the activation of its precursor, plasminogen, by several activators. In man, there are two plasminogen activators (PAs), urokinase-type PA (uPA) and tissue-type PA (tPA). Receptors for plasminogen, uPA and tPA are present on cell surfaces, facilitating the assembly of the system. The proteolytic activities of plasmin and the PAs are modulated by their respective inhibitors. Plasmin inhibitors include α_2-antiplasmin and α_2-macroglobulin as well as α_1-antitrypsin, α_2-antiplasmin, C1 inhibitor and antithrombin. PA inhibitors (PAIs) include the type 1 (PAI-1), type 2 (PAI-2) and type 3 (PAI-3, identical to the inhibitor of activated protein C) and protease nexin. In addition, the fibrinolytic system may also be inhibited by a protein that is activated during clotting. This protein was recently described and termed thrombin-activatable fibrinolysis inhibitor (TAFI) (Juhan-Vague et al. 2000). It is a carboxypeptidase B derived from the liver. When cleaved by thrombin, it is converted to the active form (TAFIa) as carboxypeptidase U. TAFIa stabilizes fibrin and inhibits the lysis of fibrin by preventing the binding of plasminogen to fibrin.

Triggering of fibrinolysis occur when the plasminogen activator, plasminogen, and fibrin are close together. Both plasminogen and its activator bind avidly to fibrin as the clot forms. This close association prevents inhibition of plasmin activity by the inhibitor, and allows proteolysis of the fibrin to proceed after the production of plasmin. Plasmin attacks fibrin reducing its size such that it no longer has hemostatic activity. Many fragments are formed during this process, and a few retain the capacity to polymerize, thus some of the early degradation products can compete with fibrinogen for thrombin and act as inhibitors of clot formation. This may prevent the clot being removed before the tissue is repaired.

Hemodynamic and Rheologic Factors in Thrombus Formation

Platelet – vessel wall interactions can involve transient adhesion and permanent adhesion. Reaction rates for the interactions between platelets and walls are generally very small except on damaged vessels and some artificial surfaces (Richardson et al. 1981). As reported by (Ramos and Nyska 2007, Einav and Bluestein 2004), the rheological properties of blood can augment the thrombotic process by creating a turbulent flow that accelerates the enzymatic reactions between platelets and the vessel wall, and turbulence can slow blood flow creating local hypoxemia.

Abnormal Hemostatic Process in Cancer and Angiogenesis

Haemostatic abnormalities associated with malignancy have been described since the 1877 by the French internist Armand Trousseau (1877), who was the first to note that venous thrombosis events (VTE) frequently preceded the diagnosis of cancer by months or years (Kwaan and Vicuna 2007). Approximately 20% of venous thrombosis events are associated with cancer, and according to some studies, cancer patients have from 6 to 10 fold higher risk for developing VTE (Horton 2005, Heit et al. 2002, Blom et al. 2005). The reasons for an association between abnormalities of coagulation and cancer are multiple as shown in Fig. 3.4 and are attributable to the following factors: (a) activation of the primary and secondary hemostatic systems; (b) activation of the fibrinolytic system, (c) activation of procoagulant activity by cancer therapies; (d) miscellaneous factors (Caine 2002, Nijziel et al. 2006).

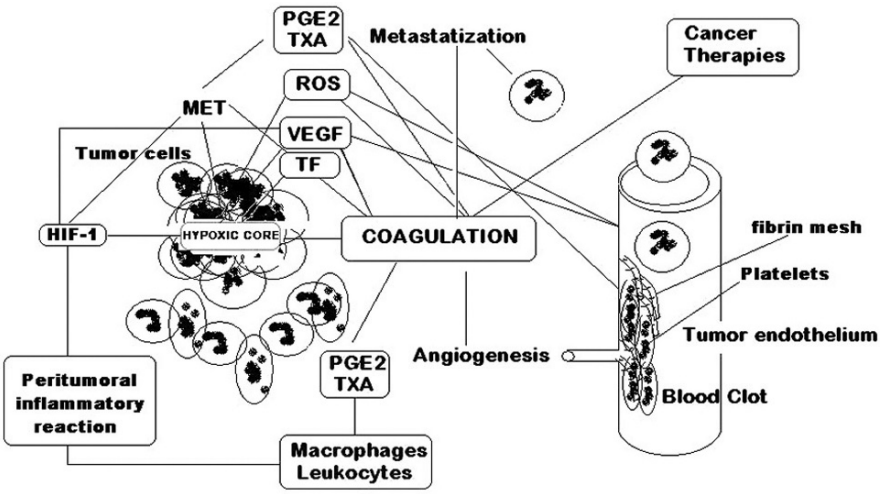

Fig. 3.4 The various factors that contribute to the coagulation abnormalities in cancer are illustrated

Activation of Primary Hemostatic System

Platelets in cancer are activated and release significant levels of VEGF (Gunsilius et al. 2000). VEGF in turn increases the adhesion and the activation of platelets through the release of Tissue Factor (Verheul et al. 2000). The role of VEGF and TF in angiogenesis and metastasization has recently been confirmed by several authors (Gasic 1984, Jurasz et al. 2004). As shown in Fig. 3.5, the hypoxic tumor cells produce several prothrombotic factors such as: VEGF, urokinase plasminogen activator receptor (uPAR) (Denko and Giaccia 2001) tumor-derived VEGF induces the formation of TF and Weibel-Palade bodies by endothelial cells. Weibel-Palade bodies contain P-selectin and VWF and in presence of Calcium initiate the accumulation of platelets on the endothelium surface (Brock et al. 1991, Andre et al. 2000). The result of platelet-endothelium interaction is the formation of thrombin, which provides a provisional mesh of fibrin matrix. This will provide neovascularization via protease-activated receptors, (PAR)-1 and PAR-2 (Nierodzik and Karpatkin 2006, Nagy et al. 1989). Furthermore, as reviewed by Nierodzik, thrombin activates tumor cell adhesion to platelets and endothelial cells, enhancing

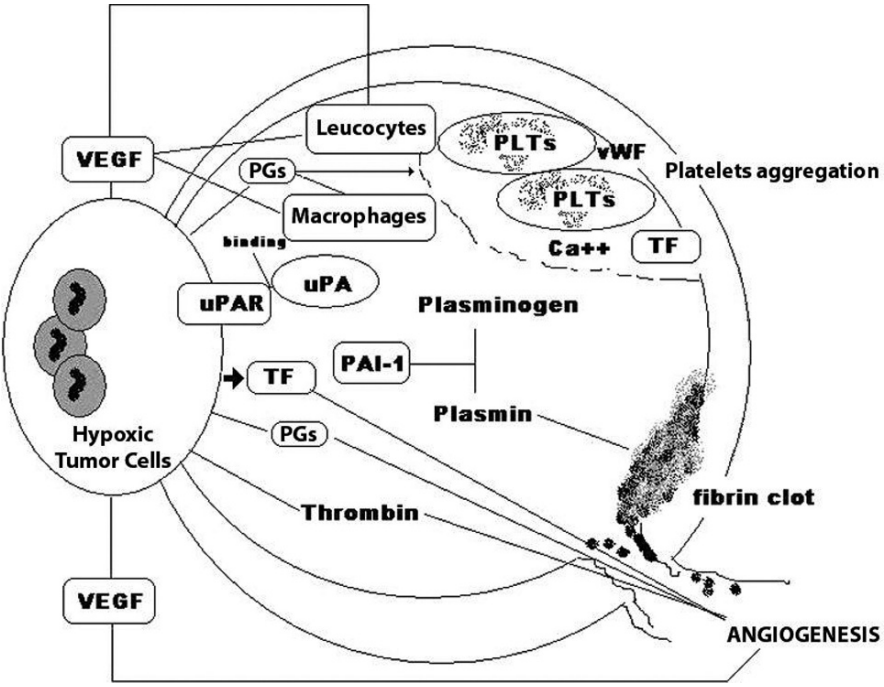

Fig. 3.5 The various factors induced by the hypoxic tumor cells and by the vascular wall that contribute to the angiogenic and thrombotic processes are illustrated. TF: tissue Factor, VEGF: vascular endothelial factor, PG: prostaglandins, uPA: urokinase-type Plasminogen Activator, PAI-1 Plasminogen activator inhibitor-1, uPAR: Urokinase receptor

tumor cell growth, angiogenesis and accelerate tumor cell seeding and metastasization (Nierodzik and Karpatkin 2006, Sierko and Wojtukiwiewicz 2004). Between the 10 to 57% of cancer patients show thrombocytosis. This epiphenomenon is associated to an increased production of thrombopoietin and other cytokines such as IL-6, IL-1 and VEGF (Sierko and Wojtukiwiewicz 2007). The increased number of platelets associated to a stagnant blood flow through the tumor capillary create favorable conditions for thrombus formation, in accordance with the Virchow triad (Chung and Lip 2003/2004). In fact, three particular local flow conditions, described by Virchow (1856) for favoring thrombosis, are concomitantly present in the internal lumen of tumor capillaries and are: blood stasis, hypercoagulable state and endothelial damage (Lowe 2003/2004, Chung and Lip 2003/2004, Jain 1988, Virchow 1856).

Activation of Fibrinolytic System

The fibrinolytic system is involved not only in hemostasis but participates in many other physiologic and pathologic conditions, including development, cell migration, wound healing, angiogenesis, tumor growth and metastasis (Kwaan 1992a, b, Vassalli et al. 1991, Soff et al. 1995; Myohanen and Vaheri 2004).

The various components of the fibrinolytic system are found in most tumors and their expression signifies not only their function but also carries a prognostic value. Their expression is in turn modulated by cytokines and growth factors, many of which are up-regulated in cancer. Though both plasminogen activators, tPA and uPA, are expressed in tumor cells, uPA with its receptor (uPAR) is mostly involved in cellular functions, while tPA with its receptor Annexin II on endothelial surface regulates intravascular fibrin deposition. Among the inhibitors of fibrinolysis, PAI-1 is a major player in the pathogenesis of many vascular diseases as well as in cancer (Liao 2007). Its expression in tumors in experimental animals had been found to inhibit tumor growth and metastasis (Soff et al. 1995), but paradoxically, because it inhibits apoptosis, it may also promote tumor growth (Kwaan et al. 2000). In cancer patients, increased plasma levels of PAI-1 has been associated with increased metastasization and angiogenic process (Czekay and Loskutoff 2004, Noel et al. 2004). Furthermore, patients with elevated serum levels of PAI-1 have a poor prognosis and show an increased risk of thrombosis (Noel et al. 2004). Tumor cells and activated monocytes have an increased expression of urokinase-type plasminogen activator receptor (uPAR) permitting the binding of uPA and the conversion of plasminogen in plasmin. This initiates the cleavage of fibrin mesh with degradation of extracellular matrix allowing tumor cells to invade the surrounding tissues (Nijziel et al. 2006).

Activation of Procoagulant Activity by Cancer Therapies

Changes in the quantitative and qualitative aspects of coagulation have been noted in patients undergoing surgery, radiotherapy, chemotherapy and immunotherapy. The risk of thromboembolism by different mediators of coagulation are illustrated

Table 3.1 Drugs and cancer therapies associated with TVE and mechanism of action

Agent	Patients	Activation of coagulation	Impairment of natural anticoaugulantion	Effects on endothelium
Estrogenic agents	Prostate		+	
Tamoxifen	Breast	+	+	
5-Fu	Breast Colorectal cancer	+		+
Cyclophosphamide	Breast		+	
Doxorubicin	Multiple cancer types	Not known		
Thalidomide	Renal, myeloma		+	+
Cisplatin	Ovarian carcncer			+
Mitomycin-c	Multiple cancer types			+
Bleomycin	Multiple cancer types			+
L-asparagine	Leukemia	+		
Surgery	Multiple cancer types	+		+
Radiotherapy	Multiple cancer types	+		+
Immunotherapy	Melanoma, renal cancer	+		

in Table 3.1. These therapeutic measures, except for surgery, are associated with apoptosis of the tumor cells. In vitro studies of apoptosis had revealed that the cell membrane procoagulant, tissue factor, is activated in this process by phosphatidylserine. This renders apoptotic cells thrombogenic, and explains why thrombotic risk is higher in tumor cells undergoing apoptosis (Wang et al. 2001).

Surgery

Post-operative venous thromboembolism is an important cause of death in hospitalized patients undergoing major elective surgery. A study of autopsy-proven pulmonary embolism in hospital patients showed that venous thromboembolism accounted for 10% of deaths and that recognition of non-fatal thromboembolism continues to be a problem (Kakkar and Lorenzo 1998). Oncological surgery is associated with an increased risk of thrombosis and depends on the type of surgical intervention and on the diagnostic methods (Desauw). The incidence varies from the 16 % in gynecological cancer to 25 % in gastric cancer, and is higher, 50%, in orthopedic tumors (Desauw).

Radiotherapy

Radiation damages endothelial cells (EC) of malignant and normal tissues. In particular, pro-thrombotic and pro-inflammatory alterations to endothelial cells phenotype have been described following radiotherapy. Studies by Smith have shown that von Willebrand factor (VWF) and factor VIII are released according to the radiation dose (Smith et al. 1989).

Chemotherapy

Some chemotherapeutic drugs have been demonstrated to alter or activate coagulation leading to pro-thrombotic state (Letai and Kuter 1999, Zakarija and Kwaan 2007). Combined therapy for breast cancer may determine thrombosis,. Patients receiving the CMF regimen (cyclophosphamide, methotrexate and fluoruracil) have alterations of coagulative and fibrinolytic parameters. Thirty eight patients have been studied with coagulation parameters determined before and after CMF regimen; in all patients a decrease in protein C (PC) has been found along with elevated plasminogen activator inhibitor (PAI-1) (Rella et al. 1996).

Tamoxifen is an estrogen antagonist used in the treatment of breast cancer. However in certain tissues (i.e. hepatic tissue) it show a progestinic effects and enhances hepatic coagulation factor synthesis (Chang et al. 1996, Vitseva et al. 2005). Despite the clear association with enhanced venous thrombosis (Duggan et al. 2003), some studies have failed to demonstrate biochemical signs of activation of coagulation and fibrinolysis (Mannucci et al. 1996). The mechanisms that generate the thrombophilia are unknown. According to Lox the coagulative alterations of tamoxifen are due to an increase in the fibrinolytic factors, whereas Erman believed that *it is due* to a reduction of tissue factor pathway inhibitors (TFPI). Vitseva et al. attributed it to the ROS induced by tamoxifen the cause of the increased platelet aggregation. Notwithstanding these studies, the mechanism of tamoxifen thrombophilia remains elusive (Erman et al. 2004, Lox et al. 1997, Vitseva et al. 2005).

5-Fluoruracil causes direct endothelial damage impairing the antioxidant defense capacity of endothelium and increasing the lipid peroxidation of the endothelium itself. This damage is prevented by probucol a lipid regulating drug (Ramot). L-aspariginase used in the treatment of acute lymphoblastic leukemia increases thrombotic events by inhibiting the production of fibrinogen, plasminogen, protein–C, and protein-S and ATIII (Lee and Levine 1999). The thrombotic complications often manifest themselves as seizures or as strokes and arise in 1%–14% of patients treated.

Thalidomide alone or in combination with anticancer drugs can increase the risk of thrombosis as demonstrated by Desai for renal carcinoma (Desai et al. 2002). Lenalidomide, a less toxic derivate of thalidomide, increases VTE risk too, but, as for thalidomide, the mechanism of action is not known. According to van Heeckeren the disruption of the function and/or integrity of vascular endothelium can be attributed to the increased risk for thrombosis and/or hemorrhage found in patients receiving these antiangiogenic drugs (van Heeckeren et al. 2007).

Cancer patients often receive treatment and nutrition by central venous access catheters. These devices have been documented to be thrombogenic in 38% to 62% patients. Recent surverys, however, revealed that the incidence may be lower (Freytes 2007). Position (left versus right subclavian access), composition (double or triple lumen catheter), line of insertion (one versus two punctures) and type of fluid infused may influence the risk of thrombosis (Letai and Kuter 1999).

Immunotherapy

Several monoclonal antibodies (mABs) have been approved in USA and Europe for treating many different cancers, an example is Bevacizumab (Avastin), a recombinant humanized monoclonal antibody directed against vascular endothelial growth factor (Gerber). Generally, mABs act on molecular targets expressed on cancer cells, sometimes these targets are expressed both in normal and cancer cells and this factor may explain the different grades of toxicity, resulting from the disruption of normal cellular function. In general, targeted molecular therapies have good toxicity profiles, but some patients are exquisitely sensitive to these drugs and can develop particular and severe toxicities (Widakowich et al. 2007). For example, Avastin has shown to improve the survival of patients with colorectal cancer in combination with chemotherapy. Notwithstanding the improvement in survival, the addition of Avastin has been found to increase arterial thromboembolic events (19% treated patients versus 9% control arm), and this complication could not be prevented by aspirin use (Scappatici, Widakowich et al. 2007).

Miscellaneous Factors

Quantitative and qualitative abnormalities of platelets have been demonstrated in cancer patients. Increased or reduced spontaneous aggregation or impaired adhesion are frequently found (Jurasz et al. 2004, Wojtukiewicz et al. 2001). Treated cancer cells undergoing apoptosis can release microparticles. These membrane microparticles are shed by apoptotic cells and by platelets. They are formed by phosphatidylserine activating tissue factor to form a lipid complex with pro-coagulant activity (Morel et al. 2005, 2008, Wang et al. 2001). According to Morel, these microparticles are also responsible for platelets leukocytes interaction (Morel et al. 2005, 2008). This interaction is also mediated by other factors produced by tumor cells such as: platelet derived growth factor (PDGF), Transforming Growth factor-β or by an excessive production of reactive oxygen species (ROS) (Uchiyama et al. 1991, Karihtala and Soini 2007, Wojtukiewicz et al. 2001).

Other factors that increase the pro-coagulative tendency of cancer are fibrinogen and increased plasma viscosity (Jain 1988, Baronzio et al. 2003). Fibrinogen is usually elevated in disseminated malignancy and has profound effect on plasma viscosity (Jain 1988). In fact, von Tempelhoff observed that fibrinogen is a major plasma protein and in cancer capillaries it is associated to a local hemoconcentration due to liquid loss, increasing blood rheology and hence producing stasis and hypoxia (von Tempelhoff et al. 2002). He believed that a high blood viscosity to be a marker of hypoxia.

Hypothesis on the Genesis of Hemostasis and Neoangiogenesis in Cancer

Tumor growth leads to tissue hypoxia; tissue hypoxia, in turn, is a strong stimulus for expression of genes encoding factors that promote tumor growth (von Tempelhoff et al. 2002). Several studies (Yu et al. 2004, Rak, Denko and Giaccia

2001, Boccaccio and Comoglio 2005, Varki 2007) suggest that the link between tumor microenvironment and thrombosis is caused by the concomitant activation of certain oncogenes (K-ras, EGFR, PML-RARα, and MET) followed by the inactivation of tumor suppressors (e.g., P53 or PTEN). This ratio may increase the risk of thrombosis by inducing the expression of tissue factor, a potent pro-coagulant molecule, and plasminogen activator inhibitor-1(PAI-1), a fibrinolysis inhibitor (Yu et al. 2004, Rak).

In 2001 Denko and Giaccia suggested that hypoxia was the trigger for the increased expression of genes that facilitate coagulation and angiogenesis. The genes induced by hypoxia are those encoding for TF, PAI-1, uPA and uPAR, VEGF (Denko and Giaccia 2001). They believed that hypoxia, thrombosis and metastasization share common pathways (see Fig. 3.5).

Another theory for explaining the pro-coagulant activity of tumor is that elaborated by Boccaccio and Comoglio (Boccaccio and Comoglio 2005). They suggested that hypoxia induced, in particular, MET and COX2 regulatory oncogenes (see Fig. 3.4). These oncogenes cooperates with factors present in the tumor microenvironment such as hypoxia and inflammation, for promoting pro-coagulant substances: cytokines (Il-6, IL-8, IL-10), thromboxanes and PAI-1. Hypoxia created by the anomalous tumor perfusion triggers many biochemical and functional events present in the tumor microenvironment, including thrombosis (Denko and Giaccia 2001).

Therapeutic Implications

The presence of TVE is associated with negative prognostic events in breast cancer such as a decrease in life expectancy and an increase in the tumor relapse, (von Tempelhoff et al. 2002). It is not excluded that these results may be applied to other kind of human cancers. The advent of low-molecular-weight heparins (LMWH) has resulted in important changes in the treatment and prophylaxis of venous thromboembolism. Low-molecular-weight heparin preparations reduce the overall incidence of deep vein thrombosis in general surgery by at least 70% (Kakkar and Lorenzo 1998). Unfractionated heparin and LMWH are the anticoagulants of choice when a rapid anticoagulant effect is required (Hirsh et al. 2001). Both are glycosaminoglycans consisting of chains of alternating residues of glucuronic acid or iduronic acid, with molecular weight ranging from 30000 to 3000 D (Weitz 1997). The treatment of thrombotic events in cancer patients is similar to the management in non-cancer patients (Andrea and Ansell 2003). After an initial infusion of 5000 units of unfractionated heparin (UFH) as a bolus, the patients must receive approximately 30,000 units of heparin for 24 h. maintaining the partial thromboplastin time (aPTT) between 1.5–2 times the control value (Loreto et al. 2000, Levine and Lee 1999). The use of unfractionated heparins require the patient hospitalization, whereas the recent introduction of LMWH avoids the hospitalization and permits an administration without the control of aPTT. Both UFH and LMWH act by catalyzing the effect of antithrombin on the inhibition of thrombin and factor Xa; they differ in their bioavailability and binding to plasma proteins, in fact each of the

low-molecular-weight heparins has different cumulative effects, and each product exhibits a distinct profile (Hirsh et al. 2001). UFH and LMWH differ also for their effects on angiogenesis. These differences have been observed in vitro and in vivo and are due to a modulation of adhesion molecules (P-Selectin, L-selectin), and the release of pro-fibrinolytic and anti-thrombotic mediators from the blood vessels (Collen et al. 2000, Stevenson et al. 2005). The inhibitory action on adhesion molecules (P-Selectin, L-selectin) can also modulate the metastasization as documented in many animal models (Stevenson et al. 2005). The administration of LMWH should be followed by oral anticoagulant drug, for preventing recurrence (Loreto et al. 2000). The oral anticoagulant of choice is warfarin. (Andrea and Ansell 2003). For the first case of VTE the duration of treatment is of 3 months, for patients with high risk of thrombosis anticoagulation it must be given for a longer period and as long as the cancer is present (Loreto et al. 2000; Andrea and Ansell 2003). The risk of bleeding in these patients has been calculated to be higher than in noncancer patients (21.6% vs 4.5%) (Andrea and Ansell 2003). As reported by Zacharski, LMWH seem superior to warfarin regarding the secondary prevention of VTE and the risk of bleeding. In the nonsmall cell lung cancer patients, dalteparin, a new class of LMWH, has shown improved response rates and survival (Zacharski et al. 2005). Also, the risk of thrombosis in therapy with thalidomide or lenalidomide has been shown to be reduced by aspirin (Hirsh 2007).

Conclusion

The presence of hypoxic regions within solid tumors is associated with a more malignant phenotype and worse prognosis. To obtain a blood supply and to protect themselves against cellular damage and death, oxygen-deprived cells in tumors alter gene expression, resulting in resistance to therapy. Hypoxia is sensed at the cellular level, leading to the activation of molecular pathways to cope with this stress. The key mediator of hypoxia response is HIF1α, a member of the hypoxia-inducible factor (HIF) family of proteins. This protein is a transcription factor that stimulates the expression of a multitude of genes important for adaptation to hypoxia, including those encoding angiogenesis. Angiogenesis is stimulated by vascular endothelial growth factor (VEGF), a HIF target gene, to increase blood flow toward oxygen-deprived tissues. This regulation of angiogenesis by hypoxia and HIF is crucial during embryonic development, but also for recovery after ischemic injury. Angiogenesis is one of the physiological responses to hypoxia (Simon 2007). Nevertheless, angiogenesis also has deleterious effects by favoring tumor growth. Fluid movement across capillaries in tumor and in normal states is the result of a balance between filtration and reabsorption due to changes in capillary and tissue hydrostatic and oncotic pressures. Any disturbance in this balance favors an increased filtration and/or decreased reabsorption that results in edema or in TIF formation. Cumulation of TIF results not only from a disruption of Starling equilibria, but also to a disturbance of other factors not completely elucidated, such as interstitial matrix

contraction and compositions (Heldin et al. 2004). Thus, the precise contribution of the Starling forces and matrix composition for tumor warrants further study.

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Chapter 4
Cancer, Stem Cells and the Neoplastic Niche

Christopher R. Cogle

Abstract Conventionally, cancer is treated as a homogenous mass of highly proliferative cells, with therapeutics designed to destroy rapidly dividing cells. However, not only are cancers heterogeneous, but also small subsets are endowed with the ability to initiate cancer formation and metastasis. The resistance of cancer initiating cells to current therapies may explain high relapse rates. Our inability to eradicate cancer may be due to misrecognition of the proper cancer target. Designing novel therapeutics for cancer eradication will require understanding specific pathways involved in cancer initiating cell self-renewal, differentiation and homing. Furthermore, targeting the distinctions of the cancer initiating cell microenvironment may be key to effective cancer therapies.

Keywords Stem cells · Cancer stem cells · Cancer initiating cells · Bone marrow · Neoplastic niche · Metastatic niche

Normal Stem Cells and Their Niche

Two functional features define stem cells: self-renewal and multi-lineage differentiation. This functional definition takes into account more than just stem cells, but also complex interactions between stem cells and their environment. The stem cell niche, or surrounding microenvironment, acts as a habitat for stem cells to reside and reproduce. The niche also regulates how stem cells participate in tissue generation, maintenance and repair. By balancing stem cells between states of proliferation and quiescence, the stem cell microenvironment attempts to meet the needs of the organism. Niche factors alter gene expression, which lead to stem cell proliferation, differentiation or quiescence. Certain provocations, such as injury or degeneration, promote niche signaling for stem cell proliferation in an effort to repair tissues. Niche factors include secreted elements like Wnt proteins, cell-cell contact,

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extracellular matrix (ECM) glycoprotein contact, binding to stromal cell adhesion molecules, oxygen tension, pH, and ionic strength (Scadden 2006). Each tissue has its own cellular components in its stem cell niche. For example, in the adult human, the hematopoietic stem cell (HSC) niche is composed of endosteal osteoblasts, sinusoidal endothelial cells, vascular pericytes, multipotent mesenchymal stromal cells (MSC), sympathetic nerves, fibroblasts, adipocytes, ECM and basement membrane (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003; Kiel, Yilmaz et al. 2005; Katayama, Battista et al. 2006). In the brain, vascular endothelial cells and pericytes serve as stem cell niche components (Palmer, Willhoite et al. 2000). The complexity of stem cell and niche interactions is underscored when considering the difficulty in maintaining the undifferentiated state of stem cells in ex vivo culture.

In more ominous settings, the stem cell niche may induce pathology by imposing abnormal function on stem cells. Deregulation of proliferation signaling from the stem cell niche results in cancer formation. For example, stem cell microenvironments secrete Wnt proteins (19 identified to date) to promote the maintenance and proliferation of stem cells (Clevers 2006). After Wnt proteins bind to cell surfaces of stem cells, intracellular ß-catenin is freed to promote nuclear transcription of Wnt target genes (e.g., *c-MYC*, *PPARδ*, *survivin*) (He, Sparks et al. 1998; He, Chan et al. 1999; Zhang, Otevrel et al. 2001). Abnormal Wnt signaling results in many development disorders, degenerative diseases and cancers. For instance, overproduction of Wnt results in mammary tumors.(Tsukamoto, Grosschedl et al. 1988) Underproduction of the Wnt inhibitor, APC, results in colon cancers (Kinzler, Nilbert et al. 1991). And abnormalities in the downstream Wnt signaling molecule, beta-catenin, in combination with several other events, results in leukemias (Jamieson, Ailles et al. 2004; Zhao, Blum et al. 2007). Whereas, intense research has focused on malignant consequences of aberrant downstream Wnt signaling pathways, little is known about Wnt secretion in neoplasia. Identifying the source of Wnt secretion in the stem cell microenvironment and mechanisms of secretion may represent targets for future cancer therapies.

Cancer Initiating Cells and Their Niche

Cancer Initiating Cells

Cancer initiating cells have been defined as “a small subset of cancer cells within a cancer that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor” (Clarke, Dick et al. 2006) (Fig. 4.1).

The concept of cancer initiating cells was first proposed in 1855 when Virchow suggested that cancer arises from embryonic remnants in adults (Virchow 1855). Several investigators subsequently echoed this embryonal rest hypothesis, all generally suggesting that cancer is a problem of developmental biology (Cohnheim 1867;

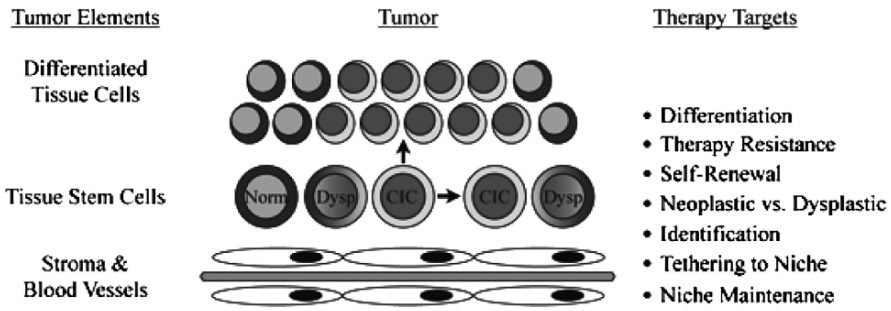

Fig. 4.1 A cancer initiating cell perspective of cancer. The cancer initiating cell niche is made up of stromal elements, including fibroblasts, extracellular matrix and blood vessels. The niche maintains stem cells and cancer initiating cells by secreting factors, providing cell-cell contact and regulating microenvironment pH, oxygen tension and ionic concentrations. Cancer initiating cells, supported by the stem cell niche, remain in an undifferentiated state capable of asymmetric division. Dysplastic cells surrounding neoplastic stem cells harbor genetic abnormalities but do not transform to frank neoplasia until influential promoting signals. Several therapeutic opportunities are identified with this new perspective

Durante 1874; Rotter 1921). Certainly, the existence of teratocarcinoms which contain cells of all three germ layers and afflict young adults along midline migration pathways between gonads to brain, corroborate the embryonal rest theory.

Whereas, the embryonal rest hypothesis may explain teratocarcinomas which arise in children, the hypothesis does not fit well with other acquired cancers which arise in mid- to late-adulthood. Hematologic malignancies, which usually arise in the seventh and eighth decades of life, provide the clearest example of adult cancer initiating cells. The first reports of leukemia initiating cells were made in the 1930s when Furth and Kahn transplanted leukemia from one mouse to another via a single undifferentiated leukemia cell (Furth and Kahn 1937). It wasn't until the 1990s that John Dick's laboratory took cues from normal hematopoiesis and enriched for leukemia initiating cells by selecting for $CD34^{+}CD38^{-}$ acute myelogenous leukemia (AML) cells (Lapidot, Sirard et al. 1994). Using this selection technique, Dick et al. determined that the AML initiating cell exists at a frequency of 1/250,000 $CD34^{+}CD38^{-}$ AML cells. In leukemia initiating experiments, when replacing the SCID mouse strain with a more immunocompromised NOD/scid strain, the xenotransplanted human AML cells were able to repopulate secondary mice, finally demonstrating in vivo self-renewal. In the future, use of more even more immunocompromised mice will help identify new subpopulations of cancer initiating cells. For example, the NOG strain (NOD/scid/IL2R-gamma knockout mice) exhibits severe deficiencies in innate and adaptive immunity (Ito, Hiramatsu et al. 2002). These mice are more tolerant to xenotransplanted human cells, resulting in higher human cell engraftment efficiencies and permitting lower detection limits of cells initiating cancer or organ regeneration (Ikoma, Yamazaki et al. 2005; Fujino, Hiramatsu et al. 2007).

Table 4.1 Cell surface protein expression of subpopulations of cancer cells which can re-initiate cancer

Malignancy	Cell surface proteins	References
Acute myeloid leukemia (AML)	$CD34^+$ $CD38^-$	(Bonnet and Dick 1997)
Acute lymphoblastic leukemia (ALL)	$CD34^+$ $CD38^-$ Philadelphia chromosome$^+$	(Cobaleda, Gutierrez-Cianca et al. 2000)
Breast	$CD44^+$ $CD24^-$ ESA^+	(Al-Hajj, Wicha et al. 2003)
Brain	$CD133^+$	(Singh, Hawkins et al. 2004)
Colon	$CD133^+$	(O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007)
	$CD44^+$ $EpCAM^{high}$ $CD166^+$	(Dalerba, Dylla et al. 2007)
Head and Neck	$CD44^+$	(Prince, Sivanandan et al. 2007)
Multiple myeloma	$CD138^-$	(Matsui, Huff et al. 2004)
Prostate	$CD44^+$	(Collins, Berry et al. 2005; Patrawala, Calhoun et al. 2006)
Melanoma	$CD20^+$	(Fang, Nguyen et al. 2005)
Pancreatic	$CD44^+$ $CD24^+$ ESA^+	(Li, Heidt et al. 2007)
Lung	$CD34^+$ $CD45^+$ $Sca1^+$ $PECAM^-$	(Kim, Jackson et al. 2005)
Osteosarcoma	$CD44^+$ $Stro1^+$ $CD105^+$	(Gibbs, Kukekov et al. 2005)

Subsequent to the seminal leukemia initiating cell discoveries, investigators have followed suit by identifying other cancer initiating cells based on cell surface protein expression (Buzzeo, Scott et al. 2007) (Table 4.1).

In human gliomas, Steindler identified neurosphere-forming cells which exhibit self-renewal and multi-lineage differentiation (Ignatova, Kukekov et al. 2002). Dirks then went on to use $CD133^+$ selection to enrich for glioma initiating cells (Singh, Hawkins et al. 2004). In breast cancer, Al-Hajj and Clarke identified tumorigenic cells from metastatic breast carcinomas by selecting for $CD44^+CD24^{-/low}ESA^-$ cells (Al-Hajj, Wicha et al. 2003). In osteosarcoma, Gibbs et al. identified sarcospheres which express pluripotency genes Oct3/4 and Nanog (Gibbs, Kukekov et al. 2005). In colorectal carcinomas, investigators have found two methods of enriching for colon cancer initiating cells. The laboratories of Dick and De Maria have used $CD133^+$ selection and Clarke has used $CD44^+EpCAM^{high}$ selection to enrich for colon cancer initiating cells (Dalerba, Dylla et al. 2007; O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007). In pancreatic cancer, $CD44^+CD24^+ESA^+$ selection and $CD133^+$ selection have been used to enrich for pancreatic cancer initiating cells (Li, Heidt et al. 2007).

The preference of "cancer initiating cells" versus "a cancer stem cell" in this work is based on the availability of several methods to enrich cancer initiating cells.

The term "cancer stem cell" implies a single entity, when in fact, there is no evidence of a single entity and, quite contrary, several neoplastic sub-populations demonstrate cardinal features of self-renewal, differentiation and invasion.

Semantics aside, one of the cautionary notes in the aftermath of identifying cancer initiating cells is for the cancer community to scrutinize conventional techniques of analyzing tumors. Currently, most researchers and clinical pathologists analyze bulk tumors. However, the preponderance of tumors is comprised of differentiated cells which are different than the sub-elements harboring more malignant and metastatic potential: cancer-initiating cells (Nuciforo and Fraggetta 2004). Better and real-time characterization of cancer initiating cells within whole tumors may be needed to fully appreciate prognosis, treatment response, and relapse potential.

Cancer Initiating Cell Resistance to Conventional Therapies

Identification of cancer initiating cells also begs the question of cancer relapse origin. Are cancer initiating cells responsible for cancer relapse? If so, cancer initiating cells must be less sensitive to conventional therapies than their differentiated progeny.

Given the important role of multiple drug resistance (MDR) transporters in stem cells (family of at least 48 human ATP binding cassette (ABC) transporters discovered to date), this mechanism has been suggested as cause for cancer initiating cell resistance to conventional chemotherapies (Donnenberg and Donnenberg 2005). In younger patients with AML, MDR1 is less frequent, which may explain better responses to therapy (Leith, Kopecky et al. 1999). Administration of MDR inhibitors as adjuvant therapy do bring about improvements in remission rates (Leith, Kopecky et al. 1999; Chauncey, Rankin et al. 2000). However, it is not clear whether the more effective response rates are due to MDR inhibition in cancer cells and increased sensitivity to chemotherapy or pharmacokinetic increases in circulating chemotherapy levels due to altered chemotherapy metabolism.

In chronic myeloid leukemia (CML), the disease clinically manifests in the granulocytic lineage, but the initiating cell is though to arise from a malignant hematopoietic stem cell given that its pathogenetic fusion gene (*BCR-ABL*) can be found in all blood elements except T lymphocytes (Fialkow, Jacobson et al. 1977). Imatinib directly targets the *BCR-ABL* encoded tyrosine kinase activity in CML leading to decreased proliferation of myeloid progenitors. However, despite cytogenetic responses as measured by FISH, molecular eradication of the disease as measure by more sensitive quantitative PCR is difficult and the current standard of care is to keep patients on imatinib indefinitely or until disease relapse or progression. The persistence of molecular disease is generally ascribed to evasion or resistance by quiescent CML initiating cells (Jiang, Zhao et al. 2007). Several strategies are now being developed to target resistant CML initiating cells.

For patients with glioblastomas, radiation therapy is a cornerstone of treatment. However, remissions are transient and glioma relapses are frequent. Given the earlier findings that gliomas are a heterogenous mixture of cells and that $CD133^{+}$ glioma

cells enrich for the glioma stem cell, Bao et al. questioned whether the radioresistance of gliomas is due to a resistant glioma initiating cell (Bao, Wu et al. 2006a). In both cell culture and xenografts, $CD133^+$ glioma initiating cells resisted ionizing radiation as compared to more differentiated parts of the tumor. Glioma initiating cells are thought to use DNA repair mechanisms in response to radiation-induce DNA damage. In additional experiments, inhibiting checkpoint kinases, Chk1 and Chk2, increased sensitivity of radiation in $CD133^+$ gliomas initiating cells. Future adjuvant strategies to radiation therapy may use radiosensitizers focused on gliomas initiating cell resistance.

Targeting the undifferentiated state of glioma initiating cells, Piccirillo et al. demonstrated that bone morphogenetic proteins (BMPs), which normally mature neural precursor cells, also differentiate $CD133^+$ glioma initating cells (Piccirillo, Reynolds et al. 2006). By forcing this maturation, glioma initiating cell tumor formation was impaired. These results pave the way for new strategies of glioma differentiation therapy. In the future, differentiation agents may be applied to gliomas to make them more amenable to surgery, radiotherapy, and/or chemotherapy.

Origin of Cancer Formation

Identification of cancer initiating cells has shed new light on oncogenesis. For example, a stem cell perspective on cancer etiology suggests that stem cells residing in adult tissues give rise to cancer after epigenetic changes and accumulation of genetic mutations. Tissue stem cells are ideal targets for oncogenesis given their longevity, self-renewal and multilineage differentiation. In comparison, short-lived differentiated cells are less likely to accumulate the necessary epigenetic and genetic mutations required for oncogenesis.

To illustrate, in skin cancers, the type of cancer depends on the differentiation stage of the affected skin stem/progenitor cell (Perez-Losada and Balmain 2003). Skin stem cells usually reside in bulges of hair follicles. These stem cells give rise to other skin precursors such as basal cells in the epidermis and transit-amplifying cells. These precursors then give rise to terminally differentiated keratinized cells. Neoplastic transformation of bulge stem cells give rise to trichoepitheliomas, which contain neoplastic elements of hair follicles, basal regions and keratotic regions. Oncogenic activation of more differentiated precursors leads to basal cell carcinomas, squamous cell carcinomas and papillomas. More convincing is the two-step model of skin carcinogenesis: initiation and promotion (Friedewald and Rous 1944). DNA damaging agents or factors applied to the skin (e.g., UV irradiation, chemotherapy) may initiate genetic mutations; however cancer will not arise unless the mutated stem/progenitor cell is promoted to proliferate. Given that complete skin turnover takes about 2 months in humans and that skin cancer often arises several years after initial toxin exposure, it is likely that self-renewing skin stem/progenitor cells are responsible for skin carcinogenesis.

Neoplastic transformation of adult stem cells is further supported by the observation of field cancerization (also known as condemned mucosa) (Slaughter, Southwick

et al. 1953). Often, neoplastic regions are surrounded by dysplastic areas. Toxins (e.g., UV irradiation, local chemotherapy, smoking, alcohol) applied on to epithelial tissues are thought to initiate cancer over wide expanses of exposed epithelia. Dysplastic patches of epithelia surrounding neoplastic regions may contain genetic mutations of TP53 or loss of heterozygosity (Braakhuis, Tabor et al. 2003). If stem cells in the surrounding dysplastic tissue are promoted to proliferate, then a population of high risk cells may undergo neoplastic transformation. The therapeutic consequence of this concept is to not only treat the obvious neoplastic elements, but to also treat the surrounding cancerized field, if possible.

In gliomas, evidence seems to indicate that gliomas arise from a malignant neural stem cell migrating from the sub-ventricular zone rather than an abnormal brain niche dysregulating glial cells into a cancer-initiating phenotype (Sanai, Alvarez-Buylla et al. 2005). In rodents and canines, avian sarcoma virus or systemic exposure to *N*-ethyl-*N*-nitrosourea leads to formation of gliomas in the subventricular zone rather than in non-proliferative regions of the brain (Lantos and Cox 1976). Furthermore, experiments by Vick et al. in dogs favors the stem cell transformation theory by demonstrating that intraventricular injections of avian sarcoma virus led to periventricular gliomas which then spread to distant white matter without any apparent histologic connection (Vick, Lin et al. 1977).

However, not all cancers arise from a tissue stem cell. For some cancers, it is possible that cancer initiating cells may arise from progenitor cells which lack the ability to self-renew. As an example, chronic myeloid leukemia (CML) is thought to arise from hematopoietic stem cells which express the *BCR-ABL* fusion (Sirard, Lapidot et al. 1996). However, recent studies have found that transformation of this chronic leukemia to acute myeloid leukemia does not involve the malignant hematopoietic stem cell (Jaiswal, Traver et al. 2003). Moreover, when Weissman et al. tested acute myeloid leukemias arising from CML, they found mutations enhancing nuclear beta-catenin in granulocyte-macrophage progenitor cells, which subsequently endowed the progenitor cells with high self-renewal, heightened proliferative capacity and resultant blast crisis (Jamieson, Ailles et al. 2004). Furthermore, some cancer cells usurp developmental signaling cascades such as bone morphogenic protein (BMP), Sonic hedgehog (SHH) and Notch (Bailey, Singh et al. 2007). Activation of these pathways endow cells with self-renewal, proliferation, invasion and metastatic potential.

Microenvironmental Effects on Cancer Initiation

Normally, the stem cell niche maintains a balance between stem cell quiescence and proliferation (Li and Neaves 2006). Wnt/beta-catenin growth promoting signals are counterbalanced by BMP anti-growth signals. If the dynamic balance is egregiously altered, then dominant signaling such as Wnt/beta-catenin activation or loss of BMP can lead to cancer formation within hematopoietic, epidermal and gastrointestinal systems (Gat, DasGupta et al. 1998; Haramis, Begthel et al. 2004; He, Zhang et al. 2004; Jamieson, Ailles et al. 2004).

Conversely, the microenvironment can also act as a tumor suppressor. For example, injection of the teratocarcinomas in normal mouse blastocysts leads to chimeric animals without cancer (Brinster 1874; Mintz and Illmensee 1975; Papaioannou, McBurney et al. 1975; Martin 1980). Injection of leukemia into placentas of 10-day-old mice results in normal blood development (Gootwine, Webb et al. 1982). Similarly, the embryonic microenvironment also re-differentiates neuroblastoma and melanoma, thereby suppressing cancer formation (Podesta, Mullins et al. 1984; Gerschenson, Graves et al. 1986).

Proliferation, differentiation and apoptosis occur during embryonic development. Incubating cancer cell lines in extracts from zebrafish embryos during their differentiation phase led to slowed cancer cell proliferation (Cucina, Biava et al. 2006). When cancer cell lines were incubated in extracts taken from zebrafish embryos during their replicative phase, the cancer cells continued to proliferate, uninhibited. Whereas the specifics of the extracts have yet to be defined, the general phenomenon that dysregulated cancer cells can be controlled and induced to differentiate indicates great potential for redifferentiation therapy.

These incidences of cancer suppression, albeit in experimental settings, begs the question of how often cancer initiation and promotion are naturally stymied by non-permissive microenvironments. Defining these incidences and their mechanisms would not result in therapeutic consequences, but staging of cancer would have to change. Currently tumor, node and metastasis (TNM) staging systems do not take into account the microenvironment. If differences in microenvironment are deemed important then this could be added to TNM staging. After all, cancer genotype often does not always equal phenotype when treating patients.

Cancer Initiating Cell Dependence on Microenvironment

Focusing on the normal stem cell niche may give clues to critical mechanisms in the cancer initiating cell microenvironment. Normally, the stem cell niche contains vascular elements, which balance stem cells between quiescence and proliferation, and shelter from apoptotic stimuli. Endothelial cells, in particular, secrete factors that promote stem cell survival, self-renewal and proliferation.

Recently, Calabrese et al. found that glioma initiating cells reside in vascular niches and that disrupting these niches may be key to brain tumor eradication (Calabrese, Poppleton et al. 2007). Presence of endothelial cells were critical to glioma initiating cell-derived tumor formation. Treatment of tumor-bearing mice with inhibitors of VEGF or ERBB2 reduced tumor blood vessels and diminished tumor size. Interestingly, these drugs had little impact on cancer cell survival and proliferation, which suggests that the anti-vascular effects specifically acted on glioma initiating cells. Together, these results highlight the importance of the vascular niche on tumor initiation. For example, brain cells which acquire epigenetic or gene mutations might be more likely to be promoted into neoplastic transformation when juxtaposed to the vascular niche. Recent evidence also suggests a mutual relationship between glioma initiating cells and vascular niche maintenance.

Bao et al. showed that glioma initiating cells secrete high levels of VEGF, which stimulates endothelial cell survival and tube formation (Bao, Wu et al. 2006b). Future therapies targeted at disrupting the glioma initiating cell vascular niche using anti-angiogenic agents and vascular disrupting agents may be critical for brain tumor eradication.

Independence of Cancer Initiating Cells

Cancer initiating cells are a consequence of oncogenesis and are promoted by niche signaling. In turn, cancer initiating cells may also help maintain the cancer niche. For example, in patients with acute myeloid leukemia, circulating endothelial cells (CECs) harbor the same cytogenetic abnormalities as the leukemic blasts (Rigolin, Mauro et al. 2007). FISH analysis of CECs revealed mutations such as the t(15;17) translocation (*PML-RARA* fusion) which was present in the patient's leukemic blasts. The malignant CECs also expressed endothelial surface proteins CD144, CD146, VEGFR2, VWF, lacked CD45 and bound Ulex Uropeus lectin 1 (UEA-1).

Similarly, in patients with multiple myeloma, CECs have been found harboring the myelomatous 13q14 deletion(Rigolin, Fraulini et al. 2006). Subsequent analysis using polymerase chain reaction showed that the CECs also carried the same immunoglobulin rearrangement as the malignant plasma cells.

Analysis of CML patients demonstrated leukemia-derived blood vessels harboring a t(9;22) translocation (*BCR-ABL* fusion) (Gunsilius, Duba et al. 2000). In these studies, the *BCR-ABL* fusion gene was directly observed in the endothelium of myocardial blood vessels, demonstrating that these endothelial cells were functionally capable of undergoing angiogenesis. Similar findings have also been observed in patients with B-cell lymphoma (Streubel, Chott et al. 2004).

Together, the significance of these findings indicate that hematologic malignancies seem to contribute to their own niche and that blood vessels may be a sanctuary site for later relapse. Targeting hematologic malignancy hemangioblast activity may represent a target for future therapies.

Blocking Cancer Initiating Cell Interaction With the Microenvironment

The endosteal niche in bone marrow maintains hematopoietic stem cell self-renewal and survival (Lord, Testa et al. 1975; Taichman 2005). Stem cells express CD44 receptors which tether to stromal adhesion molecules like hyaluronic acid, osteopontin, collegens and matrix metalloproteinases. A number of cancer initiating cells also express CD44 isoforms. Taking cues from normal hematopoietic stem cell biology, investigators have blocked CD44-stroma binding and found impairments in leukemogenesis. The first major finding was that CD44 expression is upregulated in *BCR-ABL* leukemia stem/progenitor cells (Krause, Lazarides et al. 2006). When Krause et al. then mutated the CD44 receptors of *BCR-ABL* leukemia cells,

leukemia formation was impaired. Interestingly, direct injection of the CD44 deficient *BCR-ABL* leukemia into bone marrow (via intrafemoral injectio) did result in leukemogenesis, further suggesting the importance of CD44 in homing and migration to the supportive endosteal niche. Finally, Krause used antibodies to CD44, which impaired *BCR-ABL* leukemogeneis in mice. Additionally, Dick et al. used antibodies to CD44 which also blocked human acute myeloid leukemia engraftment in immunocompromised mice (Jin, Hope et al. 2006). These results indicate a potential for interfering between cancer initiating cell interactions with the stem cell niche.

Stem cells also express migration receptors CXCR-4 and VEGFR-1 (Muller, Homey et al. 2001; Kaplan, Riba et al. 2005; Ratajczak, Zuba-Surma et al. 2006). Future research may seek to identify the degree of CXCR4 expression on putative cancer initiating cells. Moreover, the idea that VEGFR-1^+ hematopoietic progenitor cells prepare the metastatic "soil" for immigrating cancer initiating cells highlights opportunities to interfere with tumor invasion and spread. Already, small molecule inhibitors of the SDF-1/CXCR4 axis inhibits metastases in preclinical experiments and anti-VEGF antibody therapy has shown clinical responses (Muller, Homey et al. 2001; Hurwitz, Fehrenbacher et al. 2004).

Bone Marrow Stem and Progenitor Cell Contribution to Cancer

Cancers require new blood vessel formation for growth, survival and metastasis. The origin of cancer blood vessels may be from angiogenesis, vessel intussusceptions, vascular mimicry, and/or malignancy-derived. Recent studies have also purported that bone marrow contributes to tumor neovascularization via vasculogenesis (Lyden, Hattori et al. 2001; Bolontrade, Zhou et al. 2002; Dwenger, Rosenthal et al. 2004; Peters, Diaz et al. 2005; Duda, Cohen et al. 2006; Hammerling and Ganss 2006; Lee, Bolontrade et al. 2006; Santarelli, Udani et al. 2006; Nolan, Ciarrocchi et al. 2007). Accordingly, bone marrow produces vasculogenic progenitor cells which emigrate to sites of cancer growth, proliferate, liberate proangiogenic factors and differentiate into endothelial cells which support cancer growth and metastasis. The phenomenon is far from settled, as other investigators have found little to no marrow contribution to cancer blood vessels (De Palma, Venneri et al. 2003; Gothert, Gustin et al. 2004; Ziegelhoeffer, Fernandez et al. 2004; Larrivee, Niessen et al. 2005; Shinde Patil, Friedrich et al. 2005; Udani, Santarelli et al. 2005; Zentilin, Tafuro et al. 2006). With controversy as the quantity of bone marrow stem and progenitor cells contributing to cancer neovessels, the clinical and therapeutic significances have been called into questions.

Recently, we found evidence that bone marrow contributes to lung cancer blood vessels and that cytokines which affect hematopoietic cell mobilization also affect marrow-derived tumor vasculogenesis. Specifically, mobilizing mouse marrow with granulocyte colony stimulating factor (G-CSF) plus stem cell factor (SCF) increases

tumor growth rate and amplifies the number of marrow-derived blood vessels. The therapeutic significance is that marrow recovery after chemotherapy or while patients receive mobilizing cytokines like G-CSF may actually support tumor rebound due to marrow-derived vasculogenesis. We also tested the affects of blocking SDF-1 by administering antibodies after lung cancer inoculation in mice. Antibodies to SDF-1 inhibited tumor growth and resulted in decreased numbers of marrow-derived tumor microvessels. The controversy over why some investigators have found marrow contribution to cancer blood vessels, whereas others have not, may rely upon differences in cancers, marrow cells, host animals and detection techniques. Some cancers may liberate more chemokines than others. Nonetheless, in the setting of lung cancer, we found that altering cytokines involved in leukocyte trafficking also affects tumor growth and vasculogenesis. Future anti-cancer strategies may target marrow contribution to tumor vasculogenesis by interfering with marrow homing and migration.

When metastatic cancers switch from micro-metastatic (dormant) to macro-metastatic (active), new blood vessels are required. Recruitment of endothelial progenitor cells (EPCs) are likely responsible for this angiogenic switch. When Gao et al. tracked impaired EPCs (inhibited Id1 transcription factor normally promoting vasculogenesis), tumor formation was also impaired (Gao, Nolan et al. 2008). Impairment of Id1 did not affect lung cancer metastasis to distant organs, but it did decrease the rate of micrometastatic lesions coverting to macrometastatic lesions. Phenotyping ECPs is difficult (Aicher and Heeschen 2007; Case, Mead et al. 2007), thus additional studies are needed to confirm whether marrow-derived EPCs truly flip the switch.

Marrow not only contributes to cancer vasculogenesis, it also seems to contribute directly to cancer. Research indicate that bone marrow-derived cells contribute to cancer arising from the stomach mucosa (Houghton, Stoicov et al. 2004). Transplantation experiments performed in mice with chronic gastritis due to *Helicobacter* infection showed that consequent gastric carcinomas contained marrow-derived dysplastic and neoplastic glands. This study primarily emphasizes the importance of chronic inflammation in recruiting marrow cells, which then, by unknown mechanisms, progress from dysplastic and then neoplastic morphology. The observation of marrow-derived cells within gastric neoplasia led investigators to conclude that bone marrow can be a primary source of epithelial cancer.

These studies prompted us to more rigorously investigate marrow contribution to epithelial cancers in mice and humans (Cogle, Theise et al. 2007). We addressed the clinical relevance by showing human data of marrow cells expressing cytokeratin proteins within tumors. No fusion events were found using karyotype analysis and confocal microscopy. Marrow-derived neoplastic cells were low in number and sporadic, suggestive of developmental mimicry rather than marrow acting as a primary source of neoplasia. To validate our clinical findings, we employed animal models of cancer, which demonstrated bone marrow contribution in gastrointestinal neoplasias and lung cancers of mice. Again, marrow contribution was few in number and sporadic suggestive of developmental mimicry rather than marrow as a seed of cancer. We also questioned which cell in the marrow directly participates in

contribution to cancer. Since the HSC has been observed to adopt the phenotype of several extramedullary tissues (Goodell 2003; Herzog, Chai et al. 2003), we questioned whether the HSC is the source of developmental mimicry in cancer. Using single HSC-transplanted mice bearing lung cancer we showed that the progeny of HSC can incorporate in cancer at low levels without evidence for stable fusion. Follow-up studies have confirmed our findings (Avital, Moreira et al. 2007).

Conclusions

The root of cancers can be found in developmental biology. Discovery of cancer initiating cells has illuminated mechanisms of oncogenesis, treatment resistance, relapse and metastasis. Up to now, therapies have simply focused on hindering the proliferative potential of the whole tumor. With the enlightened position of stem cell biology, the near future of cancer will target cancer initiating cell resistance mechanisms like checkpoint kinases, differentiation cues like BMPs, and multi-drug resistance genes. Understanding that small subsets of cancer are endowed with self-renewal, multi-lineage differentiation and invasive properties, empowers cancer investigators to move beyond brute force cytotoxicity and closer to strategic strikes.

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Part II
Tumor Microenvironment, Therapeutic Perspectives and Strategies for Normalization

Chapter 5
Barriers to Drug Delivery in Cancer: Clinical Implications

Gianfranco Baronzio, Isabel Freitas, Attilio Baronzio, Miriam Baronzio, Elisabetta Crespi and Paolo Antonio Netti

Facts are the air of scientists. Without them you can never fly.
Linus Pauling

Abstract A drug to be effective must satisfy two important requirements. First, it must act on diseased tissue sparing healthy tissue, second it must reach target tissue at adequate concentration to perform its therapeutic effect. As reported by several authors, conventional cancer treatments do not achieve this aim, resulting in suboptimal activity and inability to eradicate tumor tissue. The reasons for this failure are multiple and partially due to the unique physiology of tumor tissue. Tumors develop drug barriers, including high pressure zones and collapsed blood vessels, that make it difficult for blood-borne drugs to reach the tumor's inner core. In this review, we describe tumor physiology resulting in barriers and forces that govern drug distribution inside tumor regions. Furthermore, we present strategies to overcome these impediments.

Keywords Barriers · Drug delivery · Tumor interstitium · Tumor interstitial fluid · Convective forces · Tumor blood flow

Introduction

Every tumor is different and appears microscopically as a nonuniform tissue with regions well oxygenated and metabolically active near regions hypoxic and dormant (See Fig. 5.1). This chaotic 3D architecture is dictated by the need of nutritive substances and oxygen. Hypoxic and undernourished tumor cells trigger signals for obtaining new blood vessels (neoangiogenesis) to satisfy their demands. This neovasculature is however inefficient and does not take pace with tumor growth, so, new hypoxic areas are created. A vicious circle is triggered that leads to metabolically compromised microenvironments. Different metabolic and oxygenation states

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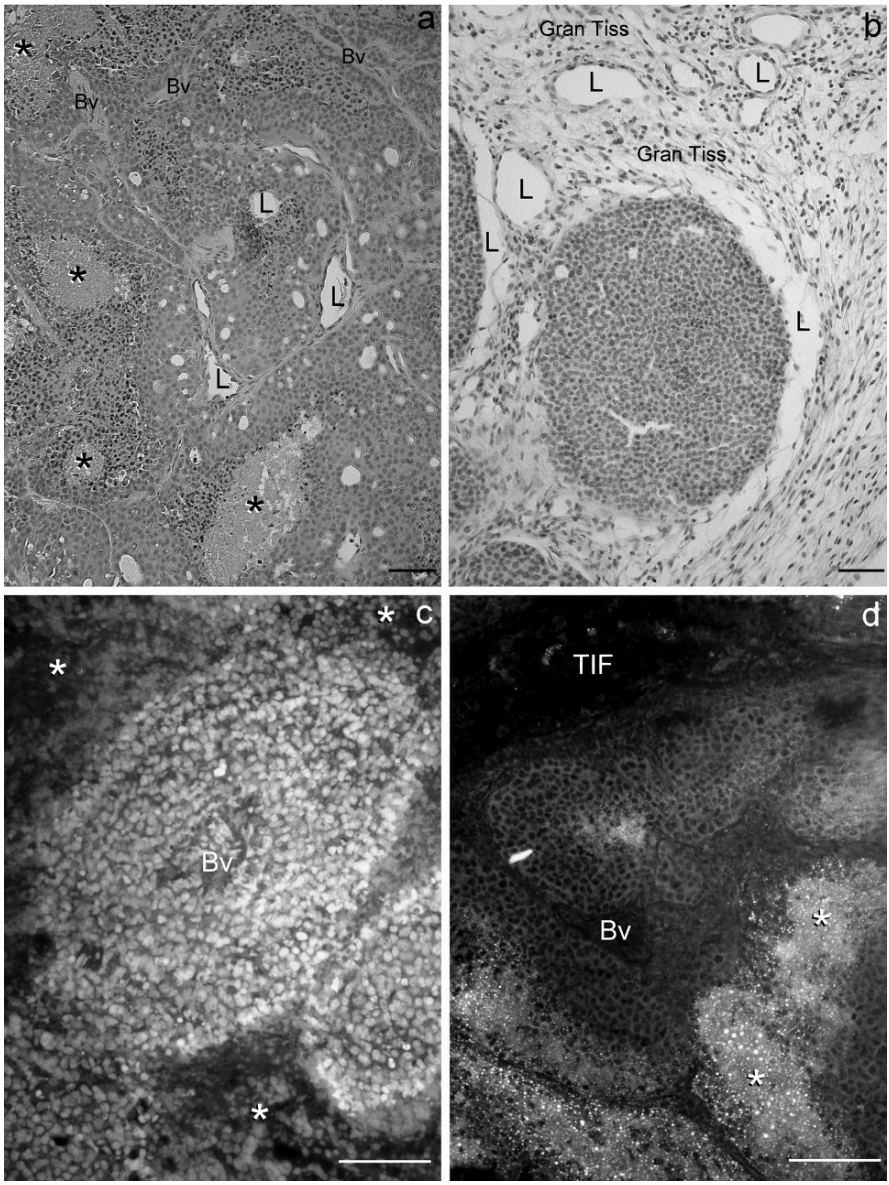

Fig. 5.1 The microenvironment of a carcinoma. (**a**), (**b**) and (**d**) Mammary carcinoma in MMTV-*neu* (*erbB-2*) transgenic female mice; (**c**) Ehrlich carcinoma inoculated in the skeletal muscle of the hind leg of Swiss mice. (**a**) Tumor cuffs supported by central blood vessels (Bv) or lymph vessels (L); necrosis (asterisks) develops in the central regions with hypoxic cells likely located at the necrosis interface. Hematoxylin and Eosin. (**b**) A tumor cord with external lymph vessels (L) surrounded by granulation tissue (Gran Tiss). Hematoxylin. (**c**) Tumor cuffs around blood vessels (Bv) with external necrosis (asterisks). Fluorochromization with Acridine Orange. (**d**) Tumor cuffs with inner necrosis (asterisk). Neutral lipids in hypoxic cells and in necrotic areas (asterisks) fluoresce yellow-gold with Nile Red. An external area of accumulatin of tumor interstitial fluid (TIF)

coexist together. These areas have different metabolic activity and growth kinetics, moreover, during their progression change daily and rearrange continuously their structures. These morphological differences compromise drug effectiveness. Most blood vessels inside tumor are highly disorganized as they take tortuous turns and many of these twisted blood vessels near the center of tumor are crushed due to the irregular growth of tumor in a confined space. Also due to the elevated vascular permeability of tumor vessels along with the absence of a functional lymphatic network, in this confined space interstitial fluid is collected more abundantly than in normal tissue, creating another impediment to drug distribution inside tumor mass. The accumulation of this fluid in fact carries an increase in the interstitial fluid pressure that decreases from the core toward tumor periphery. The altered composition of extracellular fluid, associated with increased interstitial pressure and microcirculatory nonhomogeneity hinders macromolecular drug distribution and therefore the efficacy of chemotherapy.

Tumor Hypoxia, Angiogenesis, Angioarchitecture and Tumor Microenvironment Genesis

Tumor hypoxia is a multifactorial event responsible for the generation of many tumor microenvironment alterations (Baronzio et al. 2003, Höckel and Vaupel 2001). Tumor initially is a rapidly expanding mass (a cluster of thousands of altered cells) originating from a small subset of transformed cells. These cells obtain their nutritive substances and oxygen from host vessels by diffusion, and likely utilize waste products, accumulated in the interstitium for growth signaling. When cancer cells reach a mass of 10^6 cells metabolic strains develop. In fact, cells in the core of this growing cluster are distanced by 100–200 μm from nutritive sources. This interval is a limiting distance for oxygen diffusion (Vaupel 1993) and cancer cells living at pO_2 levels below 2.5–10 mmHg become hypoxic and anoxic. In an attempt to resolve this suffering situation, a non-specific stress response is triggered to overcome the nutritive deprivation through adaptation or escape from the "hostile" environment. (Kunz and Ibrahim 2003, Denko et al. 2003, Höckel and Vaupel 2001). Hypoxia – inducible factor (HIF)-1, is one of these hypoxia regulating mechanisms and is constitutively up-regulated in several tumor types (Zhong et al. 1999). The up-regulation of HIF-1 in several human tumors is associated with an increased over-expression of vascular endothelial growth factor (VEGF), which binds to cognate receptor tyrosine kinases (VEGFR 1 and VEGFR2) located on the surface of vascular endothelial cells. The receptor ligation triggers a cascade of intracellular signaling pathways that initiate angiogenesis (Semenza 2001). Several experimental results demonstrate that hypoxia induced angiogenesis and stroma modifications are tightly associated in a

Fig. 5.1 (continued) carrying isolated tumor cells can be noticed at the periphery. Neutral lipid droplets accumulation (steatosis) is a typical hypoxia-associated process, since free fatty acids that cannot be β-oxidized in mitochondria when oxygen is lacking, react instead with glycerol, derived from glycolysis, giving triglycerides. Scale bar: 50 μm

coordinated process and that the persistence of peritumoural inflammation leads to a pathologic vicious cycle that disrupts normal repair processes. These observations led Dvorak to conclude that tumors are wounds that never heal (Dvorak 1986). The persistence of angiogenic signals build a tumor vasculature with special characteristics. In fact, many tumors reveal chaotic networks of tortuous and distended veins, venules and venous capillaries along with intertwining capillaries branching from arterioles and veins. Irregularities of vascular wall structures in tumors have also been described (McDonald, Baluk 2002, Warren 1979). The resulting intratumoral circulation is characterised by tortuous microvessels lacking the normal hierarchical arrangement of arterioles, capillaries and venules. Within this altered microenvironment, blood flow is sluggish with unstable rheology, anomalous and generally stagnant. As a result these characteristics lead to heterogeneous perfusion with hypoxia and acidity in low-flow regions (Gillies et al. 1999). In more aggressive tumors, areas of increased microvessel density are juxtaposed to areas of necrosis, hypoxia and low metabolic rate, consequently maintaining areas more resistant to chemotherapeutics (see. Fig. 5.2) (Gillies et al. 1999, Sutherland 1988, Vaupel et al. 1989).

Poor perfusion, tortuous and permeable vasculature, and uncontrolled growth are physiologic properties of tumors that limit drug extravasation, deposition, and retention. Unique critical barriers created by the tumors include tumor perfusion, transvascular transport , tumor interstitium composition, tumor interstitial pressure, and cell density (Jain 1994, Minchinton and Tannock 2006).

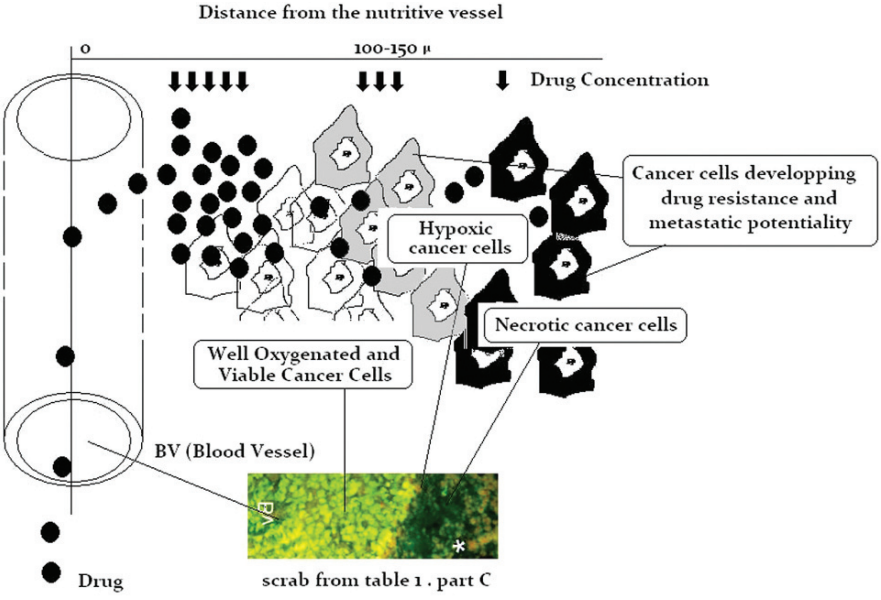

Fig. 5.2 Tumor mass is built by a multilayer of cancer cells overlapping or living distant from nutritive blood vessels (BV). Cancer cells growing around a nutritive central blood vessel (BV) are depicted. The concentration of drugs have a similar behaviour, decreasing penetration within cancers

Tumor Perfusion

The rate at which a drug reaches tumor tissue depends on blood flow to that region. As the cardiovascular system is the means of transport of endogenous and exogenous substances, blood flow fraction destined to each organ determines the relative mass of solute in plasma and the percent of drug that reaches that region. Another aspect that decreases efficacy of drugs is short circulation time in vivo.

Measurements of tumor perfusion are difficult and prone to error. Different techniques have been used clinically including magnetic resonance image (MRI), volume computed tomography (VCT) , positron emission tomography (PET) and ultrasound. The common principle used by each of these devices is quantification of capillary perfusion as a rate constant. With the advent of anti-vascular agents used therapeutically in cancer clinics, both laboratory scientists and clinicians are exploiting new device technologies (Delorme and Krix 2006, Miles 2003, Tozer 2003, Rehman and Jayson 2005). Clinical and experimental evidence demonstrate existence of a compromised and anisotropic blood supply to many tumors, as well as temporal heterogeneity in blood perfusion (Jain 1988). Dewhirst and Jain (Dewhirst et al. 1989, Sevick and Jain 1989) using various techniques have shown that there is an increase in geometric resistance and viscosity in tumor capillaries. Geometric resistance is a measure introduced by Sevick (Sevick and Jain 1989) and is in part due to the tortuosity and disorganization of the vessels. Blood viscosity within tumors is also increased, dependent on hematocrit and related to the low shear rate of blood in the tumor capillaries (Sevick and Jain 1989).

Blood flow decreases generally as tumors grow larger (Jain 1988). This rule is true for human tumors with some exceptions (Vaupel et al. 1989, Vaupel 1992). Blood flow in human cancers can be higher or lower than the tissue of origin and primary tumors tend to be better supplied than metastatic lesions. (Vaupel et al. 1989). Another important characteristic of tumor perfusion is intermittent or cyclic blood flow, with periods of stasis and flow reduction followed by resumption of flow, sometimes in the opposite direction (Jain 1990). Obviously all of these anomalies in perfusion affect drug delivery. The areas not reached by blood flow become deprived of oxygen and nutrients, but also are not reached by any kind of anti-neoplastic therapies (e.g., chemotherapy, immune cells, nanotherapy) (Jain 1990, Brown and Giaccia 1998). Clinical studies using blood flow modifiers have demonstrated that a selective increase in tumor blood flow is associated to an improvement in drug delivery (Jirtle 1988). These methods are described later when clinical strategies able to modify drug delivery are considered.

Transvascular Transport Barrier and Tumor Interstitium

Whenever a blood-borne agent reaches the target tissue, its travel has not finished. In fact, to reach cancer cells in solid tumors, agents must enter tumor blood vessels, cross the vessel wall, and migrate through the interstitium (see. Fig. 5.2) (Jain 1987). Tumor microvessels are in general more permeable to macromolecules than normal vessels. This vascular permeability results from the presence of large pore

structures in the vessel wall (Yuan 1998). The cutoff pore size is heterogeneous and modulated by the tumor microenvironment and by the physicochemical properties of the macromolecules and of the vessel wall structure (Nagy et al. 2008, Yuan 1998). The ultrastructure of the tumor vessel wall is dynamically modulated by the tumor itself, because the transport may facilitate angiogenesis, reduce blood flow and induce interstitial hypertension in tumors (Jain 1990, Fukumura and Jain 2007, Yuan 1998). Transvascular transport is characterized by three parameters: (1) microvascular permeability to macromolecules, (2) hydraulic conductivity, and (3) the reflection coefficient (Truskey et al. 2004, Nagy et al. 2008, Jain 1987). Their relationship is mathematically expressed by the following equations. For normal capillary endothelium with a membrane semi-permeable to water and not to macromolecules, the magnitude of transvascular water movements across the endothelial barrier is defined by the Starling equation (Starling 1986) (see Fig. 5.3)

$$J_v = (L_p s)[(P_c - P_i) - \sigma(\Pi_c - \Pi_i)] \tag{5.1}$$

Where J_v is volume flux of fluid (ml/min); L_p is hydraulic conductivity (cm. min^{-1}. $mmHg^{-1}$); s is capillary surface area (cm^2); P_c and P_i are capillary and interstitial fluid hydrostatic pressures, respectively (mmHg); Π_c and Π_i are capillary and interstitial colloid (oncotic) pressures, respectively (mmHg), and σ is osmotic reflection coefficient of vessel wall ($\sigma = 0$ if membrane is fully permeable to transport molecular species and $\sigma = 1$ if membrane is impermeable).

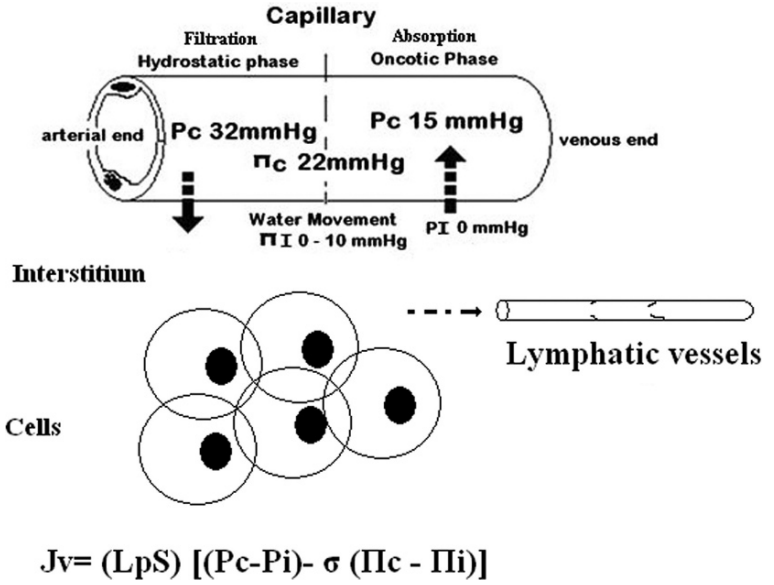

Fig. 5.3 The Starling law with the various forces acting on and across capillary wall are illustrated. Pc: capillary pressure. Pt: interstitial pressure; πc: Capillary osmotic pressure; πi: interstitial osmotic pressure

When the membrane is permeable/porous (i.e. that of tumor capillary) to both solute and solvent, and separates two solutions of different concentrations, there will be oppositely directed flows and interactions of solute and solvent (Schultz 1980, Aukland and Reed 1993). Kedem and Kaltchalsky (Kedem and Katchalsky 1958) have analyzed such systems and using the formalism of irreversible thermodynamics derived the following equation (for a complete review of these equations see Schultz 1980):

$$J_s = J_v(1 - \sigma)C_s + P_s(\Delta C) \tag{5.2}$$

Where J_s is transvascular solute flux (mg/min), P is permeability (cm/s), ΔC is the difference in solute concentration across the endothelial wall, and C_s is mean concentration of solute within the endothelial pores, J_v, S, σ are the same terms used in eq. 5.1.

Netti and Jain (Netti and Jain 2003) used the same equation of Kedem and Kaltchalsky differently expressed to outline the various coefficient that describe the transport of macromolecules across the capillary membrane and the tumor interstitium.

$$J = -D\nabla_C + {}_CR_Fu = -D\nabla_c + {}_CR_F\nabla K_P \tag{5.3}$$

Diffusion convection

Where J is the mass flux, D (cm^2/s) is the diffusion coefficient of the macromolecule, c is the interstitial concentration, R_F is the drag coefficient, u is the interstitial fluid velocity , K (cm^2/ mm Hg.s) is the hydraulic conductivity, and p is the interstitial fluid pressure. R_F the drag coefficient may vary from 0 to values higher than unity.

The transport of macromolecules can occur by diffusion or convection (see eq. 5.3, Fig. 5.4) (Jain 1990, 2005b, Netti et al. 1995). The diffusion coefficient (D), and the hydraulic conductivity (K) provide a measure of the interstitial transport resistances and hence the hindrance encountered by macromolecules moving through tumor interstitium (Jain and Gerlowski 1986). Before discussing parameters of diffusion, hydraulic conductivity and physiochemical properties of macromolecules, it is important to describe tumor interstitium. Initial studies on the extracellular compartments of solid tumors have been conducted by Gullino at the National Cancer Institute from 1960 to 1970, using innovative methods for collecting tumor interstitial fluid and studying tumor blood perfusion (Gullino 1970). Gullino found two important aspects of tumor interstitial fluid (TIF): (1) TIF has a different composition in quantity and quality compared to normal interstitial fluid, and (2) TIF pressure is much higher than normal interstitial fluid (i.e., TIF pressures can reach values $\geq$ 25–30 mmHg (Gullino et al. 1965, Gullino 1966) as compared to normal interstitial fluid pressure which is usually 0 mmHg or less (Guyton and Hall 2006). A simplified way of characterizing tumor composition is by three principal components: cancer cells that account for the 50% of tumor volume (TV), blood

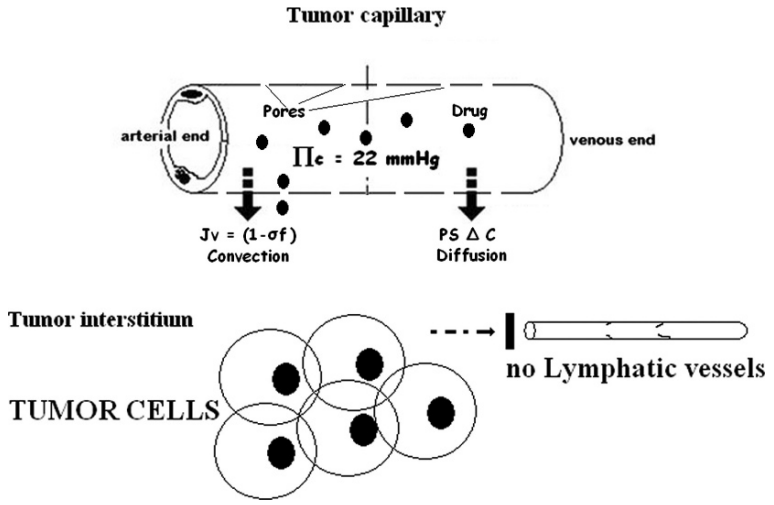

Fig. 5.4 The distribution of a blood borne drug is illustrated as the method of transport by convection and diffusion

vessels that account for 1–10% of TV, and interstitium than occupies the remaining of TV (Kuszyk et al. 2001).

The interstitium, defined as the space located between capillary walls and the cells, occupies more than the 40% of TV, and is composed by an extracellular matrix (ECM) plus a liquid phase. The extracellular matrix of tumor provides nutritional support for cancer cells and stromal cells; it is generally composed by fibrous proteins (e.g. collagen, elastin) and polysaccharides (e.g. hyaluran and proteoglycan) and their quantity and quality is formed by fibroblasts upregulated by cancer cells (Egeblad et al. 2005, Vernon and Sage 1995). The presence of collagen and glycosaminoglycans (GAG) seem to play an important role in drug distribution and tumor progression (Gullino and Grantham 1962, Swabb et al. 1974, Ziche et al. 1989). Thus, tumor ECM within the interstitium plays an influential role on agent access to cancer cells.

The vascular space of tumors gives us an idea of the distance that a drug must cross before reaching cancer cells. Gullino estimated interstitial space volume and found it increased compared to the normal interstitial space. In hepatomas, the interstitial space is twice that of normal liver (Gullino and Grantham 1964). Reasons that determine the accumulation of liquid inside the tumor mass are several: a) capillary morphology and physiology abnormalities; b) hydrostatic and osmotic pressure differences; and c) no lymphatic drainage.

Capillary Morphology

Quantitative and qualitative blood vessel analyses in different tumors demonstrate that tumor blood vessels differ from normal counterparts in shape and morphology

(Hori, Konerding 1995, 1999, Tsunenari et al. 2002, Vaupel 1997, Vaupel et al. 2000, Warren 1979). In fact, endothelial cells lining tumour vessels do not form normal monolayers but are irregularly shaped and disorganized, with some overlapping one another (McDonald and Baluk 2002). These cells are loosely connected and leave focal intercellular openings. The sizes of the openings are generally less than 2 μm in diameter (McDonald and Baluk 2002). Pericytes, which are juxtaposed to endothelial cells, normal serve important roles in the regulating vascular formation, stabilization, remodelling and function (Armulik et al. 2005). However, in tumor tissues, pericytes also support neoplastic growth and metastasis (Morikawa et al. 2002). Within tumors, pericytes deposit increased quantity of extracellular membrane (ECM) components, reside in loose association with endothelial cells and extend their cytoplasmatic processes deep in tumour tissues. These characteristics support consequent cancer angiogenesis and metastasis (Morikawa et al. 2002, Xian et al. 2006).

The final result of these morphological abnormalities is a tumor endothelium more permeable and with an increased pore cutoff compared to the normal endothelium (Nagy et al. 2008).

Hydrostatic and Osmotic Pressure Differences

The physiological consequence of increased permeability belies the Starling law. We can interpret the Starling law as the result of two opposing forces on the opposite sides of the endothelial wall, specifically the hydrostatic pressure difference (P_c–P_i) acting to filter water out of the endothelium and the oncotic pressure difference (Π_c–Π_i) acting to reabsorbs water (see Fig. 5.5).

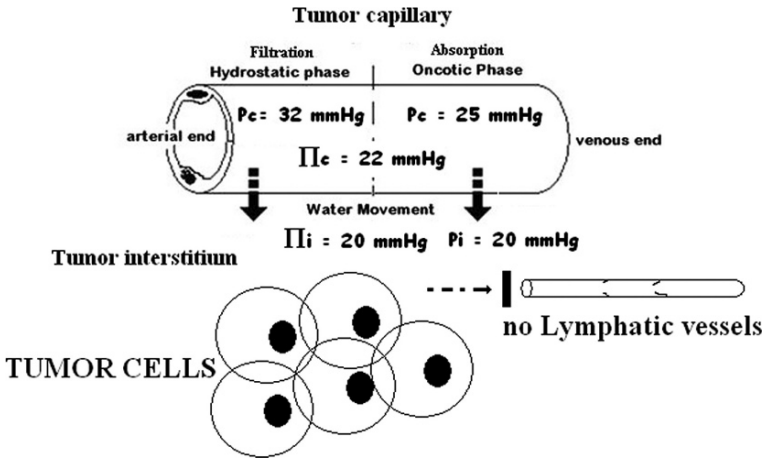

Fig. 5.5 The non-obedience of Starling Law in tumor is outlined as the various forces acting across the tumor vascular wall. Pc: capillary pressure. Pi: interstitial pressure; πc: Capillary osmotic pressure; πi: interstitial osmotic pressure

The capillary pressure (P_C) is the principal driving force in regulating fluid filtration, it is generally between 28–35 mm Hg with an axial decreases of 15–20 mm Hg. This drop is called arterial venous pressure difference (a–v Δp). This difference in tumor capillaries is less than in normal capillaries, and P_C at the arterial end results near equal to that at the venous end with a median drop of 8–10 mmHg (see Fig. 5.5) (Heldin et al. 2004, Jain 1987). This difference permits a gradient at the venous end for the intravascular oncotic pressure (Π_I) compared to P_I. The reasons for this behavior are not completely understood and can be ascribed to greater number of arteriovenous anastomoses, to an increased tortuosity of tumor neovasculature and to a stagnant blood flow (Jain 1988). Furthermore, tumor vessel leakiness allows the loss of a greater quantity of macromolecules toward the interstitium, compared to normal physiologic situation (McDonald and Foss, Jain 1987). Albumin, which generates 70% of oncotic pressure is lost in greater quantity and collected in the interstitium. This loss of albumin in tumor microcirculation results in an increased oncotic pressure (Πi)in the interstitium (Stohrer et al. 2000a, Butler et al. 1975, Gullino 1966). As a consequence, an even greater gradient develops at the venous end in favor for Π_I compared to P_I, with a decrease in water reabsorption at the venous end (see. Fig. 5.5).

The Lymphatic Drainage

Tumors also lack functioning lymphatic systems and consequently water reabsorption mechanisms are altered (Freitas et al. 1997, Gullino 1966, Jain and Fenton 2002). Under normal conditions, interstitial pressure ranges between 0 to -6 mmHg and the excess of fluid is removed by lymphatics (Guyton and Hall 2006). The excessive accumulation of liquid in tumors, creates a progressive increase of tumor interstitial fluid pressure (TIFP) from the periphery to the center of the tumor (Freitas et al. 1997, Gullino 1966, Jain 1987, 1988). TIFP increase has been associated to poor prognosis, and to decreased response to therapy in cervical cancer (Milosevic et al. 1998, 2001). Animal studies have demonstrated that increased TIFP correlates with decreased drug uptake (e.g., brain tumors) (Navalitloha et al. 2006). On the other hand, lowering TIFP reduces mechanical stretch, and consequently decreases tumor proliferation (Hofmann et al. 2006) increasing chemotherapy efficacy (Heldin et al. 2004, Salnikov et al. 2003).

From a mechanical point of view, tumor ECM is a hydrophilic gel structure (Jain 2005b). It forms a complex 3D network, and the content of collagen and GAGs limit space available for plasma proteins and other macromolecules simply because two materials cannot occupy the same space at the same time (volume exclusion) (Swartz and Fleury 2007). Collagens impart structural integrity to tumor tissue, whereas GAGs act as the major excluding agent of the tumor interstitium, primarily responsible for poor delivery of monoclonal antibodies and of LAK and TILS cells (Jain and Gerlowski 1986, Jain 1990).

Several parameters influence interstitial transport of drugs into cancer tissues. The first parameter to analyze is transvascular transport. The rate of tumor transvascular transport is characterized by the microvascular permeability coefficient

(Truskey et al. 2004). Permeability is a measure of the property of capillary endothelium that allows for the selective exchange of substances or whole cells (e.g. lymphocytes) between the blood and surrounding tissues. Small lipid-soluble molecules such as carbon dioxide and oxygen move freely by diffusion. Water-soluble molecules cannot passively pass through endothelial walls and are dependent on membrane pores. These pores are channels that selectively restrict passage of macromolecules depending on their size, shape, and electrical charge (Renkin 1985, Tuma).

According to pore theory normal endothelium has a cutoff pore size of 20 nm, whereas in tumors pore sizes are heterogeneous and can be up to 2 microns in diameter (Truskey et al. 2004, Yuan 1998). A study by Roberts and Palade clearly demonstrated that VEGF transforms a continuous endothelium in a fenestrated one (Roberts and Palade 1995). Given the increased expression of HIF-1 and VEGF in hypoxic tumor tissues, this could explain the increased venular and capillary permeability in tumor endothelium. However, what is not clear is the persistence of these fenestrations over time and how VEGF can mediate both angiogenesis and permeability (Roberts and Palade 1997). Tumor permeability and pore cutoff change with tumor microenvironment as demonstrated by Jain and his coworkers who analyzed transport pathways by implanting human tumors subcutaneously or intracranially (Hobbs et al. 1998). The relationship between molecular weight (MW) and vascular permeability has been studied and found to have a negative inverse relationship between molecular weight increase and permeability, but a positive relationship between MW and plasma half- life concentration (Dreher et al. 2006). The increase in plasma half-life is purported to provide a greater time of extravasation with increased tumor tissue penetration. As an example, MW between 40–70 KDa accumulate more deeply into tumor tissues as compared to macromolecules with MW between 3.3–10 KDa.

Two other parameters describing tumor resistance to drug penetration include tumor interstitial pressure (TIFP) and tumor interstitium composition. Boucher was the first to show that TIFP increase is dependent on microvascular pressure (MVP) and is not the result of increased tumor permeability or lymphatic absence (Boucher and Jain 1992). TIFP is an important obstacle to tumor treatment because it blocks uptake of therapeutic agents (Jain 1994, Heldin et al. 2004). In the center of a tumor, pressure is high, but not homogeneously so. The outward efflux of liquid from the tumor center versus the tumor periphery block the convection of therapeutic agents such as monoclonal antibodies (Jain 1988, Boucher et al. 1990). It has been calculated by Jain (Jain 1987) and Butler (Butler et al. 1975) that velocity of efflux of interstitial fluid is on the order of 0.1– 2 μm /sec, and for adequate tumor infiltration macromolecules at the tumor periphery must overcome this outward flux. Recently, Netti (Netti et al. 1995) has tried to understand the mechanisms regulating TIFP, to develop strategies for overcoming this barrier. From these experiments emerge that a correlation exist between tumor blood flow (TBF) and TIFP, in fact TIFP varied with a delay of the order of 10 s varying TBF. Netti has also found, that the change of blood pressure, chronically or acutely, was not followed by an appreciable increase of macromolecular uptake; whereas, uptake was higher with a periodic

modulation of blood pressure. From these experiments emerged the concept that use of vasoactive agents in a periodic administration may augment agent uptake into tumor tissue.

Netti et al. (Netti et al. 2000) also studied the effect of tumor ECM to drug penetration. Normally, GAGs regulate distribution of solute and water. However, in solid tumors, presence of GAGs alone cannot explain the resistance of ECM to macromolecular transport (Swabb et al. 1974). Netti et al. measured in situ the IgG movement in four tumors and found greater resistance to the penetration of immunoglobulins in tumors with higher collagen contents. They concluded that collagen influences tissue resistance to IgG penetration and that collagenase significantly increased the IgG interstitial diffusion rate.

As accounted for in eq. 5.4, diffusion and hydraulic conductivity also help to better explain movement of macromolecules in the tumor interstitium. Diffusion, D (cm^2/s) of a macromolecule has been quantified with invasive and non-invasive methods. However, the most appropriate technique of measurement of D seems to be the measure of diffusion coefficient of fluorescently labeled molecules, where D is calculated by the relaxation of fluorescent gradient or by adapting the method of fluorescence recovery after photobleaching measured in small region of tissue ($\leq 40\,\mu m$) (Gribbon and Hardingham 1998, Netti and Jain 2003). The diffusion coefficient depends upon molecular weight of the diffusing substance and can be described by a power law

$$D = a(MW)^{-b} \tag{5.4}$$

Where MW is the molecular weight and a and b are two experimental constants. The coefficient b depends on the sizes and shapes of macromolecules (Netti and Jain 2003, Dill and Bromberg 2003, Pluen et al. 1999).

For better realizing, the correlation between D and molecular weight we will do a brief diversion. The relation between viscosity and the diffusion coefficient is given by the Einstein-Stokes equation

$$D = \frac{kT}{f} \tag{5.5a}$$

where k is the Boltzmann constant, T is the absolute temperature, f is the frictional coefficient (Einstein 1956, Berg 1992). In the simplest case, when the molecule is roughly spherical in shape eq. 5.5 becomes

$$D = KT/6\pi\eta r \tag{5.5b}$$

(Einstein 1956). Qualitatively, the Einstein-Stokes equation shows that the viscosity and diffusion coefficient are inversely related, however as outlined, by Chang, eq. 5.5b is an idealized expression because most solute molecules

are solvated in solution and the measured radius tends to be greater than the true radius (Chang 2000). The Stokes law also applies to nonspherical molecules of the same volume and eq. 5.5 can be written as

$$D = KT/f/f_0 \tag{5.5c}$$

Where f_0 stays for friction coefficient for a spherical molecule and the f/f_0 is a ratio that gives the divergence from the ideal spherical solution (Van Holde et al. 2006, Dill and Bromberg 2003). In the ideal situation, a sphere has a weight proportional to its volume $m \approx r^3$, and this occurs with particularly compact molecules such as proteins, for more flexible molecules the mass becomes proportional to the square of the radius $m \approx r^2$.

Studies of agent diffusion in different tumors have demonstrated variances depending on the tumor type. For example, the diffusion coefficient according to agent molecular weight is greater in glioblastoma and sarcoma than in carcinoma and in normal tissue (Netti et al. 1995, Pluen et al. 1999). Associated with molecular weight, steric hindrance to the movement of macromolecules, the electrical charge of the macromolecules itself and of the interstitium have a roles in agent distribution (Wiig et al. 2005, Pluen et al. 1999, Taylor and Parker 2003).

The hydraulic conductivity (K) is a another property that describes the ease with which water moves through pores (Darcy law). K depends upon temperature and tissue structure (Truskey et al. 2004). The temperature dependence is determined mainly by the viscosity μ, the tissue dependence is related to the tissue concentration of GAGs (g/100 tissue) and has been experimentally expressed by the following formula

$$K = 4.6 \times 10^{-13}[GAG]^{-1.202}(\mu_{37}/\mu) \tag{5.6}$$

Where μ_{37} is the viscosity of the interstitial fluid at 37°C and the unit of k is Cm^4 dyne $^{-1}$ sec^{-1} (Swabb et al. 1974, Truskey et al. 2004). K is very sensitive to change in tissue composition, in fact K gives us geometric information about the medium (Baxter and Jain 1989, Truskey et al. 2004, Raghavan et al. 2006). However, not all authors agree that tumor K is GAG dependent. According to Netti, tumor ECM is similar to other soft tissue (i.e., cartilage) with similar composition and water content. This investigator purports that hydraulic conductivity in tumor is not dependent on GAG concentration but on fibrillar connective tissue content and tissue hydration (Netti et al. 2000, Netti and Jain 2003). In this case, the hydraulic conductivity is described by an exponential law

$$K = K_0 e^{\beta E} \tag{5.7}$$

where β is an experimental parameterand K_0 is the hydraulic conductivity at zero tissue deformation (Netti and Jain 2003). K is a measure of interstitial fluid velocity that in tumor is contrasted by a retardation factor. This retardation factor is not only the opposite current mediated by interstitial fluid pressure but also a phenomenological parameter dependent on tissue hydration, and on solute properties. The K value of colon (Boucher et al. 1998), mammary (Netti et al. 2000), sarcoma (Zhang et al. 2000) and glioma (Smith and Humphrey 2007) are higher compared to normal tissue.

Tissue Penetration (Tumor Cell Density)

The efficacy of many available anti-neoplastic agents is hindered not only by low blood perfusion and TIF composition, but also by TIFP increase which blocks tumor tissue penetration. For cytotoxic chemotherapeutic agents to be effective, the agents must reach most of the cancer cells (Minchinton and Tannock 2006, Tannock et al. 2002). Failure to deliver optimal quantities of antineoplastic agents and achieve uniform drug exposure results in recurrence, metastasis, and development of therapeutic resistance (Davis and Tannock 2000, Minchinton and Tannock 2006, Tannock et al. 2002, Trédan et al. 2007). Successful cancer treatments require that all malignant cells must be killed. According to Michinton there are at least three reasons why cells distant from nutritive vessels become resistant to chemotherapy: (1) non-proliferating cells present inside the tumor core are less responsive to anticancer drugs, (2) some drugs are less active at acidic pH, and (3) tumor cells distant to nutritive vessels are exposed to low drug concentrations (Minchinton and Tannock 2006). The Tannock group has built several methods to demonstrate in vivo and in vitro drug penetration (Tannock 2001, Tannock et al. 2005, Kyle et al. 2004). From these experiments appear that both paclitaxel and taxanes exhibit limited tumor penetration. In fact, these drugs did not penetrate further than 100–200 μm into the tumor tissue (Kuh et al. 1999, Tunggal et al. 1999). In, conclusion tumor cell density poses a formidable barrier to effective treatment of solid tumors (Tannock et al. 2002).

Putting it All Together

Delivery of conventional chemotherapeutic agents inside tumor tissues remain a significant challenge. Furthermore, notwithstanding high values of D and K for macromolecules, these agents do not distribute uniformly in tumor tissues. For chemotherapeutic agents to exert their cytotoxic effects they must enter tumor blood vessels, cross the vessel wall, and migrate through the interstitium. Heterogeneous tumor perfusion, vascular permeability, increased cell density, and increased interstitial pressure are critical barriers that limit penetration of drugs into tumor tissues. Moreover, TIFP blocks convection currents within tumors which reduce drug inflow. TIFP is such a challenge that it affects penetration of chemotherapy (Salnikov

et al. 2003), effectiveness of radioimmunotherapy (Jain et al. 2007a), inflow of monoclonal antibodies (Jain 1988, Thurber et al. 2008), infiltration of immune competent cells (e.g., TIL, LAK) (Jain 1990), and perfusion of nanopharmaceuticals (Campbell 2006).

Clinical Methods to Overcome Impediments to Drug Delivery

Heterogeneous tumor perfusion, vascular permeability, cell density, and increased interstitial pressure represent critical barriers that limit penetration of anti-neoplastic agents. Strategies aimed to improve drug delivery and penetration in tumors are of outstanding clinical importance. The amelioration or the attempt to normalize tumor vasculature (Fukumura and Jain 2007, Jain 2005a) can be obtained or using antineoplastic agents in right sequence or using adjuvant agents to augment chemotherapeutic penetration.

The Concept of Vascular Normalization

Recently, Jain purported that drugs which induce vascular normalization alleviate hypoxia and increase efficacy of conventional therapies (Jain 2005b). In a recent study, vascular normalization after anti-angiogenic therapy brought about improved cancer cell kill with cytotoxic chemotherapy. A mathematical model has also been constructed to validate the effects of normalization not only on TIFP behavior but also on interstitial fluid velocity (IFV). The model demonstrates that decreased interstitial pressure is associated to an increase flux convection toward tumor core with a decrease of convective loss of growth factors (e.g., VEGF) into the surrounding tissue. This has dual effects of increased drug penetration and decreased angiogenesis and lymphangiogenesis (Jain et al. 2007b). Nakahara et al. studied the effects of a VEGF inhibitor (AG-01376) on the distribution of antibodies and found that antibody transport increases with a reduction of tumor vascularity by 86% (Nakahara et al. 2006). Another study of Winkler et al. demonstrated that VEGF inhibition enhances response to radiation through increasing oxygenation (Winkler et al. 2004).

Attempts to Modify Tumor Perfusion

Clearly an increase of flow into tumor tissues would augment delivery of chemotherapeutic agents to desired sites of tumor growth (Jirtle 1988). In animal models of gliomas and pancreatic cancer, administration of angiotensin-II, a potent vasoconstrictor in normal tissue, before delivering chemotherapy resulted in greater anti-neoplastic effects. (Ishikawa et al. 2007, Tokuda et al. 1990). Angiotensin II is an octapeptide (MW 1046 D) product of renin-angiotensin system and is one of the strongest physiological vasoconstrictors (Oparil and Haber 1974). Angiotensin II vasoconstrictive activity is 20–30 times more potent than that of norepinephrine and

exert its activity binding on specific receptors present in vascular smooth muscles (Jirtle 1988). The principles behind delivering angiotensin-II before chemotherapy rests upon three important observations: (1) tumor blood vessels often do not contain smooth muscle cells, as opposed to normal tissues which do contain smooth muscle cells, (2) angiotensin-II preferentially constricts blood vessels in normal tissue, and (3) chemotherapeutic agents will be shunted from normal tissues to malignant tissues. (Jirtle 1988, Suzuki et al. 1984). Clinical follow-up of vasoconstrictor-chemotherapeutic delivery have been reported by Hoshi and Ohigashi for gastrointestinal cancers (Hoshi and Sato 1995, Ohigashi et al. 2003). Although pre-clinical experiments and early clinical trial results are encouraging, clinical feasibility of administering angiotensin II will require much more testing. The reason is that the steal phenomenon is due to the vasculature disposition of normal tissue and tumor tissue. The vasculature of normal tissue and of tumor tissue can be in series or in parallel. In the first case of tumor vasculature in series with normal vasculature the use of a vasoconstrictor decreases both normal and tumor blood flow I in the second case when the two circuits are in parallel, the use of a vasoconstrictor decreases normal blood flow, which increases tumor blood flow. In the case of using vasodilatators, such as hydralazine, we get the opposite effect (Vaupel 1993). With the advent of MRI it will be possible to determine non – invasively the structure of tumor and normal microcirculation and to know in advance which kind of therapy to use (Neeman and Dafni 2003).

Attempts to Decrease TIF and TIFP

(a) Nicotinamide

Nicotinamide is the amide form of Vit B_3 and has been investigated for its capacity to radiosensitize murine tumors (Horsman et al. 1987). In the late 1990s, Lee discovered a decrease in TIFP following administration of Vit B_3 to CH3 mice bearing FSaII tumors (Lee et al. 1992). The mechanism is linked according to Hirst to a reduction of vascular resistance by up to 30%. These direct effects on vascular resistance together with the reduction of interstitial fluid pressure could be combined to improve the homogeneity of tumor perfusion (Hirst et al. 1995). Peters studied the time course of this effect and found that nicotinamide at a dose of 500–1000 mg kg^{-1} reduced the TIFP within 20 min from the administration. The effect was abolished 80 mon after the administration (Peters et al. 1997).

(b) Hyperthermia

Hyperthermia uses heat for treating tumors. It is not a new therapy and many biological effects have been recently elucidated (Habash et al. 2006). Among these biological effects, that on TIFP has been studied by Leunig et al. in1992. These

investigators discovered that hyperthermia treatment at 43°C for 30–60 min was able to decrease interstitial fluid significantly (Leunig et al. 1992).

(c) Chemotherapy

Chemotherapy is the use of different chemotherapeutics alone or in sequence with the aim on reducing TIFP. Experimental studies on murine mammary carcinoma (MCA-IV) and human soft tissue sarcoma (HSTS-26T) treated with taxanes (paclitaxel; Docetaxel) showed a reduction on microvascular pressure and TIFP. The same effect was not obtained on glioblastoma U87. A change on neoplastic density too was observed (Griffon-Etienne et al. 1999). A confirmation to these experimental studies come from the clinical work conducted on breast carcinoma by Taghian et al. They randomized 54 patients into two groups: a neoadjuvant sequential chemotherapy group, consisting of doxorubicin and paclitaxel. In the group one paclitaxel followed doxorubicin, in the group 2 paclitaxel preceded doxorubicin administration. The group 2 treated with paclitaxel before doxorubicin manifested a decrease in TIFP of 36% and an improvement in tumor pO2 by almost 100%. In, contrast doxorubicin had no significant effect on either parameters (Taghian et al. 2005).

(d) VEGF Inhibition

Studies by Nakahara and Winkler, previous reported are two examples on the use of VEGF inhibition for decreasing TIFP (Nakahara et al. 2006, Winkler et al. 2004). As reported by Minchinton anti VEGF therapy acts by pruning immature vessels, by improving perivascular cell coverage and structure of basement membrane. These mechanisms lead to the normalization of tumor vessels. The effect is temporary, so a period of application in conjunction with other therapies is necessary for exploiting it (Minchinton and Tannock 2006).

(e) PDGF

Imanitib a selective-low molecular weight inhibitor of Platelet derived growth factor (PDGF) has been demonstrated to lower TIFP in KAT-4 thyroid carcinoma grown in immunocompromized mice and in PROb colon carcinoma grown in syngenic rats (Pietras et al. 2001). As for VEGF inhibition PDGF was followed by an increased uptake of anticancer drugs, peculiarly of taxol and 5-FU (Pietras et al. 2002).

(f) Hyaluronidase

Hyaluran is a component of ECM and forms large network with GAGs and can retain extracellular water (Heldin et al. 2004). Injection of Hyaluronidase in human

osteosarcoma xenografts induced a 20–40% decrease of TIFP after one hour from injection. TIFP returned to the normal level after 48 hs (Brekken et al. 2000).

(d) Dexamethasone

Kristjansen evaluated the effect of dexamethasone on interstitial hypertension in a human colonic adenocarcinoma. (Kristjansen et al. 1993). The authors studied the TIFP value on 68 tumors and found that the effect of dexamethasone was dose dependent and lasted for 4 days followed by a slightly increase in systemic blood pressure.

(e) PGE_1

Rubin et al. used prostaglandin E_1 methyl ester (PGE_1), into the s.c. tissue surrounding transplanted rat colonic (PROb) carcinomas or chemically-induced rat mammary carcinomas, and lowered TIFP by 30%. Transcapillary transport of EDTA into the interstitium of PROb tumors quantified by microdialysis increased by 39.6% (Rubin et al. 2000). Salnikov used the same method on two experimental tumors in rats and showed a decrease in TIFP and ian improvement on antitumor effect of 5-FU (Salnikov et al. 2003).

Conclusion

The transport of effective doses of anti-cancer tumor drugs to solid tumors is challenging due to the barriers imposed by the tumor vasculature, interstitium and tumor cell layers. Therefore, developing effective drug delivery mechanisms needs to have a thorough understanding of tumor unique physiology and of these barriers. The better knowledge of these parameters will in a near future ameliorate the arrival of conventional cancer therapy.

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Chapter 6
Hypoxia, Hyperthermia, Chemotherapy: Interactions and Opportunities

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Abstract Hypoxia characterizing tumour milieu and represents a key aspect in tumor progression and response to treatment. Many researchers are evaluating the differences between normal and neoplastic cells at biomolecular properties. The physiology of tumours is substantially different from that of normal tissues from which we have a unique and selective target for cancer treatment.

Keywords Hypoxia · Hyperthermia · Oxaliplatin · Docetaxel

Introduction

The peculiar features of hypoxia and acidity characterizing the tumor microenvironment represent key factors in tumor progression, metastasis and response to therapies. Nonsurgical approaches to cancer treatment, primarily radiation therapy and chemotherapy, are almost exclusively based on agents that kill cells. The main problem with these current treatments, however, is that they do not have specificity for cancer cells. For antineoplastic drugs, it is primarily the rapid proliferation of many of the cancer cells that makes them more sensitive to cell killing than their normal cellular counterparts. For radiation therapy, a degree of specificity is achieved by localizing the radiation to the tumor and its immediate surrounding normal tissue. However, both treatments are limited by their toxic effects on normal cells. To, achieve greater efficacy, many researchers are attempting to stress differences between normal and malignant cells at the cellular milieu and biomolecular properties. The physiology of solid tumors at the microenvironmental level is sufficiently different from that of the normal tissues from which they arise to provide a unique and selective target for cancer treatment.

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Hypoxia in Tumor Microenvironment

It is commonly believed that hypoxia and related acidity are a consequence of the chaotic and heterogeneous microvasculature structure of solid tumors (Brown and Giacca 1998). During the period of carcinogenesis, solid tumors are highly proliferative and are separated several hundreds of microns away from blood vessels. Blood vessels are the primary mode for delivery of glucose and O_2, and for the removal of metabolic H_+ and lactate (Schornack and Gillies 2003). Oxygen concentrations decrease with distance from a capillary, and tumor cells are even more distant from their blood supply. Tumor cells generally are highly proliferative and likewise require more nutrients than normal tissues. Therefore, pre-malignant lesions will inevitably develop hypoxic regions with low blood supply. Hence there is a clear requirement for new microvasculature in the growth of tumors. In recent years there is evidence that tumor cells secrete angiogenesis factors and the most important of these factors in tumors is vascular endothelial growth factor (VEGF). VEGF mRNA levels dramatically increase within a few hours after exposing cell cultures to hypoxia and return to background when normal oxygen support is resumed. Tumor hypoxia contributes to progression by the activation of genes associated with those promoting angiogenesis. Moreover, it has profound effects on therapy: oxygen helps to stabilize radiation damage in DNA, while hypoxic cells show considerable (about five-fold) resistance to radiotherapy; this is considered the major cause for the failure of radiotherapy in some tumors. Attempts to overcome this effect include the use of hyperthermia, oxygen-mimetic "radiosensitizers" and conformal radiotherapy to allow re-oxygenation of tumor tissue. Radiosensitizers are drugs designed to act similar to oxygen in fixing radiation damage in DNA, but they are less rapidly metabolized, and are therefore more widely distributed in tumor tissues. There are also good reasons why, and considerable evidence to show how, hypoxic cells in tumors limit the efficacy of anticancer drugs.

The pioneering work of Gray et al. (1953) demonstrated that the sensitivity to radiation damage of cells and tissues depends on the presence of oxygen at the time of irradiation. The histological studies on human lung adenocarcinomas by Thomlinson and Gray (1955) provided an explanation of the mechanism by which cells could become hypoxic in tumors. They postulated that, because of their unrestrained growth, tumor cells would be forced away from vessels, beyond the effective diffusion distance of oxygen in respiring tissue, thereby becoming hypoxic and eventually necrotic.

There are two important consequences of reducing oxygen concentration: (a) the fraction of proliferating cells and/or (b) the rate of cell proliferation decreases as a function of distance from the vascular supply, a phenomenon that is largely the result of decreasing oxygen levels (Gray et al. 1953, Thomlinson and Gray 1955, Moulder and Rockwell 1987). An important consequence of this hypoxia-induced inhibition of proliferation is that, because most anticancer drugs are primarily effective against rapidly dividing cells, their effectiveness would be expected to fall off as a function of distance from blood vessels. This has been shown experimentally

(Schornack and Gillies 2003, Gillies et al. 1999, Gray et al. 1953, Thomlinson and Gray 1955, Moulder and Rockwell 1987, Brown 1999, Graeber et al. 1996) and (b) since hypoxic cells are the ones most distant from blood vessels, they will be exposed to lower concentrations of drug primarily because the metabolism of such agents through successive cellular layers.

Hypoxia in solid tumors, however, has an important consequence in addition to conferring a direct resistance to radiation and chemotherapy (Moulder and Rockwell 1987, Brown 1999, Graeber et al. 1996). Graeber showed that low oxygen levels caused apoptosis in minimally transformed mouse embryo fibroblasts and that this apoptosis depended to a large extent on wild-type p53 genotype. They further showed, using these same cells growing as solid tumors in immune-deprived mice, that apoptosis co-localized with hypoxic regions in tumors derived from p53 wild-type mice. In tumors derived from p53 null cells, there was much less apoptosis and no co-localization with tumor hypoxia. These findings provide evidence that hypoxia, by selecting for mutant p53, might predispose tumors to a more malignant phenotype.

Clinical data support this conclusion. Studies on both soft tissue sarcomas and on carcinomas of the cervix indicate that hypoxic tumors are more likely to be metastatic.

However, others have proposed that tumor hypoxia can occur in a second way, by temporary obstruction or cessation of tumor blood flow: acute hypoxia model. Definitive evidence for this type of acute hypoxia arising from fluctuating blood flow, has come from elegant studies with transplanted tumors in mice using diffusion limited fluorescent dyes. Because fluctuating blood flow has also been demonstrated in human tumors, it is likely that this type of hypoxia is also present in human tumors. The consequences of acute hypoxia will be similar to those of the diffusion-limited hypoxia. Any cells surrounding a closed blood vessel will be resistant to radiation killing because of their lack of oxygen at the time of radiation and will be exposed to lower levels of anticancer drugs than those surrounding blood vessels with a normal flow. This would be expected to lead to differences in response to anticancer agents, as has been observed in experimental tumors.

The low oxygen levels in tumors can be probably turned from a disadvantage to an advantage in cancer treatment. Such a possibility was proposed 20 years ago by Lin et al. (1972), who reasoned that compounds based on the quinone structure of mitomycin – C might be more active in hypoxic tumors. It was known that mitomycin C required metabolic reduction of the benzoquinone ring to produce the cytotoxic bifunctional alkylating agent. Lin reasoned that a lower oxidation reduction (redox) potential for tumor tissue, relative to most normal tissues, could increase reductive activation of these quinone derivatives in tumors. Although this was not the correct mechanism for the increased cytotoxicity of mitomycin C and certain analogues toward hypoxic cells (much lower levels of hypoxia are needed to change cellular redox potential), these studies were important in suggesting the potential of hypoxia-activated drugs and led to the concept of selectively killing the hypoxic cells in solid tumours (Guadagni et al. 2001, 2002).

Hypoxia and Chemotherapy Agents

Four classes of compounds have been developed since this concept was first proposed: quinones, nitro aromatics, aliphatic and hetero aromatic N-oxides. All share two characteristics: (1) they require hypoxia for activation and (2) this activation is dependent on the presence of specific reductases. The most effective compounds have shown the ability to enhance the anti-tumour efficacy of agents that kill better-oxygenated cells, i.e. radiation and standard cytotoxic chemotherapy agents such as cisplatin and cyclophosphamide. Tirapazamine (TPZ) is the most widely studied of the lead compounds. After successful pre-clinical in vivo combination studies it entered clinical trial; over 20 trials have now been reported. Although TPZ has enhanced some standard regimens, the results are variable and in some combinations toxicity was enhanced. Banoxantrone (AQ4N) is another agent that is showing promise in early phase I/II clinical trials; the drug is well tolerated, is known to locate in the tumour and can be given in high doses without major toxicities. Mitomycin C (MMC), which shows some bioreductive activation in vitro, has been tested in combination trials. However, it is difficult to assign the enhancement of its effects to targeting of the hypoxic cells because of the significant level of its hypoxia-independent toxicity (Zaffaroni et al. 2001). More specific analogues of MMC, e.g. porfiromycin and apaziquone (EO9), have had variable success in the clinic. Other new drugs that have good pre-clinical profiles are PR 104 and NLCQ-1; data on their clinical safety/efficacy are not yet available.

In the quinone class, the three principal agents of current clinical interest are mitomycin C, porfiromycin and E09. All are structurally similar and require reductive metabolism for activity. Each of them is converted by reductive metabolism to a bifunctional alkylating agent and probably produces its major cytotoxic activity through the formation of DNA interstrand cross-links.

Mitomycin C, considered to be the prototype bioreductive drug, was introduced into clinical use in 1958 and has demonstrated efficacy towards several tumors, in combination with other selective drugs whose toxicity toward hypoxic cells is modest, with values for hypoxic cytotoxicity ratios (the ratio of drug concentration to produce equal cell kill for aerobic and hypoxic cells) of 1 (no preferential toxicity) to approximately 5. However, based on this activity, mitomycin C has been combined with radiotherapy in two randomized trials of head and neck cancer, the pooled results of which gave a statistically significant disease-free survival benefit (Moulder and Rockwell 1987, Brown 1999, Graeber et al. 1996, Lin et al. 1972).

The third drug in this series, E09, is a much more efficient substrate for DT-diaphorase than either mitomycin C or porfiromycin and shows high toxicity to both aerobic and hypoxic cells with high DT-diaphorase levels. Cells with low DT-diaphorase levels are much less susceptible to killing by E09 under aerobic conditions, but this drug shows a high, up to 50-fold, preferential toxicity toward hypoxic cells. However, the pharmacokinetics of this agent work against its clinical utility, and phase I clinical studies have shown little activity of this drug.

A second class of bioreductive agents is that of the nitroimidazoles, the first two of which, metronidazole and misonidazole, have been extensively tested as hypoxic radiosensitizing agents. Further drug development by Adams and Stratford (1994) produced a compound, RSU1069, which has been shown to be a highly efficient cytotoxic agent with activity both in vitro and in vivo. RSU1069 has an hypoxic cytotoxicity ratio of some 10–100 for different cell lines in vitro, and it, or its prodrug, RB6145, has shown excellent activity with mouse tumor models when combined with irradiation or agents that induce hypoxia. Unfortunately, however, clinical testing of RB6145 has been aborted due to irreversible cytotoxicity toward retinal cells.

Tirapazamine (TPZ) is the first, and thus far, only representative of the third class of hypoxia-selective cytotoxins (Brown 1999). The mechanism for the preferential toxicity of TPZ toward hypoxic cells is the result of an enzymatic reduction that adds an electron to the TPZ molecule, forming a highly reactive radical. This radical is able to cause cell killing by producing DNA damage leading to chromosome aberrations. Moreover, DNA damage occurs only from TPZ metabolism within the nucleus. TPZ produces specific potentiation of cell kill by radiation and cisplatinum. Specifically, the synergistic cytotoxic interaction observed when TPZ and cisplatinum are given in sequence depends on the TPZ exposure being under hypoxic conditions. In fact, there is no interaction when TPZ is given under aerobic conditions. It has also been demonstrated that the cytotoxic activity of TPZ under hypoxia is independent of p53 gene status of tumour cells. This drug has 100-fold differential toxicity toward hypoxic vs aerobic cells.

Based on experimental studies that evaluated the responsiveness of tumour cells under aerobic and hypoxic conditions, Teicher et al. (1990) classified chemotherapeutic agents into three groups: (1) preferentially toxic in aerobic conditions (bleomycin, procarbazine, streptonigril, actinomycin D, vincristine and melphalan); (2) preferentially toxic under hypoxic conditions (mitomycin C and adriamycin); (3) no major preferential toxicity to oxygenation (cisplatinum, 5-fluorouracil and methotrexate).

Hypoxia Inducible Factor 1 Alpha and Nitric Oxide

Hypoxia, exists in solid tumor tissues due to abnormal vasculature, vascular insufficiency, treatment or malignancy related anemia, and low intra tumor blood flow. Hypoxic status, in solid tumor promotes accumulation of hypoxia-inducible factor-1 alpha, which is promptly degraded by proteasomal ubiquitination under normoxic conditions. However, under hypoxic conditions, the ubiquitination system for HIF-1 alpha is inhibited by inactivation of prolyl hydroxylase which is responsible for hydroxylation of proline in the oxygen-dependent degradation domain of HIF-1 alpha. HIF-1 alpha is an important transcriptional factor that codes for hundreds of genes involved in erythropoiesis, angiogenesis, induction of glycolytic enzymes in tumor tissues, modulation of cancer cell cycle, cancer proliferation, and cancer

metastasis. Hypoxia and accumulation of HIF-1 alpha in solid tumor tissues associate with resistance to chemotherapy, radiotherapy, and immunotherapy and poor prognosis. Production of vascular endothelial growth factor (VEGF) in cancer cells is regulated by the activated HIF-1 mediated system. An increase in VEGF levels subsequently induces HIF-1 alpha accumulation and promotes tumor metastasis by angiogenesis. Recently, angiogenesis targeting therapy using humanized VEGF antibody and VEGF receptor tyrosine kinase inhibitors have been used in solid cancer therapy.

Nitric oxide (NO) is a unique chemical gaseous molecule that plays a role as a chemical messenger involved in vasodilator, neurotransmitter, and anti-platelet aggregation. In vivo, NO is produced and released from three different isoforms of NO synthase (NOS) and from exogenously administered NO donors. In oncology, NO has been mainly discussed as an oncogenic molecule over the past decades. However, NO has recently been associated with cancer cell apoptosis, cancer cell cycle, cancer progression and metastasis, cancer angiogenesis, cancer chemoprevention, and modulator for chemo/radio/immuno-therapy. The presence and activities of all the three isoforms of NOS and were detected in cancer tissue components such as cancer cells, tumor-associated macrophages, and vascular endothelium. Overexpression of iNOS in cancer tissues has been reported to associate with poor prognosis in patients with cancers. On the other hand, NO donors such as nitroglycerin have been demonstrated to improve the effects of cancer therapy in solid cancers. Nitroglycerin has been used safely for a long time as a potent vasodilator for the treatment of ischemic heart diseases or heart failure. Therefore, some experts support the clinical use of nitroglycerin as a novel cancer therapy in combination with anticancer drugs for improvement of cancer therapeutic levels. Yasuda (2008) demonstrates the unique physiological characteristics of malignant solid tumors, several factors in solid tumors resulting in resistance for cancer therapies, and the effects of NO from NOS or exogenous NO-donating drugs on malignant cells. Furthermore, they refer to promising therapeutic roles of NO and NO-donating drugs for novel treatments in solid tumors.

Expression of Hypoxia-Inducible Factor 1 Alpha Gene and Outcome in Ovarian Cancer

Shimogai et al. (2008) conducted a study to determine whether and how expression of the HIF-1 alpha gene relates to outcome in patients with epithelial ovarian cancer. A total of 66 patients with epithelial ovarian cancer, who underwent primary surgery followed by platinum-based chemotherapy, were entered into this study. The study confirmed the expression of HIF-1 alpha and vascular endothelial growth factor (VEGF) by immunohistochemistry. To determine the quantity of HIF-1 alpha and VEGF expression, messenger RNA of each gene was measured by real-time reverse transcription-polymerase chain reaction. Protein expressions of HIF-1 alpha and VEGF were highly cancer cells. Threshold value of HIF-1 alpha and VEGF

gene expression was 6.0 and 3.0, respectively. Expression of HIF-1 alpha did not relate to clinical stage, but tumors with low VEGF gene expression was observed more frequently in stage I patients. Response rate to chemotherapy did not differ between high and low expression of either genes. Overall survival for patients with high expression of the HIF-1 alpha gene was significantly lower, but disease-free survival did not differ between high and low expression of HIF-1 alpha, whereas both overall and disease-free survival for patients with high expression of the VEGF gene were significantly lower. Multivariate analysis revealed that FIGO stage and HIF-1 alpha expression were independent prognostic factors but that VEGF was not. The present study suggest that the expression level of HIF-1 alpha could be an independent prognostic factor in epithelial ovarian cancer.

Hyperthermia and Schedule Administration Chemotherapy

Pre-clinical thermo-chemotherapy studies have given valuable information on the schedule of the cytotoxic interaction between the different agents and on the molecular mechanisms responsible for the potentiating effect. Several studies have demonstrated that the cytotoxic activity of various chemotherapeutic agents is enhanced by mild or moderate hyperthermia (40.5 – 43°C) (Urano et al. 1999). There are data regarding doxorubicin, the platinum compounds cisplatin and carboplatin, the bifunctional alkylating agent melphalan and the antimetabolite methotrexate which indicate that in each case maximal cytotoxicity occurs when the drug is administered simultaneously with hyperthermia (Urano et al. 1999, Zaffaroni et al. 1989, Kusumoto et al. 1995, Bates and Mackillop 1998, Zaffaroni et al. 1992, Orlandi et al. 1995, 1996, Rietbroek et al. 1997).

The mechanisms responsible for the effect of hyperthermia on cell killing by anticancer drugs are not entirely understood. For example, with melphalan, which is widely used in experimental and clinical thermo-chemotherapy studies, different putative mechanisms of potentiation have been suggested including an increase in melphalan influx leading to a higher intracellular drug accumulation (Bates and Mackillop 1998).

The alteration of the DNA quaternary structure, which favours alkylation; the interference with drug-DNA adduct metabolism and inhibition of repair (Zaffaroni et al. 1992) the stabilization of drug-induced G2 phase cell accumulation (Zaffaroni et al. 1992) through the inhibition of $p32^{ctlc2}$ kinase activity (Orlandi et al. 1995, 1996). In regard to cisplatin, the cytotoxic activity of this compound, and that of the platinum derivatives lobaplatin and oxaliplatin, is increased under hyperthermic conditions as the consequence of an enhanced formation of DNA-platinum adducts (Rietbroek et al. 1997).

Pre-clinical studies have also significantly contributed to the proposition of potential cellular determinants of response to individual and combined treatments. The relevance of cell kinetic and DNA ploidy characteristics as indicators of thermoresponsiveness has been determined in primary cultures of human melanoma

(Orlandi et al. 1993). Results from this study showed that the median 3H-thymidine labeling index of sensitive tumors was four-fold that of resistant tumors. Moreover, thermosensitivity was found more frequently in tumors with a diploid nuclear DNA content than in those with DNA aneuploidy.

Since heat and drug sensitivity may be related to the ability of tumor cells to mount a stress response, the relationship between constitutive (and inducible) levels of heat shock proteins (HSPs) and thermosensitivity has been evaluated in testes and bladder cancer cell lines (Richards et al. 1995). No correlation between constitutive levels of HSP90 or HSP72/73 and cellular thermoresponsiveness was found. However, results suggest that low HSP27 expression might contribute to heat sensitivity.

Hyperthermia and Antineoplastic Drugs Selection

The most effective agent(s) at elevated temperatures have yet to be determined. Some studies suggest that the drug of choice at elevated temperatures may be different from that at physiological temperature, and that alkylating agents may be most effective at elevated temperatures. To further investigate these possibilities, the effect of chemotherapeutic agents were compared by Takemoto et al. (2003). He studied these agents: cyclophosphamide, ifosfamide, melphalan, cisplatin, 5-fluorouracil, mitomycin C and bleomycin. Three tumours (mammary carcinoma, osteosarcoma and squamous cell carcinoma) were used. They were transplanted into the feet of C3H/He mice. When tumours reached 65 mm, a test agent was injected intraperitoneally. Tumours were immediately heated at 41.5ºC for 30 min, and the tumour growth (TG) time was studied for each tumour. Using the TG times, the TG-50 (the time required for one-half of the total number of the treated tumours to reach the volume of 800 mm from 65 mm was calculated. Subsequently, the tumour growth delay time (GDT) and the thermal enhancement ratio (TER) were obtained. The GDT was the difference between the TG-50 of treated tumours and that of non-treated control tumours. The TER was the ratio of the GDT of a group treated with an agent at 41.5ºC to that of a group treated with the agent at room temperature. Results showed that the top three effective agents tested at 41.5ºC were solely alkylating agents cyclofosphamide, ifosfamide and melphalan for each kind of tumour. A GDT of cisplatin was smaller than those of the alkylating agents. The smallest TER, 1.1, was observed for 5-fluorouracil, which was given for mammary carcinoma, and for mitomycin C, which was given for squamous cell carcinoma. It could be concluded that the alkylating agents at elevated temperatures might be the drugs of choice for many types of tumors.

Alkylating Agents and Oxaliplatin

Urano and Ling (2002) studied the effects of various agents on animal tumours with different histopathology at elevated temperatures. His studies indicated that

alkylating agents were most effective to all tumours at a moderately elevated temperature. Cisplatin was also effective to all tumours, but its effectiveness at 41.5°C was less than that of alkylating agents. To quantitatively study these findings, the magnitude of thermal enhancement of melphalan, an alkylating agent, and that of oxaliplatin, a new platinum compound, were studied by this author, at 37 – 44.5°C by the colony formation assay. The dose of each agent was kept constant, and cell survival was determined as a function of treatment time. The cell survival curve was exponentially related with treatment time at all test temperatures, and the T(0) (the time to reduce survival from 1 to 0.37) decreased with an increasing temperature. These results suggested that the cytotoxic effect of these agents occurred with a constant rate at 37°C, and the rate was facilitated with an increasing temperature. This suggests that heat can accelerate the cytotoxic chemical reaction, leading to substantial thermal enhancement. The thermal enhancement ratio (TER, the ratio of the T(0) at 37°C to the T(0) at an elevated temperature) increased with an increase in the temperature. The activation energy for melphalan at moderately elevated temperatures was largest among the agents tested in the laboratory and that for oxaliplatin was approximately half of the melphalan activation energy. This suggests that the thermal enhancement for the cytotoxicity of melphalan or alkylating agents might be the greatest.

Docetaxel

Recent studies suggest that docetaxel may show improved response at elevated temperatures. Factors that may modify the thermal enhancement of docetaxel were studied by Mohamed et al. (2004) to optimize its clinical use with hyperthermia. The tumor studied was an early-generation isotransplant of a spontaneous C3Hf/Sed mouse fibrosarcoma, Fsa-II. Docetaxel was given as a single intraperitoneal injection. Hyperthermia was achieved by immersing the tumor-bearing foot into a constant temperature water bath. Four factors were studied: duration of hyperthermia, sequencing of hyperthermia with docetaxel, intensity of hyperthermia, and tumor size. To study duration of hyperthermia tumors were treated at 41.5°C for 30 or 90 min immediately after intraperitoneal administration of docetaxel. For sequencing of hyperthermia and docetaxel, animals received hyperthermia treatment of tumors for 30 min at 41.5°C immediately after drug administration, hyperthermia both immediately and 3 hr after docetaxel administration and hyperthermia given only at 3 hr after administration of docetaxel. Intensity of hyperthermia was studied using heat treatment of tumors for 30 min at 41.5 or 43.5°C immediately following docetaxel administration. Effect of tumor size was studied by delaying experiments until three times the tumor volume (113 mm^3) was observed. Treatment of tumors lasted for 30 min at 41.5°C immediately following drug administration. Tumor response was studied using mean tumor growth time. Hyperthermia in the absence of docetaxel had a small but significant effect on tumor growth time at 43.5°C but not at 41.5°C. Hyperthermia at 41.5°C for 90 min immediately after docetaxel administration significantly increased mean tumor growth time ($P = 0.0435$)

when compared to tumors treated with docetaxel at room temperature. Treatment for 30 min had no effect. Application of hyperthermia immediately and immediately plus 3 hr following docetaxel was effective in delaying tumor growth. Treatment at 3 hr only had no effect. No significant difference in mean tumor growth time was observed with docetaxel and one half hour of hyperthermia at 41.5 or 43.5°C. For larger tumors, hyperthermia alone caused a significant delay in tumor growth time. Docetaxel at 41.5°C for 30 min did not significantly increase mean tumor growth time compared to large tumors treated with docetaxel at room temperature. Docetaxel shows a moderate increase in anti-tumor activity with hyperthermia. At 41.5°C the thermal enhancement of docetaxel is time dependent if hyperthermia is applied immediately following drug administration. With large tumors docetaxel alone or docetaxel plus hyperthemia showed the greatest delays in tumor growth time in the experiments.

Hyperthermia and Gene Therapy

Li reported the activity of adenovirus-mediated heat-activeted antisense Ku70 expression radiosensitizers tumor cells in vitro and in vivo (Li et al. 2003). Ku70 is one component of a protein complex, Ku70 and Ku80, that functions as a heterodimer to bind DNA double-strand breaks and activates DNA-dependent protein kinase. The previous study of this group with Ku70 null and Ku80 null mice, and cell lines have shown that Ku70- and Ku80-deficiency compromises the ability of cells to repair DNA double-strand breaks, increases radiosensitivity of cells, and enhances radiation-induced apoptosis. In this study, Li examined the feasibility of using adenovirus-mediated, heat-activated expression of antisense Ku70 RNA as a gene therapy paradigm to sensitize cells and tumors to ionizing radiation. First, they performed experiments to test the heat inducibility of heat shock protein (hsp) 70 promoter and the efficiency of adenovirus-mediated gene transfer in rodent and human cells. Replication-defective adenovirus vectors were used to introduce a recombinant DNA construct, containing the enhanced green fluorescent protein (EGFP) under the control of an inducible hsp70 promoter, into exponentially growing cells. At 24 h after infection, cells were exposed to heat treatment, and heat-induced EGFP expression at different times was determined by flow cytometry. The data by Li clearly show that heat shock at 42°C, 43°C, or 44°C appears to be equally effective in activating the hsp70 promoter-driven EGFP expression (>300-fold) in various tumor cells. Second, the authors have generated adenovirus vectors containing antisense Ku70 under the control of an inducible hsp70 promoter. Exponentially growing cells were infected with the adenovirus vector, heat shocked 24 h later, and the radiosensitivity determined 12 h after heat shock. Our data show that heat shock induces antisense Ku70 RNA, reduces the endogenous Ku70 level, and significantly increases the radiosensitivity of the cells. Third, the author has performed studies to test whether Ku70 protein level can be down-regulated in a solid mouse tumor (FSa-II), and whether this results in enhanced radiosensitivity in

vivo, as assessed by in vivo/in vitro colony formation and by the tumor growth delay. Their data demonstrate that heat-shock-induced expression of antisense Ku70 RNA attenuates Ku70 protein expression in FSa-II tumors, and significantly sensitizes the FSa-II tumors to ionizing radiation. Taken together, these interesting results suggest that adenovirus-mediated, heat-activated antisense Ku70 expression may provide a novel approach to radiosensitize human tumors in combination with hyperthermia.

Conclusions

Several aspects of tumor physiology seem to be directly responsive to the low oxygen environment within the tumor and the comprehension, that hypoxic tumours are resistant to radiotherapy led to the concept that cancer cells might be resistant because of their poor oxygen supply and subsequent hypoxia.

Tumour hypoxia is seen as a mechanism of resistance to many antineoplastic drugs, as well as a predisposing factor toward increased malignancy and metastases.

The key to effective use of this knowledge is to recognize the changes that are going on within the tumor in response to the given agent.

However tumour hypoxia is a unique target for hyperthermia and cancer bioreductive therapy that could be exploited for therapeutic use. A hypoxic cell is unable to maintain a stable pH; this increases the permeability of the cell membrane so that antineoplastic agents can easily move through the membrane improving the global concentration of the drug both inside and outside the cell. Hyperthermia seems a favorable opportunity to enhance these phenomena.

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Chapter 7
Effects of Molecularly Targeting Hypoxia in Oncology

Part A: Theory With Renal Cell Carcinoma as Model

Giammaria Fiorentini

Abstract The interaction between neoplastic cells and vasculature results in low oxygen tension, low extra-cellular pH and high interstitial fluid pressure. New multi-target tyrosine kinase inhibitors are new therapeutically options in solid tumours. They share the capacity of modulate the hypoxia inducible factor (HIF)-VEGF-VEGF receptor sequence that plays a predominant role in the development of solid tumours.

Keywords Neoplastic vasculature · Hypoxia · VEGF · Hypoxia inducible factor

Introduction

The microenvironment of solid tumours has several characteristics that distinguish it from the corresponding normal tissues. These different aspects are thought to be due to the interaction between the poorly formed tumour vasculature and the physiologic characteristics of the cells within the tumour. The interaction between the cancer cells and vasculature results in three well known micro-environmental hallmarks of solid tumours: low oxygen tension, low extra-cellular pH and high interstitial fluid pressure. To overcome these challenges new therapy for renal cell carcinoma (RCC) took a major step forward with the approval of sorafenib and sunitinib in 2005 and 2006. These two multi-targeted tyrosine kinase inhibitors are the first in a growing list or molecularly targeted therapy in RCC. They all share the ability to modulate the hypoxia inducible factor (HIF)-VEGF-VEGF receptor axis that plays a significant role in the development of many tumours. Several aspects of tumor physiology seem to be directly responsive to the low oxygen environment through the activity of the HIF-1 transcription factor. HIF-1 is therefore characterized as responsible

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for adaptive changes in the hypoxic regions within the tumour. There are many of genes that HIF-1 can transactivate, including VEGF, glycolitic enzymes, ion channels, protease regulators, and mitochondrial regulators. The possibility of HIF-1 blockade therefore represents an interesting strategy for modifying the tumour physiology. HIF-1 is part of the loop responsible for the physiologic condition within the tumour. The tumour vasculature leads to hypoxia , this leads to HIF-1 gene expression changes. Several examples of specific HIF-1 target genes fit well into this model of explaining the changes observed in the tumour microenvironment.

Renal Cell Carcinoma: A Model.

Cancers of the kidney and renal pelvis represent a significant health problem. In the United Kingdom, about 7000 people were diagnosed with the disease in 2003 and there were 3500 deaths. Unlike the majority of tumours, both mortality and incidence rates continue to rise (UK Kidney Cancer statistics). In common with other tumours, earlier presentation correlates with improved prognosis, those patients who present with locally confined stage I or stage II disease (approximately 56% of all cases) have a 5-year survival over 90%. In contrast to the 25%, of patients who present with distant metastases (stage IV) the overall prognosis is much worse with a median 9-month survival for patients, and a 5-vear survival rate of 10%.

The near complete resistance to chemotherapy or radiotherapy is well described for RCC and as a result no therapy has challenged immunotherapy as the standard treatment for advanced disease, despite response rates that can be described as modest at best, until 2005 (Sutphin et al. 2007). Complete and durable responses are seen only in a minority of patients. One of the approved cytokine agents, interleukin2 (IL-2), was accepted by the United States Food and Drug Administration in 1992 based on phase 2 data that showed prolonged and complete remission in about 7% of patients. The other most often used cytokine, interferon, produces similar response rates.

Decades of research are finally having an impact on the outlook for patients with advanced RCC.

The realization that RCC is a both a highly vascularized disease and an acutely hypoxic tumour has led researchers to pursue anti-angiogenic strategies. Approved agents include sorafenib and sunitinib as well as temsirolimus, a mammalian target of rapamycin (mTOR) inhibitor. Bevacizumab, an antibody that targets vascular endothelial growth factor (VEGF), also shows promising clinical activity in RCC. While these 4 agents have different biologic targets they all share the ability to modulate the hypoxia inducible factor (HIF)-VEGF-VEGF receptor axis that plays a significant role in the development of RCC (Sosman et al. 2007).

Hypoxia Inducible Factor in the Biology of RCC

The disease of Von Hippel-Lindau (VHL) is an autosomal dominant, hereditary condition that stems from the inactivatation of the VHL tumour suppressor gene located on chromosome 3p25 (Latif et al. 1993). VHL disease is defined by an increased

risk for a variety of tumours including those of the central nervous system, hemangioblastomas, pheochromocytomas, and clear cell RCC (Alleman et al. 2004). The VHL gene has been implicated in sporadic clear cell RCC where it is inactivated by mutation in about 60% of cases (Gnarra et al. 1994) and by hypermethylation in a further 10% to 20% of cases (Herman et al. 1994). VHL protein is a gene product with multiple functions, one of which is regulation of the HIF. The HIF-1 is a transcription factor consisting of an unstable alfa subunit (HIF-alfa) that degrades in the presence of oxygen and a stable beta subunit (HIF-beta) as a heterodimer (Carroll and Ashcroft 2006). The human genome contains three HIF-alfa genes, although only HIF-1 alfa and HIF-2 alfa have been well-documented as activating transcription under physiological conditions (Ibrahim et al. 2005).

Both HIF-1 alfa and HIF-2 alfa appear to share many physical characteristics. In the presence of oxygen, HIF-alfa is quickly hydroxylated to create a binding site for pVHL. Von Hippel-Lindau protein is a key component of the ubiquitin ligase complex that marks HIF-alfa for proteasomal degradation. This results in HIF-alfa being present in low, transient amounts under normoxic conditions in normal cells (Semenza 2003).

Under hypoxic conditions, HIF-alfa is not hydroxylated and degraded. Instead it accumulates in cells and binds with a HIF-beta subunit. The HIF-1 heterodimer migrates to the nucleus where it binds to specific DNA sequences to activate genes that are involved in the acute or chronic adaptation to hypoxia. HIF-1 up-regulates at least 65 genes which have been found to be essential for successful cellular adaptation to hypoxia (Semenza 2003).

The same genes can support cancer cell development by modulating glucose metabolism, angiogenesis, as well as those involved in aspects of tumour invasion and apoptosis resistance.

Pathologically, under normoxic conditions, excess HIF-1 heterodimer accumulates due to the lack of functional pVHL and allows accumulation of HIF-alfa. This mimics hypoxia, combining the HIF-beta subunit to produce HIF-1 heterodimer. This biochemical shift favours the development of RCC. The inefficient vasculature found in most tumours hampers the delivery of oxygen and produces intermittent hypoxia, which stimulates further accumulation of HIF-alfa. Overall tumor growth is stimulated by activation of HIF responsive genes that promote adaptation to hypoxia, survival, angiogenesis, and metastases.

Given that pVHL and HIF are ubiquitously expressed, a full explanation as to why human clear cell RCC specifically results from aberrations in this pathway has yet to be elucidated. Differences have been shown between renal and other epithelial cell types. For example proliferation is stimulated via cyclin D1 under hypoxic conditions leading to HIF stimulation in renal cells and it is known that portions of the kidney, particularly medulla, are hypoxic at rest (Wykoff et al. 2004). Overall renal epithelium is better able to proliferate in a hypoxic environment, the corollary being the cells which are more susceptible to the oncogenetic effects of HIF over-activation.

Preclinical evidence for HIF-1 as an anticancer target has been contradictory, at least in part due to the complexity of the system and a lack of adequate models in which to test hypotheses. Results have varied depending on the cell line used, which

HIF subunit used, site of tumour injection in xenograft models, and timing of HIF inhibition. Data from patients has provided evidence for targeting HIF in cancer. HIF-1 alfa has been shown to be over-expressed in may solid tumours and associated with mortality and worse treatment outcomes. Finally multiple receptor tyrosine kinase pathways implicated in human cancers merge on HIF-1 (Melillo 2006)

Whatever the underlying causes of RCC sensitivity and reactivity to pVHL, HIF, its subunits, and the pathways it modulates, HIF has emerged as a major target of therapeutic interest in clear cell RCC.

HIF-1 Inhibition: The Selective

As a transcription factor, HIF-1 is not a conventional target for anticancer drug therapy. However several groups have attempted to design specific HIF-1 inhibition strategies.

Creighton-Gutteridge and colleagues (Creighton-Gutteridge et al. 2007) have reported preclinical data for NSC 644221, a HIF-1 alfa inhibitor identified from the open synthetic repository of the United States National Cancer Institute (NCI) database. The compound inhibited HIF-1 alfa not HIF-1 beta in a dose-related and time-related manner in (pVHL lacking) RCC4 ce11s. HIF-1 translation was inhibited in the presence of topoisomerase II, but that inhibitory activity was highly cell line dependent. Originally developed as a less toxic topoisomerase Il inhibitor compared to available compounds, NSC 644221 lacks activity as a cytotoxic agent which might be a desirable clinical feature if chronic dosing were necessary to inhibit HIF-1 (Melillo et al. 2006).

Another group reported on PX-478, a novel agent, that suppresses constitutive and hypoxia induced HIF-l alfa in cancer cells. Xenograft studies confirmed antitumour activity in a variety of models that were apparently related to HTF-1 expression. Pharmacokinetics were acceptable and toxicology studies revealed neutropaenia and weight loss in mice. Pharmacodynamic (PD) studies showed HIF-1 levels and expression of HIF-1 target genes, including VEGF and glucose transporter-1 (Gut-1) to be inhibited (Welsh et al. 2004).

They noted that tumour growth inhibition correlated with diminished glucose uptake via Glut-1 inhibition rather than decreases in VEGF and an anti-angiogenic mechanism. In addition, HIF-1 related glucose metabolism has been implicated in apoptosis resistance by others (Fulda and Debatin 2007).

HIF-1 Inhibition: The Non Selective

While clinical testing of specific HIF-1 inhibitors has yet to he undertaken, a number of non selective approaches are already in clinical trial. For example, targeting the mTOR pathway, using temsirolimus in combination with interferon, has been shown to be efficacious in patients with poor prognosis RCC (Hudes et al. 2007).

mTOR regulates HIF-1 protein expression dependent on the cellular context (Hudson et al. 2002) and preclinical data were consistent with HIF being a determinant of cell line sensitivity to mTOR inhibition (Thomas et al. 2006). These investigators also used FDG-PET scan as a biomarker of HIF inhibition that could be included in future clinical trials (Thomas et al. 2006).

Geldanamycin, an HSP9O antagonist and naturally occurring anasamycin antibiotic has been shown to affect HIF-1 stability and function. HSP9O is a molecular chaperone protein that ensures the proper conformation, localization, and function of its client proteins such as HIF-l alfa as well as other transcription factors and signalling kinases. The inhibition of HSP90 results in client proteins being targeted for degradation by the ubiquitin-proteosome system. Given the multiple client proteins that in tract with HSP90 there is hypothetical potential to interfere with all 6 of the hallmark traits of cancer (Powers and Workman 2006):

- sustained angiogenesis (VEGF, VEGF receptor, HIF-1)
- Growth factor independence (Raf, ErbB 2)
- Resistance to antigrowth signals (cyclin-dependent kinase 3)
- Unlimited replication potential (human telomerase reverse transcriptase)
- Tissue invasion and metastasis (metalloproteinase,matrix metalloproteinase 2)
- Avoidance of apoptosis (survivin)

Inhibition of this single target allows both horizontal and vertical blockade of HIF related pathways (Tan et al. 2005). Clearly HSP90 is an attractive anticancer target in many tumour types. Interest in renal cancer was generated by observations regarding the interaction between HSP90 and HIF-1. For example, studies by lsaacs and colleagues (Isaacs et al. 2002) in RCC lines C2 and C6 showed that HSP90 inhibition led to degradation of HIF-1 alfa via rapid proteasomal degradation under both normoxic and hypoxic conditions and interfered with HIF-I alfa transcriptional activity, including the downstream expression of VEGF. The clinical use of geldanamycin was limited by its side effects profile, but derivatives including 17-allylamino-17-demethoxygeldanamycin and a water soluble form, 17-dimethylaminoethylamino-l7-demethoxy-galdanamycin (17DMAG) are available (Ronner et al. 2006).

Integration Approaches

At present there are several molecularly targeted drugs available for testing in RCC. These include antibodies such as bevacizurnab (anti-VEGF) or cetuximab (anti-epidermal growth factor receptors (EGFR) and panitumumab that have been developed to block ligand binding to EGF-R1. Other agents act as receptor tyrosine kinase inhibitors against VEGF-receptor 1 (VEGF-R1), 2 (VEGF-R2) or 3 (VEGF-R3) signalling. Most currently available tyrosine kinase inhibitors were designed to inhibit specific receptors, but the final products typically bind to and inhibit multiple receptors. These so-called dirty receptor tyrosine kinases (RTK)

inhibitors include sunitinib, which blocks VEGF-R2 and platelet-derived growth factor receptor (PDGFR) as well as c-Kit and Flt3 receptors. Sorafenib, which was designed to bind Raf kinases, is also an effective inhibitor of VEGF-R2, PDGF-R, c-Kit and Flt3. Other RTK inhibitors target EGFR (erlotinib) and ErbB-1 (gefitinib), either alone or in combinations of ErbB-1 and ErbB-2 (lapatinib) or ErbBl-1 and VEGF-R2 (AZD6474) (Gullob et al. 2006). Small molecule inhibitors also include the mTOR inhibitors such as temsirolimus. mTOR is a key component of intracellular pathways that regulate orderly progression through the cell growth cycle and angiogenesis.

During initial development of these drugs as monotherapy, up to 75% of patients treated had attained stable disease or tumour regression. Typically these responses have lasted between 3 to 24 months, depending on the drug and the trial (Sosman et al. 2007). While response levels remain higher than those typically expected from cytokine treatment, targeted therapies developed to date rarely, if ever, produce complete or near-complete responses that are also durable in nature. To further improve outcome, many research groups are focusing their attention on combination therapy.

Considering HIF-1 and associated pathways there are 2 potential routes to develop rational drug combinations. Horizontal blockade attempting to inhibit multiple, parallel pathways (Shin et al. 2000). Targets that have been identified to date are found in different cell types such as the tumour cells (EGFR), endothelial cells (VEGF-R2), and pericytes (PDGF) The alternative is vertical blockade in which multiple targets along a single pathway are modulated, such as VEGF and its RTK (Gnarra et al. 1994). Both strategies can be achieved by combinations of multiple targeted agents or targeted agents with existing chemotherapy, radiotherapy, or cytokine therapy.

Agents that can be used to create a horizontal blockade might include combinations of specific inhibitors of the target pathways such as bevacizumab (VEGF), erlotinib (EGFR), or imatinib (PDGF). Horizontal blockade might also include multi-targeted agents such as sorafenib and sunitinib.

Vertical blockade offers the theoretical advantage of overcoming resistance that develops through feedback mechanisms. One example of a resistance feedback mechanism would be an increase in VEGF levels as VEGFR is blocked, or an elevation in HIF-alfa as VEGF or VEGFR are inhibited. Although it is likely that other compensatory feedback mechanisms will appear (Westergaard et al. 2006).

Sosman and colleagues (Sosman et al. 2007) designed a horizontal blockade of the VEGF and EGF pathways in patients with advanced RCC. A multicentre phase 2 trial combined bevacizumab to inhibit VEGF with erlotinib to inhibit EGFR. After a median follow-up period of 15 months, median progression free survival was 11 months and the progression-free survival at 12 months and 18 months was 43% and 26%. The targeted combination appeared to be more active than either bevacizumab or erlotinib as separate agents and was well tolerated by patients with advanced RCC. However a randomized, 2-arm, phase 2 trial of bevacizumab plus erlotinib or placebo found no clinically or statistically significant differences between the two regimens (Bukowski et al. 2006). The concept of combining a VEGF-VEGFR inhibitor with an EGFR inhibitor remains appealing, if unproven.

Given the increasing number of targeted therapeutics available, there are clearly an overwhelming potential number of combinations that can he studied. Other targeted combinations under trial explore a similar strategy of using multiple agents that impact the VEGFR axis at several steps.

Conclusions

There remains a rationale to support the use of targeted therapy against HIF and its associated pathways in clear cell RCC. Approvals of first generation multi-targeted therapies, including the tyrosine kinase inhibitors sunitinib and sorafenib, and the mTOR inhibitor temsirolimus, have brought the first significant improvement in treatment results since the introduction of immunotherapy two decades ago. These agents all possess the ability to modulate HIF-1 related pathways. While we await development of specific HIF-1 inhibitors, rational combinations of these drugs might offer further improvements in patient outcomes.

Validation of HIF-1 as a target in renal cancer is yet to be completed and will require specific pharmacological tools. Current evidence suggests better knowledge of renal cell cancer biology must be used to maximise the chance of detecting clinical effects. For example phase 2 trials of HIF-1 targeting agents could be stratified on HIF-1 levels or VHL gene function in tumours. Clearly this requires adequate biomarker studies, and efforts such as those of the NCI to develop these assays need to be encouraged.

The complexity of HIF regulation and the diversity of the genes that it regulates may mean that validation of HIF as a target will have to wait until more specific agents are developed towards HIF isoforms, such that investigators could select the HIF regulated genes that are most desirable to target in cancer.

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Part B: Example of Method for Reducing Side Effects of Induced Acute Hypoxia by Tace for Treating Liver Metastases, Phase II Clinical Trial

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Abstract We performed a phase II clinical trial of intra-arterial hepatic chemoembolization (TACE) with irinotecan-eluting beads in 20 patients affected by liver metastases from colorectal cancer (CRC) as palliative setting. We observed a high response rate (80%), with reduction of lesional contrast enhancement in all responding patients. We developed an intensive treatment with intra-arterial lidocaine and post-procedure supportive therapy to reduce acute pain and toxic effects and post embolization syndrome due to acute hypoxia. Due to this support TACE was well tolerated by most patients with a median duration of hospitalization of 3 days (range 1–10). The most important adverse event was abdominal pain. Supportive treatment with antibiotic and antiemetic prophylaxis, and intravenous hydratation is strictly necessary until stabilization of serum levels of transaminases and to prevent infections. Major analgesic as morphine and intra-arterial lidocaine might be used before the procedure. Our results suggest that TACE using irinotecan-eluting beads is feasible in pretreated patients with liver metastases from CRC adopting an adequate supportive therapy to reduce side effects, particularly those linked to acute hypoxia induced by TACE.

Keywords Liver metastases · Colorectal cancer · Intra-arterial lidocaine · Hepatic angiography

Introduction

Cancer of colon and rectum is the most common tumor type presenting with liver-predominant metastases in developed countries.

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Of more than 150,000 new cases of colorectal cancer reported in the United States each year, as many as 25% will have liver metastases at presentation and another 50% will develop liver recurrence within the next 5 years. More than one third of patients presenting with liver metastases will have metastatic disease limited to the liver (Chong and Cunningham 2005).

Surgery is the standard treatment in patients with resectable liver disease. However less than 20% of the patients are candidates for radical resection (Chong and Cunningham 2005, Adam et al. 2001) and approximately 70% of these patients develop disease relapse. Chemotherapy (Adam 2003, Alberts et al. 2005 Saltz et al. 2000, Goldberg et al. 2004, Hurwitz et al. Chen et al. 2006, Cunningham et al. 2004), radiofrequency ablation (Cunningham et al. 2004, Machi et al. 2006) and trans arterial chemotherapy combined with vascular occlusion agents (TACE) have been used as palliative measures (Berber et al. 2005, Aliberti et al. 2006, Morise et al. 2006, Fiorentini et al. 2004, 2007, Popov et al. 2002, Voigt et al. 2002, Vogl et al. 2007, Hunt et al. 1990, Martinelli et al. 1994, Salman et al. 2002, You et al. 2006).

TACE is a combination of local drug infusion with selective embolization of the feeding arteries of the liver metastases. It can produce a transient, prolonged or permanent arterial occlusion, combined with antineoplastic drugs.

The advantage of delivering chemotherapy by hepatic arterial infusion is the administration of high-dose of the drug in the target (Cohen and Kemeny 2003, Fiorentini et al. 2001, 2003). The aim of this therapeutic technique is to reduce the tumor size by ischemic necrosis and direct drug effects. Largely used for the treatment of hepatocellular carcinoma, it is also used in neoadjuvant or palliative setting in patients with isolated liver metastases from neuroendocrine tumours (Fiorentini et al. 2004) and from colorectal cancer (Berber et al. 2005, Aliberti et al. 2006, Morise et al. 2006, Fiorentini et al. 2004, 2007, Popov et al. 2002, Voigt et al. 2002, Vogl et al. 2007, Hunt et al. 1990, Martinelli et al. 1994, Salman et al. 2002, You et al. 2006).

Different types and sizes of microspheres as well as collagen and gelatin sponges are used to produce a temporary arterial hepatic occlusion, while polyvinyl alcohol is used to obtain a permanent embolization (Fiorentini et al. 2007, 2001, 2003, Lewis et al. 2006, Taylor et al. 2007). Lipiodol offers a microvascular occlusive activity and a pronounced affinity to be uptaken and retained by hepatic neoplastic cells, with a combined antitumor effect when emulsified with antiblastic drugs (Morise et al. 2006, Fiorentini et al. 2004).

Mitomycin-C (MMC), melphalan, cisplatin, epirubicin and irinotecan have been most commonly used chemotherapeutic agents (Aliberti et al. 2006, Morise et al. 2006, Fiorentini et al. 2004, 2007, Popov et al. 2002, Voigt et al. 2002, Vogl et al. 2007, Hunt et al. 1990, Martinelli et al. 1994, Salman et al. 2002, You et al. 2006)

Responses were observed in about 50% of the patients. The contraindications of this treatment are extensive liver involvement more than 75%, liver insufficiency, extra-hepatic disease, portal vein thrombosis

In the latter decade many efforts have been made to provide more accurate and tolerable TACE: dosage of drug's delivery to the liver, and new agents, were tested via intra-arterial infusion. (Fiorentini et al. 2001, 2003, Lewis et al. 2006, Taylor et al. 2007).

New microspheres have been studied to obtain a more precise and selective treatment. (Lewis et al. 2006, Taylor et al. 2007).

The most common complication experienced by almost all patients undergoing chemoembolization is "post-embolization syndrome", with pain in the right upper quadrant, nausea, vomiting, fever, elevation of liver enzymes. These adverse events are less pronounced when temporary vascular occlusive agents are used. Less common complications are liver abscess, tumor rupture, acute liver failure, infarction, acute pancreatitis, and acute renal failure (Hartnell et al. 1999, Huo et al. 2004a, b, López-Benítez et al. 2007, Sakamoto et al. 1998).

Intra-arterial lidocaine and adequate supportive care have been developed at our department to reduce the symptoms after TACE.

From December 2005 we carried out a study with intra-arterial lidocaine and intensive supportive treatment to make more tolerable the application of DC beads, a new embolic microsphere product, capable of being loaded with irinotecan before administration as TACE. We believe that an adequate supportive treatment can increase the safety, feasibility and tolerability of this procedure.

In this report we studied the impact on acute toxic effects, survival rate and quality of life of treated patients.

Patients and Methods

Thirty four patients were evaluated for recruitment. Eleven refused preferring palliative care or complementary medicine. Three had early progression. Thus, twenty were considered for this clinical study.

All patients had histologically confirmed liver metastases, with size $>$ 1cm of CRC and not amenable to surgery or further chemotherapy. The measurable liver tumour burden of not more than 70% of liver volume. Performance status was 0–2 (WHO criteria), life expectancy was at least 3 months, age was $<$ 85 years. All patients presented with multiple hepatic lesions with liver substitution grade I (less than 25% of involvement) in 8 out 20 patients, grade II (less than 50%) 7/20, grade III (till 75% of substitution) 5/20.

Liver function tests were normal or no more than two fold normal value.

Exclusion criteria history of inflammatory bowel disease, significant diseases of cardiac, renal, bone marrow or pulmonary apparatus, central nervous system involvement, uncontrolled infection, liver functional test and bilirubin three-fold or more normal value, and not understanding or not giving written consent.

All patients had a CT scan of the abdomen and pelvis, baseline complete blood cell count, carcinoembryonic antigen (CEA), lactate dehydrogenase (LDH), alkaline phosphatase, AST, total bilirubin, albumin, and creatinine. CT was used to determine the percentage of liver involvement and to assess response.

Of the twenty patients enrolled in the study one of them had one metastatic lesion and one presented with four lesions. Eight patients presented with multiple lesions with a median liver substitution of 40% (range 20–70%). All patients had prior chemotherapy for metastatic colorectal cancer, eight patients progressed after a partial response to FOLFOX and two after a partial response by FOLFIRI.

Treatment

Irinotecan drug-eluting beads were administered as TACE every 3 weeks (range 1–3) at a dose of 100 mg.

Program of Intra-Arterial Lidocaine and Supportive Treatment

The prophylactic treatment to prevent renal failure was intra-venous hydration started on day −1 and continued on day 0, +1, +2 with a bag of 2000 ml (1000 ml of saline solution, 1000 ml of glucose 5%)

To reduce the risk of gastric and pancreatic toxicity ranitidine 900 mg was added to the 24 hour infusion

The prophylactic treatment against nausea included Tropisetron 5 mg, 1 vial before TACE and 1 vial at +6 hours on day 0;

Dexamethasone 8 mg at 08.00 am and 08.00 pm on day 0,+1,+2,+3,+4,+5

The prophylactic treatment against pain included morphine 10 mg, 1 vial, 30 minutes before and at +6 hours

Intra-arterial lidocaine 5 ml was infused immediately before TACE (Lee et al. 2001, Romano et al. 2003, Hartnell et al. 1999)

Prophylactic treatment against infection was based on Cefazolin 2000 mg at 08.00 am and 08.00 pm day 0,+1,+2

Supportive treatment was continued per standard practice.

TACE Administration

The irinotecan loaded microspheres solution was prepared two hours before TACE.

Diagnostic angiography (DSA) was performed under fluoroscopic guidance. A solution of 2–4 ml of 100–300 um and/or 300–500 um irinotecan drug-eluting beads mixed with non-ionic contrast medium was injected into the artery feeding the metastases. A total of 40 individual TACE were performed with 100% of technical success. We performed 25 TACE with Irinotecan 100 mg preloaded in 2 ml of 300–500 μm microsphers and 15 TACE with Irinotecan 100 mg preloaded in 2 ml of 100–300 μm and 2 ml of 300–500 μm microspheres.

Six patients received one TACE, 8 patients received two TACE and 6 received three TACE.

Evaluation of Response and Toxicity

Clinical evaluation were scheduled before procedure at 1 month and every 3 months thereafter. Each visit included: clinical status, complete serum biochemistry, dynamic computed tomography. PET was considered optional. The compilation of Edmonton Symptom Assessment System (ESAS) questionnaire was carried out at every clinical control (Vignaroli et al. 2006). Response was evaluated by computed tomography (CT) scan one month after TACE to evaluate reduction of contrast enhancement to liver metastases. Complete response was defined by the disappearance of all lesional contrast enhancement.

Results

One month after TACE we observed a reduction of the lesional contrast enhancement of the lesions in 16/20 patients 80% of patients. We also found a reduction of more than 50% of CEA levels in 12 patients who expressed it initially. Fifteen out 20 are alive, with a median survival of 200 days (range 90–380). Responder patients demonstrated an improvement of their quality of life (16/20).

Mortality was due to: one myocardial infarction, three with progressive disease, one with pulmonary embolism. 15 out 20 patients are alive with median follow up time 8.6 months (range 3–12). The median survival time has not been reached. Regarding toxicity, all patients experienced grade 2 fever (WHO criteria) for 2 days (range 1–7), grade 2 and 3 right upper abdominal quadrant pain was observed in 10 and 5 patients respectively, short lasting, 12 hours (range 3–30). Finally grade 2 nausea and vomiting was observed in all patients, for a median duration of 11 hours (range 2–48). None of the patients experienced bone marrow toxicity or renal failure. One patient developed a liver abscess two days after TACE. Prolonged antibiotic therapy for 15 day has been done as required, avoiding a surgical approach. We observed one grade 3 toxicity of acute pancreatitis with spontaneous resolution. The median duration of hospitalization was 3 days (range 1–10).

Analysis of Edmonton Symptom Assessment System (ESAS) questionnaire showed and increase of QoL in all patients after 1, 3 and 6 months.

Discussion

In a palliative setting, the prior endpoint of any treatment should be quality of life, giving a secondary relevance to cancer outcomes (response rate) and pharmacoeconomic evaluation (Tassinari 2003). For patients with metastatic disease limited to the liver, regional approaches are able to provide local control of disease with less impact on quality of life, and relatively low cost. Selected patients could benefit from RFA (Machi et al. 2006, Berber et al. 2005).

When RFA cannot be proposed for a patient, TACE is a suitable procedure for effective hepatic control. Many efforts have been made to improve the outcome of

TACE, by integrating new chemotherapeutic agents and providing a more accurate dosage of drug's delivery to the liver. The more significant problem of TACE are side effects.

Sakamoto considered a total of 2,300 TACE procedures with a 2–15-mL injection of a mixture or suspension of anticancer drugs and iodized oil, followed by administration of gelatin sponge particles. One or two chemotherapeutic drugs, including doxorubicin hydrochloride (10–30 mg), epirubicin hydrochloride (10–30 mg), mitomycin C (10–20 mg), and cisplatin (25–100 mg), were used for each procedure. Complications were encountered in 4.4% of cases (n = 102) and were related to the use of chemoembolic agents or the manipulation of a catheter or guide wire. These complications included acute hepatic failure (n = 6), liver infarction (n = 4) or abscess (n = 5), intrahepatic biloma (n = 20), multiple intrahepatic aneurysms (n = 6), cholecystitis (n = 7), splenic infarction (n = 2), gastrointestinal mucosal lesions (n = 5), pulmonary embolism or infarction (n = 4), tumor rupture (n = 1), variceal bleeding (n = 3), and iatrogenic dissection (n = 35) or perforation (n = 4) of the celiac artery and its branches.

Based on this data and our previous experiences we developed a program of supportive care to reduce post embolization syndrome and now is of crucial value to obtain good compliance from the patients (Tassinari 2003, Lee et al. 2001, Romano et al. 2003, Hartnell et al. 1999, Huo et al. 2004, b, López-Benítez et al. 2007, Sakamoto et al. 1998). In brief the pain immediately during or after the embolic particles has been treated with systemic morphine and with intra-arterial lidocaine following previous evidence of clinical improvement reported in literature.

Lee et al (Lee et al. 2001) studied the efficacy of intraarterial lidocaine administration for control of pain resulting from TACE and to evaluate the optimal timing of administration. In a prospective trial, 113 consecutive patients with HCC who underwent TACE were classified into three groups: those who received a lidocaine bolus intra-arterially immediately prior to TACE (group A, n = 30), those who received lidocaine immediately after TACE (group B, n = 46), and those who did not received lidocaine (group C, n = 37). Incidence and degree of post-procedural pain was assessed using a subjective method (visual analogue scales scored from 0 to 10) and an objective method (amount of post-procedural analgesics). The incidence of post-procedural pain in group A (16.7%) was significantly lower than that of group B (38.3%; p = 0.005). The mean pain score was 3.0 in group A and 4.8 and 3.1 in groups B and C, respectively. The mean dose of analgesic used after the procedure in group A (25.0 mg) was significantly lower than those in group B (52.9 mg) and group C (41.0 mg; p = 0.002). Lee concluded that pre-TACE intraarterial administration of lidocaine is much more effective than post-TACE administration in reducing the incidence and the severity of post-procedural pain.

Romano (Romano et al. 2003) studied the efficacy of intraarterial lidocaine on peri- and post-procedural pain and on length of hospital stay in hepatocellular carcinoma (HCC) patients undergoing chemoembolization. Twenty-eight patients (19M, 9F, age range 49–76) who underwent hepatic chemoembolization were included in the study. Group A consisted of 14 patients who received intraarterial lidocaine immediately before and during chemoembolization, while in the 14 patients

of group B lidocaine was substituted with saline solution. The doses of centrally acting narcotics (tramadol) administered periprocedurally and in the three days following the procedure were compared, as were the hospitalization times. Subjective pain was measured using the visual analogue scale. Chemoembolizations were performed with an emulsion of lipiodol, cisplatin and epirubicin followed by embolizing material (gelfoam of Contour particles) in order to achieve complete blood flow stop in the proper hepatic artery. No side effects were noted that could be due to systemic administration of lidocaine. All patients experienced some degree of post-embolization syndrome. Periprocedural, day 1 and day 2 post chemoembolization dosages of tramadol were significantly lower in group A with respect to group B patients. No group A patient required analgesia on day 3. No statistical difference was observed in time persistence of nausea and vomiting, fever and hospitalization time between the two patient groups. They conclude that intra-arterial administration of lidocaine before and during chemoembolization is a safe and effective method for preventing or reducing peri- and post-procedural pain and dosage of narcotic analgesics in patients with HCC. Hospitalization times did not differ significantly between the two groups, probably because of the other components of post-embolization syndrome, such as fever, nausea and vomiting

Hartnell et al (Hartnell et al. 1999) reported if intraarterial lidocaine reduces pain during and after chemoembolization, and whether it influences postprocedure recovery. Two patient cohorts undergoing selective hepatic chemoembolization were compared. Chemoembolization was performed without lidocaine (control group) in 27 patients and intraarterial lidocaine was used (lidocaine group) in 29 similar patients. Objective changes in patient management were assessed. Pain reduction in 31 more procedures with lidocaine (total 60) was assessed and related to tumor type. During chemoembolization, intraarterial lidocaine reduced the need for additional intravenous analgesics from 69% to 19%. After chemoembolization the mean hydromorphone hydrochloride dose in the first 24 hr was reduced from 9.5 mg to 4.15 mg; accordingly, the mean length of hospital stay was reduced from 67.5 to 53.5 hr.

During the day of chemoembolization, the mean oral fluid intake increased from 420 ml (control group) to 487 ml (lidocaine group); the percentage of patients taking solid food on the day of chemoembolization increased from 3% to 43%. Hartnell concluded that intraarterial lidocaine during chemoembolization reduces the severity and duration of pain after chemoembolization resulting in faster recovery thus reducing the length of hospitalization

Other problem TACE related is acute renal failure, we did not observe any case because adopting intensive hydration. Huo et al. (Huo et al. 2004a) reported that this complication may occur after TACE because of radiocontrast agent. To investigate the incidence, risk factors and outcome of acute renal failure, defined as increase of serum creatinine > 1.5 mg/dL, after transarterial chemoembolization, this group studied a total of 235 hepatocellular carcinoma patients with 843 transarterial chemoembolization treatment sessions were analysed. Acute renal failure developed in 56 (23.8%) patients and the estimated risk of developing acute renal failure was 6.6% in each treatment session. Comparison between the episodes of transarterial

chemoembolization with and without acute renal failure by using the generalized estimating equation disclosed that Child-Pugh class B (odds ratio: 2.6, $P = 0.007$) and treatment session (odds ratio: 1.3; $P < 0.0001$) were independent risk factors of acute renal failure. Twenty-seven patients had prolonged renal function impairment. Multivariate analysis by generalized estimating equation showed that Child-Pugh class B (odds ratio: 4.3, $P = 0.0004$) and diabetes mellitus (odds ratio: 5.2, $P < 0.0001$) were linked with prolonged acute renal failure, which independently predicted a decreased survival (relative risk: 2.3, $P = 0.002$). Huo concluded that acute renal failure after transarterial chemoembolization appears to be dose-related and is associated with the severity of cirrhosis. Patients with diabetes mellitus or Child-Pugh class B more frequently develop prolonged acute renal failure, which in turn is a poor prognostic predictor.

A complication that we report is one case of acute pancreatitis (AP). Lopez-Benitez (Lopez-Benitez et al. 2007) reported that AP is a rare complication after TACE of primary and secondary liver tumours (approximately 1.7%), but it has a significant morbidity and mortality potential if associated with other complications. It usually develops early within 24 h after the TACE procedure. They analyzed factors involved in this procedure (anatomical features, embolization materials, cytostatic drugs, technical factors). 118 TACE (bland embolization and transarterial chemoembolization) were performed. The study group included 59 patients who met the following inclusion criteria: one or more liver embolization (LE) events, with complete pre- and post-interventional laboratory studies including: serum Ca^{2+}, creatinine, blood urea nitrogen, glucose, lactate dehydrogenase, aminotransferases, alkaline phosphatase, amylase, lipase, C-reactive protein, hematocrit and leukocytes. For the statistical analysis the association between two response variables (e.g. AP after embolization and risk factor during the embolization, AP after embolization and volume of embolic material) was evaluated. The calculated frequency of AP after TACE was 15.2%. Amylase and lipase were elevated up to 8.7 and 20.1 times, respectively, 24 h after LE. they observed a statistically significantly lower incidence of AP in those patients who received 2 ml or less of embolic agent compared with those with an embolization volume of >2 ml. Although carboplatin was administered to 7 of 9 of the patients who developed AP after the embolization procedure, there was no statistical significance (Fisher's exact test = 0.197) for carboplatin as an AP risk factor when compared with all the patients who received this drug ($n = 107$). Although AP seems to have a multifactorial etiology, both the toxicity of the antineoplastic drugs as well as direct ischemic mechanisms (non-target embolization, reflux mechanisms) may be the most important causes of the inflammatory pancreatic reaction. The authors suggest that systematic measurement of serum pancreatic enzymes should be performed in cases of abdominal pain following selective TACE in order to confirm acute pancreatitis which can clinically mimic a postembolization syndrome

In our experience twenty heavily pretreated patients underwent TACE in a palliative setting, observing a high response rate (80%), with significant reduction of lesional contrast enhancement in all responding patients.

Due to intensive supportive treatment, the procedure was well tolerated by most patients with a median duration of hospitalization of 3 days (range 1–10). The most

important adverse event was abdominal pain grade 2 in ten patients and grade 3 in five patients, especially during injection of irinotecan-Dc beads. Intra-arterial lidocaine is useful to reduce the post embolization pain. Morphine 10 mg, 1 vial intravenously should be used both before and after TACE. Our results suggest that TACE using irinotecan-eluting beads is feasible and active in pre-treated patients with liver metastases from CRC. We conclude that irinotecan-eluting beads-TACE of IRI 100 mg may be an appropriate palliative therapy for patients after chemotherapy failure. Adequate supportive treatment and intra-arterial lidocaine are necessary to optimize this approach reducing acute pain.

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Chapter 8
Significance of the Tumour Microenvironment in Radiotherapy

Michael R. Horsman and Dietmar W. Siemann

Abstract The growth and development of solid tumours require that they develop a functional vascular supply. But, inadequecies of this primitive and chaotic tumour neo-vasculature result in development of oxygen deprived areas. Hypoxic cells existing in this environment are resistant to radiation therapy. Numerous approaches have been developed to deal with this hypoxia-induced radioresistance. These include increasing oxygen delivery to tumours, chemically radiosensitising hypoxic cells, or killing them with specific cytotoxins. More recent approaches have concentrated on the tumour vascular supply itself. Many of these hypoxia targeted therapies have shown benefit in clinical trials, but while additional testing is ongoing for some of them, newer agents are continually being developed.

Keywords Tumour vasculature · Microenvironment · Hypoxia · Hypoxic modification · Radiation sensitivity

Microenvironmental Characteristics of Tumours

The development of a functional vascular supply is an essential requirement for the successful growth and development of most solid tumours (Brem et al. 1976; Folkman 1986). Typically tumours can only grow to a few millimetres in size before the nutritional needs of the neoplastic cell population exceed the capacity of existing blood vessels. Subsequent growth requires the formation of a tumour initiated vascular network, primarily by the process of angiogenesis (Hahnfeldt et al. 1999; Bergers and Benjamin 2003). The neo-vasculature that arises not only provides tumour cells with oxygen and nutrients necessary for survival, but also allows for the removal of toxic waste products associated with cellular metabolism (Siemann et al. 2004), and is the principal vehicle for metastatic spread (Stoeltzing and Ellis 2006).

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The triggers that initiate this angiogenic process in tumours are not fully established. Reduced oxygenation, or hypoxia, is clearly one factor that plays a significant role (Pugh and Ratcliffe 2003), but other "non-hypoxia factors" including loss of tumour suppressor gene function and oncogene activation have also been implicated (Ferrara et al. 2003; Moeller et al. 2004; Pugh and Ratcliffe 2003). Whatever the trigger, the angiogenic process begins with the release of angiogenic factors primarily vascular endothelial growth factor (VEGF) by the tumour cells (Ferrara et al. 2003).

These growth factors then initiate a series of physical steps including local degradation of the basement membrane surrounding capillaries, invasion into the surrounding stroma by the endothelial cells in the direction of the angiogenic stimulus, proliferation of the endothelial cells and finally their organization into three-dimensional structures that connect with other similar structures to form the new blood vessel network (Bergers and Benjamin 2003). Although angiogenesis is seen with a number of other pathological conditions, such as rheumatoid arthritis, diabetes, macular degeneration, psoriasis, and cardiovascular disease (Carmeliet 2003), as well as certain physiological processes, including the female reproductive cycle, pregnancy, muscular hypertrophy and wound healing (Carmeliet 2003), the vasculature of tumours is very different from that of normal tissues (Vaupel et al. 1989; Vaupel 2004). Structurally, it is very chaotic, there is a loss of hierarchy, vascular density is abnormal, vessels have contour irregularities, and are tortuous and elongated. The vessels are also very primitive in nature, having incomplete or missing basement membranes and endothelial lining, and lacking pericytes, smooth muscle and pharmacological receptors. In addition, there are numerous functional abnormalities, including unstable speed and direction of blood flow, high vascular resistance, increased vascular fragility, red blood cell sludging, leukocyte sticking, and blockage of vessels by circulating white blood cells or tumour cells. All these factors result in a vascular supply that is unable to meet the oxygen and nutrient demands of the growing tumour mass, thus areas develop within the tumour that are oxygen deprived (hypoxic), nutrient deficient, and highly acidic (Vaupel et al. 1989; Vaupel 2004).

The first real indication that hypoxia existed in tumours was made by Thomlinson and Gray (1955). These authors demonstrated on histological sections of carcinoma of the bronchus that while typically viable tumour regions were surrounded by vascular stroma from which the tumour cells obtained their nutrients, these regions grew areas of necrosis in the tumor center. The thickness of the resulting shell of viable tissue was measured between 100–180 μm, which was within the range of the calculated distance oxygen diffused in respiring tissues. It was thus suggested that as oxygen diffused from the stroma, it was consumed by the cells, and although those beyond the diffusion distance were unable to survive, the cells immediately bordering the necrosis might be viable yet hypoxic. Later, an inverted version of the Thomlinson and Gray picture was described, with functional blood vessels surrounded by cords of viable tumour cells outside of which were areas of necrosis (Tannock 1968). This "corded" structure is illustrated in Fig. 8.1 and is the more typical picture found in most solid tumours. The hypoxia that develops

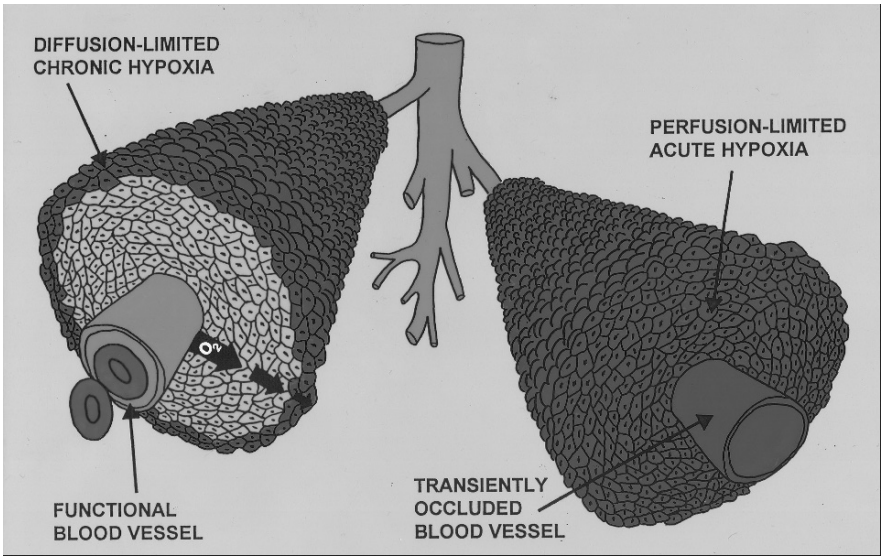

Fig. 8.1 In this schematic illustration tumour cells are seen growing as a "corded" structure around blood vessels from which the cells receive their oxygen and nutrient supply. The left side shows that as oxygen diffuses out from the vessel it is consumed thus creating an oxygen gradient, with the outmost viable cells becoming oxygen deprived or chronically hypoxic. On the right side, blood prefusion through the vessel has been temprorily stopped, thus making all the cells oxygen deprived or acutely hypoxic. Figure modified from Horsman 1998

is more commonly referred to as diffusion limited chronic hypoxia (Horsman and Overgaard 1992). A decade later, it was also suggested that hypoxia in tumours could be acute in nature (Brown 1979). However, it was not until the late 1980s that Chaplin and colleagues were able to confirm the existence of acutely hypoxic cells in tumours and demonstrate that these cells were the result of transient stoppages in tumour blood flow (Chaplin et al. 1987). This perfusion limited acute hypoxia is also illustrated in Fig. 8.1. To-date, the temporary cessations in blood flow that account for this have been observed in mouse and rat tumours, as well as human tumour xenografts, with anywhere from around 4 to 8% of the total functional vessels involved (Horsman 1995). Limited data also implicates their existence in human tumours (Powell et al. 1997). The exact causes of these transient stoppages are not known, but suggested mechanisms include plugging of vessels by circulating blood or tumour cells, collapse of vessels in regions of high interstitial pressure, or spontaneous vasomotion in incorporated host arterioles affecting blood flow in down-stream tumour vessels (Horsman 1995). The current use of "chronic and acute" to explain hypoxia in tumours is also an oversimplification of the real situation. Chronic hypoxia generally refers to prolonged and reduced oxygen concentrations that influence radiation response, but there is evidence that the oxygen concentrations in cells close to vessels can start as low as 5% (Helmlinger et al. 1997), which have no influence on radiation sensitivity, but are well below

normal physiological levels, and could influence drug activity and gene/protein regulation. Furthermore, reduced perfusion can be both partial as well as total (Kimura et al. 1996). A complete shut-down would temporarily starve cells of both oxygen and nutrients, while with a partial closure one could have plasma flow in the absence of any red blood cells, and this would result in a deficiency in oxygen, but not nutrients. It is likely that the characteristics of cells and their respsonse to therapy under these different types of acute hypoxia would be different. However we define hypoxia, its presence has now been identified in most solid animal tumour models (Moulder and Rockwell 1984) and numerous human cancers (Vaupel et al. 1989).

Influence of the Tumor Microenvironment on Radiation Therapy

The first radiobiologically oriented clinical study implicating the importance of microenvironmental parameters on the outcome of radiotherapy was reported in 1909. In an elegant experiment Schwarz demonstrated that radiation response of skin was markedly decreased if the blood flow in the irradiated area was reduced by compression (Schwarz 1909). The following year it was reported that tissues in which blood flow was stimulated by local heating showed a more prominent response to radiation (Müller 1910). Although neither study actually attributed the effects to an oxygen dependency they were clearly the first studies to demonstrate the importance of the tumour microenvironment in influencing radiotherapy. The latter study was also the first to show how radiation response could be improved by modifying the tumour microenvironment. Further experimental and clinical observations indicated the importance of sufficient blood supply to secure an adequate radiation response, which led Gray and co-workers to finally postulate the role of oxygen deficiency as a major source of radiation resistance (Gray et al. 1953).

Experimentally, radiation resistance resulting from tumour hypoxia can readily be demonstrated by clamping the tumour blood supply prior to irradiating the tumour. To determine the proportion of hypoxic cells existing in tumour models three approaches are typically employed. These are the paired survival curve assay, which involves excising tumours cells after irradiation and measuring the viability of neoplastic cells using a clonogenic cell survival assay; the clamped tumour growth delay assay, where measurements are made of the time taken for tumours to reach a specific size after treatment; and the clamped tumour control assay, in which the percentage of animals showing local tumour control at a certain time after treatment is recorded. For each technique it is necessary to produce full radiation dose-response curves under air breathing and fully anoxic (clamped tumour) conditions. The hypoxic fractions can then be calculated from the displacement of the dose-response curves. Using these assays the hypoxic fractions have been estimated to range from 1% to well over 50% of the viable tumour cells in animal tumours and human tumour xenografts (Moulder and Rockwell 1984).

Attempts to demonstrate that hypoxia could influence the radiation response of human tumours proved to be more difficult. Originally, indirect approaches were

used (Horsman 1998). These were based on estimates of tumour vascularisation, using such endpoints as intercapillary distance, vascular density and the distance from tumour cells to the nearest blood vessel (Kolstad 1968; Lauk et al. 1989; Révész et al. 1989). They showed that patients with less well vascularised tumours, and presumably more hypoxic, had a poorer outcome to radiation therapy. Later, more direct methods of hypoxia assessment were developed and tested (Raleigh et al. 1996; Horsman 1998). These included binding of exogenous markers; drugs injected into the host that in regions of hypoxia were converted to a reactive species which would subsequently bind to cellular macromolecules. Bound products could then be identified immunohistochemically from histological sections (Raleigh et al. 1996; Olive and Aquino-Parsons 2004) or non-invasively using positron emission tomography (Rasey et al. 1996; Piert et al. 2005), SPECT (Urtasun et al. 1996) or magnetic resonance spectroscopy (Seddon et al. 2003). The binding of exogenous markers has been correlated to other techniques for measuring hypoxia, and even associated with outcome following radiation therapy in head and neck carcinoma patients (Bussink et al. 2003). An alternative approach involved measuring the levels of endogenous genes/proteins that were upregulated under hypoxia. These include carbonic anhydrase IX, GLUT-1, Hypoxia Inducible Factor-1 and osteopontin (Hui et al. 2002; Airley et al. 2003; Le et al. 2003; Overgaard et al. 2005). Although the upregulation of such endogenous markers has been correlated with outcome to radiation therapy in some studies it is not a universal finding (Bussink et al. 2003), which probably reflects the fact that many of these endogenous markers are not hypoxic specific rather than any indication that hypoxia does not play a role in influencing radiation response. Probably the most direct method for estimating tumour hypoxia is the measurement of oxygen partial pressure (pO_2) distributions with polarographic electrodes (Kolstad 1968; Gatenby et al. 1988; Hoeckel et al. 1996; Brizel et al. 1996, 1997; Nordsmark et al. 1996, 2001). Using this technique the role of hypoxia in influencing both the outcome to radiation therapy and other non-radiation based treatments has clearly been demonstrated (Kolstad 1968; Gatenby et al. 1988; Hoeckel et al. 1996; Brizel et al. 1996, 1997; Nordsmark et al. 1996, 2001, 2001b).

Modification of Microenvironmental Induced Radioresistance

Increasing Oxygen Delivery

Since the oxygen supply to tumors is insufficient to meet the needs of all the tumour cells, thus giving rise to radiation-resistant hypoxia, an obvious solution to improving tumour radiation response would be to increase the oxygen supply. This has been tried, both experimentally and clinically, by simply allowing the tumour bearing host to breathe high oxygen content gas mixtures before and during irradiation.

Early experimental studies reported that both oxygen and carbogen (95% oxygen + 5% carbon dioxide) breathing could substantially enhance the response

of murine tumors to radiation and that the best effect was generally seen when the gasses were inspired under hyperbaric (typically 3 atmospheres) rather than normobaric conditions (Du Sault 1963; Suit et al. 1972). This is perhaps not surprising because hyperbaric conditions would be expected to saturate the blood with oxygen more than normobaric conditions. However, later studies indicated that the radiosensitisations produced by normabaric oxygen or carbogen are in fact quite substantial (Siemann et al. 1977; Rojas 1991; Grau et al. 1992). Such an effect is illustrated in Fig. 8.2.

Clinically, the use of high oxygen content gas breathing, specifically under hyperbaric conditions, was introduced relatively early by Churchill-Davidson (1968). Most trials were fairly small, and suffered from the applications of unconventional

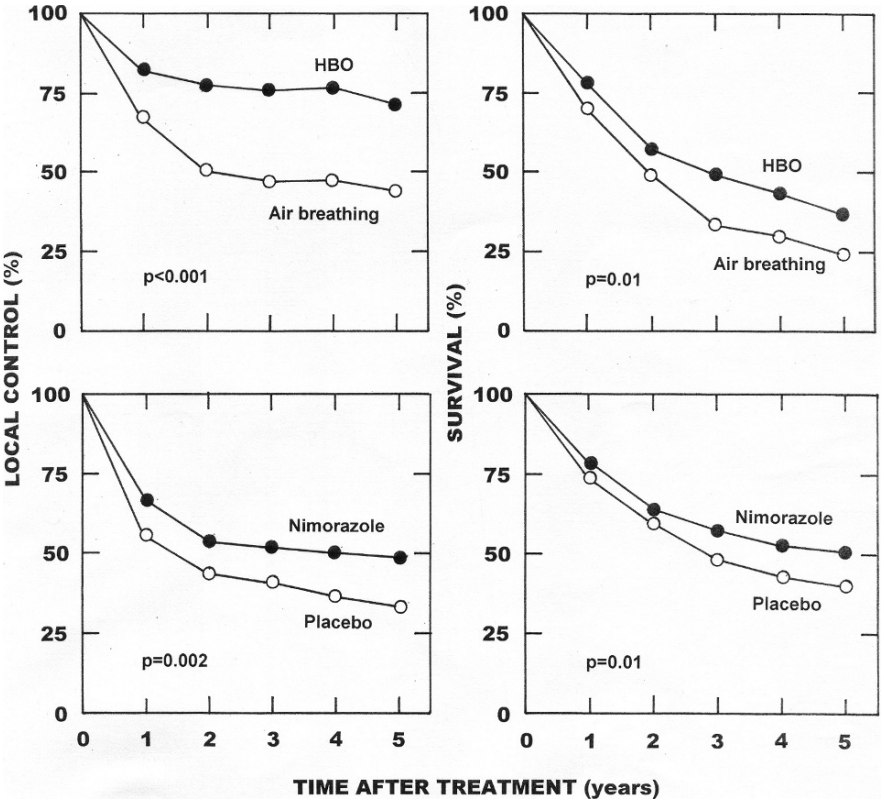

Fig. 8.2 Results of clinical randomised trials in which patients received hypoxic modification in conjunction with radiotherapy. *Top panels* – Results from the Medical Research Council hyperbaric oxygen trial showing actuarial local tumour control and survival in patients with stage III carcinoma of the cervix treated with either HBO (•; 119 patients) or air breathing (○; 124 patients); modified from Watson et al. (1978). *Bottom panels* – Results from the DAHANCA 5 study showing actuarial estimated loco-regional control and disease-specific survival rate in patients given nimorazole (•; 219 patients)or placebo (○; 195 patients). Figures modified from Overgaard et al. (1998)

fractionation schemes, but it appeared that the effect of hyperbaric oxygen was superior to radiotherapy given in air, especially when few and large fractions were applied (Churchill-Davidson 1968; Dische 1979; Dische et al. 1983). Significant improvements in local tumour control and subsequent survival were certainly seen in the large multicenter clinical trials conducted by the UK Medical Research Council for both uterine cervix (Fig. 8.3) and advanced head and neck (Dische 1979;

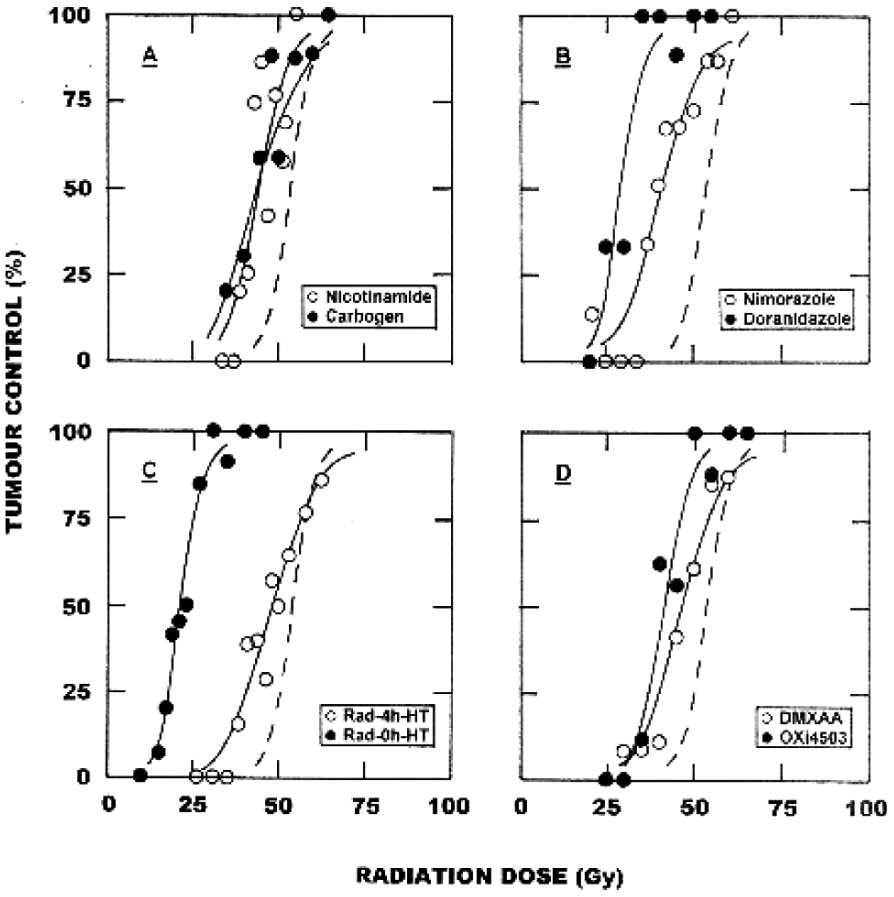

Fig. 8.3 Groups of CDF1 mice implanted with a C3H mammary carcinoma had their tumours locally irradiated with single doses of radiation. The percentage of animals showing tumour control within 90 days was recorded and the dose response curve determined following logit analysis. This is shown as the dashed line in each figure. Also shown is how this radiation response curve is modified following treatment with either (**A**) carbogen breathing for 5 minutes before and during irradiation (●) or an intraperitoneal (i.p.) injection with 1000 mg/kg nicotinamide 30 minutes before irradiating (○); (**B**) i.p. injecting 500 mg/kg doranidazole (●) or nimorazole (○) and irradiating 30 minutes later; (C) heating tumours at 42.5°C for 60 minutes either at the same time (●) or 4-hours after (○) irradiating; (D) irradiating and then within 1-hour i.p. injecting 50 mg/kg OXi4503 (●) or 20 mg/kg DMXAA (○). Data modified from Horsman et al. (1990), Grau et al. (1992), Horsman and Murata (2004), Murata et al. (2008), or unpublished observations

Henk et al. 1977; Henk and Smith 1977; Watson et al. 1978; Overgaard 1989). The same benefit was not observed in bladder cancer; nor was it repeated in a number of smaller studies (Overgaard 1989). In retrospect, the use of hyperbaric oxygen was stopped somewhat prematurely. This was partly due to the introduction of chemical hypoxic cell radiosensitizers, and partly because of patient compliance concerns.

The use of high oxygen content gas breathing under normobaric conditions, to radiosensitize human tumours, has also been tried clinically but the early studies failed to show any dramatic improvement (Bergsjø and Kolstad 1968; Rubin et al. 1979). One possible explanation may have been the failure to achieve the optimum pre-irradiation gas breathing time, since a number of experimental studies have shown this to be critical for the enhancement of radiation damage, and that it can vary from tumour to tumour (Suit et al. 1972; Siemann et al. 1977; Rojas 1991; Chaplin et al. 1993). More recent studies in head and neck using a short pre-irradiation breathing time have yielded conflicting findings. A University of Florida study failed to demonstrate any benefit of carbogen breathing (Mendenhall et al. 2005), whereas one from the University of Nijmegen was beneficial (Kaanders et al. 2002). One possible explanation for the positive results in the latter study was that carbogen was administered as part of the ARCON (Accelerated Radiation, CarbOgen, and Nicotinamide) therapy. The accelerated radiation part is included to address the issue of accelerated repopulation that is known to occur after irradiation (Petersen et al. 2001). Nicotinamide was included to deal with acute hypoxia which is not likely to be influenced by high oxygen content breathing alone. Experimental studies have clearly demonstrated that the vitamin B3 analog, nicotinamide, can enhance radiation damage in a variety of murine tumor models using both single (Fig. 8.2) and fractionated treatments (Horsman 1995). This enhancement of radiation damage appears to be dependent on the tumour type, drug dose and time of irradiation after drug administration (Horsman 1995). Nicotinamide can also enhance radiation damage in certain normal tissues, but in general the effects are less than those seen in tumours (Horsman 1995). The effect of nicotinamide seems to primarily involve the drug preventing/reducing the transient fluctuations in tumor blood flow, which normally lead to the development of acute hypoxia. This finding led to the suggestion that the optimal approach would be to combine nicotinamide with treatments that specifically overcome chronic hypoxia, and was subsequently demonstrated in combination with hyperthermia (Horsman et al. 1990), perfluorochemical emulsions (Chaplin et al. 1991), and carbogen breathing (Kjellen et al. 1991; Chaplin et al. 1993; Horsman et al. 1994).

One of the major factors influencing the delivery of oxygen to tumours is the concentration of haemoglobin. It is, therefore, not surprising that low haemoglobin concentration in general has a negative impact on tumour radiation response. In a review of 51 studies involving 17272 patients the prognostic relationship between haemoglobin concentration and local tumour control were analysed and of these, 39 studies (14482 patients) showed a correlation, while only 12 (2790 patients)

did not (Grau and Overgaard 1998). However, a large (357 patients) international multi-center study in head and neck failed to show any correlation between haemoglobin concentration and pre-treatment pO_2 measurements questioning the relationship between haemoglobin concentration and tumour oxygenation status (Nordsmark 2005).

Nevertheless, the potential benefit of increasing haemoglobin by blood transfusion prior to radiotherapy has been investigated in a number of studies (Thomas 2002). The first clinical investigation of this approach was in advanced squamous cell carcinoma of the uterine cervix (Evans and Bergsjø 1965). Transfusion to patients with low haemoglobin level resulted in an increased oxygen tension within the tumour, as measured directly using oxygen electrodes. The same study was also the first to show that transfusion to a haemoglobin level of 11 g/dL or higher was able to significantly improve survival. A Canadian retrospective study of 605 cervix cancer patients showed that the negative influence of low haemoglobin on prognosis could be overcome by transfusion (Grogan et al. 1999). However, these observations have not been supported by data from controlled randomized trials; a prospective phase III trial, from the Danish Head and Neck Cancer (DAHANCA) study group, failed to demonstrate any benefit of transfusion in head and neck cancer patients with low haemoglobin levels (Overgaard et al. 1998).

Increasing the haemoglobin concentration by stimulation with the hormone erythropoietin (EPO) has also been investigated. Several preclinical studies have shown that the induction of anemia in animals could be corrected by serial injection with EPO and that this EPO treatment also overcame the anemia-induced radiation resistance (Thews et al. 1998; Stuben et al. 2003). The concept of using EPO to correct for anemia has also been tested in a few clinical trials. However, although low haemoglobin can be effectively and safely improved by EPO (Lavey and Dempsey 1993), a number of studies in patients undergoing treatment for head and neck cancer failed to show any benefit (Henke et al. 2003; Machtay et al. 2007; Overgaard et al. 2007). More significantly, those patients that actually received EPO in conjunction with radiation therapy did significantly worse than those patients that did not receive EPO and as a result all EPO and radiation trials have been stopped.

Other "haemoglobin-related" methods for improving tumor oxygenation have been investigated. These include the use of artificial blood substances, such as perfluorocarbons (Rockwell 1985), which are small particles capable of carrying more oxygen than haemoglobin, or by manipulating the oxygen unloading capacity of blood by modifying the oxy-haemoglobin dissociation curve. This can be achieved either by increasing the red blood cell 2,3-DPG content (Siemann and Macler 1986), 2,3-DPG being one of the most important allosteric factors controlling the haemoglobin-oxygen dissociation curve, or using antilipidaemic drugs (Hirst and Wood 1991). Although each of these approaches has been shown to improve the oxygenation status of experimental tumours and/or enhance radiation damage, none of them have yet reached controlled clinical testing, thus their potential usefulness in the clinic is uncertain.

Radiosensitizers

An alternative approach to the hypoxia problem, and the one that has been most extensively investigated especially in clinical trials, is the use of chemical agents that mimic oxygen and preferentially sensitize the resistant population to radiation. The advantage of these drugs over oxygen is that they are not rapidly metabolized by the tumor cells through which they diffuse and thus can penetrate further than oxygen and so reach all cells in the tumour. In the early 1960s it was found that the efficiency of radiosensitization was directly related to electron-affinity (Adams and Cooke 1969) and that ultimately led to in vitro studies demonstrating preferential radiosensitization of hypoxic cells by highly electron-affinic nitroaromatic compounds (Asquith et al. 1974, Adams et al. 1976). Several of these compounds were later shown to be effective at enhancing radiation damage in tumours (Fig. 8.2; Overgaard 1994), and as a result they underwent clinical testing.

The initial clinical studies were with metronidazole in brain tumours (Urtasun et al. 1976) and were followed, in the latter part of the 1970s, by a series of clinical trials exploring the potential of misonidazole as a radiosensitizer (Dische 1985, Overgaard 1989 and 1994). Most of the trials with misonidazole were unable to generate any significant improvement in radiation response, although a benefit was seen in some trials, especially the DAHANCA 2 study (Overgaard et al. 1989).

While lack of benefit with misonidazole in most studies could be attributed to clinical evaluation in tumour sites lacking convincing evidence of the existing of hypoxia and the small number of patients in many of the trials, a more likely explanation is that the drug doses necessary for effective radiosensitisation also produced substantial dose-limiting clinical toxicity. As a result there followed a search for more efficient or less toxic hypoxic sensitizers. Subsequent trials evaluated pimonidazole, etanidazole and nimorazole. The European pimonidazole trial in uterine cervix was very disappointing (Dische et al. 1993), while the two other multicenter trials in head and neck cancer, using etanidazole, showed no benefit (Lee et al. 1995; Eschwége et al. 1997). On the other hand, studies with the less toxic drug nimorazole given to patients with supraglottic and pharynx carcinomas (DAHANCA 5) showed a highly significant benefit in terms of improved loco-regional tumor control and disease-free survival (see Fig. 8.3), thereby confirming the result of the DAHANCA 2 study (Overgaard et al. 1998).

Though results have been somewhat conflicting, the benefit of using hypoxic cell radiosensitizers was cleary demonstrated in a meta-analysis of all randomised clinical studies in which hypoxic radiosensitizers were given with primary radiotherapy (Overgaard and Horsman 1996). This analysis, involving more than 10000 patients in 83 randomized trials, showed that radiosensitizer modification of tumour hypoxia significantly improved the loco-regional tumor control after radiotherapy by an order of 5–10%, primarily the result of an improved response in head and neck and to a lesser extent in bladder tumours. No significant benefit was observed in other tumour sites (cervix, lung, central nervous system and oesophagus). Although the overall observed improvement in local control was small, such gains are certainly relevant, especially because they are associated with a similar improvement

in survival. In retrospect, it now seems clear that the non-significant outcome of most hypoxic sensitiser clinical trials was not due to a biological lack of importance of hypoxia, but rather a consequence of poor clinical trials methodology with a too optimistic study design and an expected treatment gain which far exceeded what was reasonable. Overall, results with nitroimidazoles add to the general concensus that if a non-toxic hypoxic modification can be applied, then such treatments may be relevant as baseline therapy with radiotherapy for certain types of cancers. Such a strategy has been adopted in Denmark where nimorazole has become part of the standard radiotherapy treatment in head and neck. In addition, there is still interest in finding new sensitizers and a number of these new agents (e.g., Sanizole and Doranidazole) have now reached clinical evaluation and the preliminary results suggest benefits (Dobrowsky et al. 2007; Karasawa et al. 2008).

One of the most effective radiation sensitizers known is hyperthermia (Horsman and Overgaard 2007). Numerous pre-clinical studies have shown both in vitro (Li and Kal 1977, Sapareto et al. 1979; Hahn 1982) and in vivo (for review see Horsman and Overgaard 2007) that irradiating tumors and heating with temperatures up to 43°C at around the same time substantially enhanced radiation response (Fig. 8.2). The exact mechanism by which heat sensitizes cells to radiation is not known, but most evidence suggests that heat primarily interferes with the cells ability to deal with radiation-induced DNA damage (Kampinga and Dikomey 2001, Roti Roti 2004). This large body of pre-clinical data showing the benefit of combining radiation and hyperthermia has resulted in the translation of this approach into a number of clinical trials. A meta-analysis of all published trials (1861 patients from 23 trials), in which patients were randomised to radiation or radiation and heat, demonstrated significant improvements in local tumour control, the most relevant endpoint for such locally applied treatments, in a number of distinct sites including chest wall, cervix, rectum, bladder, melanoma, and head and neck (Horsman and Overgaard 2007). Some of these clinical studies even reported improvements in overall survival (Horsman and Overgaard 2007).

Exploiting Tumour Hypoxia Using Hypoxic Cell Cytotoxins

The overall benefit of combining hyperthermia with radiation is not just a consequence of heat-induced radiosensitization. Part of the improvement is due to an additional indirect mechanism that results from the heat killing the radioresistant hypoxic cell population (Fig. 8.2). Several in vitro studies have reported that cells under hypoxic conditions are more sensitive to the lethal effects of hyperthermia than cells in a well-oxygenated environment (Overgaard and Bichel 1977, Suit and Gerweck 1979, Gerweck et al. 1979). Under well-defined nutrient conditions, acute hypoxia alone does not have any significant influence on the cellular response to hyperthermia (Gerweck et al. 1974, Power and Harris 1977, Gerweck et al. 1979). However, prolonged oxygen deprivation or chronic hypoxia will increase cellular heat sensitivity (Gerweck et al. 1979, Overgaard and Nielsen 1980, Nielsen 1981).

Prolonged hypoxia generally leads to metabolic changes, which in turn alter several other parameters such as acidity, and it is these changes that are responsible for the increased cell killing by heat (Gerweck et al. 1974, Hahn 1974, Overgaard and Bichel 1977, Overgaard and Nielsen 1980).

Numerous bioreductive drugs have also been developed to specifically kill hypoxic cells. These are compounds that undergo intracellular reduction to form active cytotoxic species. Much of the development of these agents arose after it was discovered that the electron-affinic radiosensitizers discussed earlier, which were relatively non-toxic to cells under normal oxygenated conditions, were reduced to a more toxic form under hypoxia (Hall and Roizin-Towle 1975). This ability to preferentially kill the radiation resistant tumour cell population makes bioreductive drugs an excellent choice for improving radiation response.

The prototype bioreductive drug is mitomycin C (Kennedy et al. 1980), which is activated by bioreduction to form products that cross-link DNA. It has been used for many years in patients as a chemoradiosensitizer, but there are also several studies in which it has been used to specifically overcome hypoxic cell radioresistance. In two randomised clinical trials in patients with squamous cell carcinoma of the head and neck, mitomycin C improved radiation-induced local tumour control without any enhancement of radiation reactions in normal tissues (Weissberg et al. 1989, Haffty et al. 1993). However, in two other trials no major influence on response or survival was seen (Dobrowsky et al. 1995, Grau et al. 2003). This absence of any improved response is perhaps not surprising since the drug was only given once during the radiation schedule and also shows little differential between aerobic and hypoxic cell killing (Stratford and Stephens 1989, Hall 1994).

The finding that misonidazole showed preferential toxcity towards hypoxic cells led to numerous efforts to find other nitroimidazole radiosensitizers that were effective as hypoxic cell cytotoxins. To that end RSU 1069 was developed. This compound has the classic 2-nitroimidazole radiosensitizing properties, but an aziridine ring at the terminal end of the chain gives the molecule substantial potency as a hypoxic cell cytotoxin. Although the drug was found to have substantial activity in hypoxic cells in vitro and in tumours in vivo (Stratford et al. 1986) preliminary clinical studies indicated dose-limiting gastrointestinal toxicity. A less toxic prodrug, RB 6145, which in vivo is reduced to RSU 1069, was also shown to have potent anti-tumour activity in experimental systems, but was dropped when toxicity studies revealed significant side effects in dogs.

Another group of bioreductives which show considerable promise are the organic nitroxides, of which the benzotriazene di-N-oxide, tirapazamine, is the lead compound. The parent moiety shows limited toxicity towards aerobic cells, but after reduction under hypoxic conditions a product is formed which has been shown to be highly toxic to both cells in vitro and can substantially enhance radiation damage in tumours in vivo (Zeman et al. 1988). Later studies demonstrated that this bioreductive drug was very effective at enhancing the anti-tumour activity of cisplatin (Dorie and Brown 1993). These findings led to clinical testing of tirapazamine, with some 9 phase I trials and 15 phase II/III trials being published (McKeown et al. 2007). Although the results from the phase II trials were promising, limited benefit was

seen in the few randomised phase II/III studies (McKeown et al. 2007). Another promising N-oxide is AQ4N, which has been shown to be effective at enhancing radiation response in animal tumours (McKeown et al. 1996) and is currently undergoing phase I/II clinical testing (McKeown et al. 2007).

Vascular Targeting Agents

The tumour vasculature not only plays a significant role in determining the microenvironment within tumours, it is also essential for the successful growth and development of those tumours (Folkman 1986). This makes the vasculature an attractive target for therapy and two major groups of vascular targeting agents (VTAs) have emerged (Siemann et al. 2005); one that involves preventing the development of the tumour vasculature by inhibiting various steps in the angiogenic process (angiogenesis inhibiting agents; AIAs) and one that damages the already established tumour vessels (vascular disrupting agents; VDAs).

Although both types of VTA mediate anti-tumour effects, they never result in tumour control, even when used in combination. This has led to the suggestion that their potential clinical application would be when combined with more conventional treatments, especially radiation. In fact, numerous pre-clinical studies have now shown that the response of tumours to radiation can be significantly improved when animals are treated with either AIAs or VDAs (Horsman and Siemann 2006). This is illustrated in Fig. 8.3 for VDAs and radiation. For both types of VTA, hypoxia has been implicated in the mechanism for this enhancement of radiation response. A number of pre-clinical studies have clearly demonstrated that AIAs can improve the oxygenation status of tumours (for review see Horsman and Siemann 2006). For one of these studies this improvement corresponded to a transient period of stabilization of the tumour vessels, in which less mature vessels were destroyed and other vessels were stabilized by the recruitment of pericytes (Winkler et al. 2004), a period that has been the "normalization window" (Jain 2001). However, this is not a universal finding because a larger number of studies reported either no improvement in tumour oxygenation status or even a significant decrease with AIA treatment (Horsman and Siemann 2006), suggesting that changes in oxygenation status may not be the only factor involved in the enhancement of radiation response by such agents. With VDAs the situation is less controversial. VDAs damage tumour vasculature and as a result cause a severe reduction in tumour blood flow leading to ischemia and cell death (Horsman and Siemann 2006). The improved antitumour responses observed when such agents are combined with radiotherapy likely reflects an additive tumour response resulting from the VDA eliminating treatment resistant hypoxic tumour cells while the radiotherapy acts against the aerobic tumour cell population. Still, the timing of VDA therapy relative to radiation treatment clearly is critical (Wilson et al. 1998; Murata et al. 2001a, b; Siemann and Rojiani 2002), since VDAs can induce hypoxia resulting in a possible "double-edged sword" phenomenon, when combining VDAs with radiation therapy.

Conclusions and Future Perspectives

In an era in which targeted therapies are proposed as the future for cancer therapy, then hypoxia must be considered the ultimate target. It is a fundamental feature of virtually all solid tumours, whether animal or human, that can be identified by a host of clinically applicable techniques. There is definitive evidence that its existence in specific tumour types will have a significant negative impact on cancer treatment, especially radiotherapy. Numerous preclinical studies have identified a range of different approaches that can reduce or eliminate hypoxia and preferentially improve tumour radiation sensitivity, and many of these have undergone successful clinical testing within substantial improvements to radiation therapy demonstrated. However, except for Denmark in which the hypoxic cell radiosensitiser nimorazole is routinely used in the treatment of head and neck cancer, none of these hypoxic modifiers have become established as a standard therapy with radiation. Whether this is a consequence of the non-glamorous nature of this target, the lack of potential profit from using easily obtainable treatments, or some other unexplained reason is not known. Nevertheless, despite this negative situation, extensive preclinical and clinical studies are ongoing in this area, so hopefully it is simply a matter of time before the hypoxia problem is eliminated.

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Chapter 9
Effects of Tumor Microenvironment on Immunity and Consequent Clinical Considerations

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The process of scientific discovery is, in effect, a continual flight from wonder

Albert Einstein

Abstract Immune response to cancer is a dynamic process in which uncontrolled growth of cancer cells is countered by various protective mechanisms. However, the progression of the disease can be interpreted as failure of the antitumor immune response. Recent studies indicate host immune response cooperates with cancer cells to promote their growth and dissemination through the production of several growth factors, cytokines, angiogenic factors, free radicals and proteolytic enzymes that are produced by stroma cells coordinated by tumor cells. Among the various microenvironmental factors hypoxia seems to play a central role in these processes and in promoting mechanisms leading to evasion of the immune system. Furthermore, recent discovery of a positive interaction with convectional therapeutic modalities, such as some chemotherapeutic drugs and radiotherapy, will permit new cocktails able to kill directly and indirectly cancer cells collaborating with the host immune system.

Keywords Tumor microenvironment · Tumor hypoxia · Immunoediting · Cytokines · Regulatory T-cells · NF-kB · Inflammation · Macrophages · ROS · HIF · Tumor immunity

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Introduction

There is considerable evidence that tumors are immunogenic and that cancer patients are capable of mounting an immune attack on their tumor cells (Houghton, Guevara-Patiño 2004). Thomas and Burnet, in 1957, proposed the theory of immune surveillance to describe the role of the immune system in controlling and mitigating neoplastic growth (Burnet 1997). The concept of immune surveillance puts forward that immune cells patrol the host for foreign and mutated cells. This theory, however, does not completely explain the appearance of tumor in the presence of an intact immune system, so the concept has been replaced by a recent theory of immune editing. Immune editing is a more complete explanation of the interaction of the immune system during the tumor development (Swann, Smyth 2007). The tumor immune editing concept comprises three main phases: elimination, equilibrium and escape. "This process is responsible for both eliminating tumors and sculpting the immunogenic phenotypes of tumors that eventually form in immunocompetent hosts" (Dunn). The inflammatory reaction that normally happens as a first line of defense is generated by the innate immune system (Stewart et al. 2007). This inflammatory reaction, generated by macrophages and natural killer cells (NKs) and coordinated by dendritic cells (DCs), is, however, incomplete or not sufficient to eradicate tumor mass (Ulrich, Sica, Stewart). Furthermore, the associated inflammation, with increased production of cytokines, chemokines and additional soluble mediators, creates an environment that facilitates tumor progression and contributes to inflammation-associated immune suppression (Ben-Baruch 2006, Salazar-Onfray et al. 2007). From these studies emerge data that demonstrate that some tumors possess qualities that effectively prevent their immune destruction (Ganss, Hanahan 1998). The inflammatory reaction is induced by the tumor stroma and hypoxia through hypoxia-inducible factor-1 (HIF-1) (Hellwig-Bürgel et al. 2005). HIF-1, not only influences metabolism of cancer cells and that of the resident normal stromal cells, but stimulates the production of gene expression of several growth factors such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and COX – derived arachidonic acid metabolites (Hellwig-Bürgel et al. 2005, Bonazzi et al. 2000, Demasi et al. 2003). Autocrine paracrine pathways are generated in areas of hypoxia as homeostatic mechanisms which tumor cells exploit in order to divide and disseminate (Arias et al. 2007). Resident macrophages and recruited marrow cells such as myeloid cells accumulate in large numbers in ischemic/hypoxic areas and this constant influx of myelomonocytic cells is required by tumors for supporting angiogenesis and stroma remodeling (Murdoch, Sica, Schmid, Kusmartsev). The generation of a new environment and hypoxia in tumor has been identified as a crucial area that can activate autocrine and paracrine signaling which promotes angiogenesis and stroma structure to support tumor growth and progression (Allavena et al. 2008, Baronzio et al. 2003, Mantovani et al. 2008).

Tumor Immune Surveillance, Immunoediting and Background on Immune Response to Cancer

Paulehrlich (1909) was the first to purport that the immune system can restrict cancer growth (Woodruff 1980). Later, Thomas and Burnet (1965) elaborated the theory of immune surveillance. This concept according to Burnet "is a tentative of generalization, that would give some logical unity to a wide range of observable phenomena." (Burnet 1997). Since his proposal a century ago, experiments with knockout mice have confirmed Burnet's initial hypothesis (Dunn). In RAG-2 deficient mice lacking T and B cells, 50% of mice developed spontaneous epithelial tumors, confirming the utility of the immune system in restraining tumor development. These results have been confirmed in another series of mice lacking the transcription factor involved in interferon gamma (INF-γ) signaling (Malmberg, Ljunggren 2006). Despite these illuminating studies which confirm the existence of immune surveillance of cancer, the theory of Thomas and Burnet does not address the fact that many cancers are progressive. At some point, the cancer or its microenvironment must hinder the immune response, causing it to fail in its surveillance. To consider the crosstalk between tumor environment and the immunity Schreiber et al. have proposed the concept of immunoediting (Dunn et al. 2002, Ochsenbein 2002). Immunoediting is a process by which a person is protected from cancer growth and the development of tumour immunogenicity is dependent on their immune system. It has three main phases: **elimination, equilibrium and escape** (Dunn et al. 2004). The elimination phase consists of different sub-phases that we briefly describe as an initial acute inflammatory sub-phase triggered by the presence of tumor or abnormal cells and supported by the innate immunity, followed by another sub-phase of adaptive immunity with an inflammatory reaction that becomes chronic.

The First Phase of Elimination

Cells of the innate immune system recognize the presence of a tumor causing local tissue damage (Borghesi, Milcarek 2007). Innate immunity against cancer is the result of inflammatory events triggered by cells, local cytokines (IL-2, IL-12, IL-18, IL-23), toll-like receptors (TRLs) and heat shock proteins (HSPs) present in tumor milieu (Lin et al. 2008). This pool of soluble mediators is released by complex interactions between morphologically and functionally distinct cells, such as dendritic cells (DCs), natural killer cells (NKs), macrophages, neutrophils and cancer cells (Diefenbach, Raulet 2002). NKs and neutrophils use pattern-recognition receptors and other cell surface molecules to directly detect and induce potent protective immunity against tumor cells. In fact, different from T cells, NKs inhibit tumor growth in a MHC-non restricted manner. Frequently tumor cells express on their

surfaces different glycoproteins (MICA and MICB), that function as ligands for NKG2D receptors on NK cells. Once activated, these receptors stimulate NK cell activity (Diefenbach et al. 2003). This is followed by the induction of inflammatory signals that are essential for recruiting cells of the innate immune system (e.g. NKs, NK T cells, macrophages and DCs) to the tumor site. During this phase, infiltrating lymphocytes such as the NKs and NK T cells begin to produce IFN-γ. In the second phase of elimination, newly synthesised IFN-γ lead to death of a limited number of cancer cells inhibiting neoangiogenesis through the production of certain chemokines such as CXCL10, CXCL9 and CXCL11 (Raman et al. 2007).

After, this phase of acute inflammation, the phase of adaptive immunity follows. In this phase, DCs, the most specialized antigen presenting cells (APCs), react against tumor specific surface antigens (TAAs). Following enulfment of the cancer cell, DCs present TAAs via major histocompatability complex (MHC) receptors to naïve T cells (Houghton, Guevara-Patiño 2004, Lord, Frelinger 1998). Activation occurs when a T cell receptor (TCR) on the naïve T cell interacts with a TAA-MHC complex on the DC. However, TCR receptor binding is not sufficient for a full activation of T cells unless co-stimulatory molecules interact with the specific ligands on the surface of APC. The presence or absence of co-stimulatory signals like B7-1 (CD80), B7-2 (CD86) and CD40/CD40L, determines whether immune response becomes anergic or tolerant. CD40/CD40L is expressed transiently following TCR activation on the surface of $CD4^+$ cells and is a key molecule in mediating the activation of B cells and in controlling $CD8^+$ T cells. Antigens can be associated to MHC I or MHC II class complexes and are presented by DCs to TCR of $CD8^+$ and $CD4^+$ T cells and their respective co-stimulatory molecules (Titu et al. 2002). Both $CD4^+$ and $CD8^+$ cells after activation and co-stimulation produce a series of cytokines that differentiate T-helper ($CD4^+$) lymphocytes in two subpopulations (TH_1, TH_2 cells). TH1 cells produce IL-2, IFN-γ, TNF-α and granulocyte macrophage colony stimulating factor (GM-CSF) that increase the activity of macrophages, and the expression of MHC class I molecules on the surfaces of $CD8^+$ cells. TH2 cells secrete another group of cytokines (IL-4, IL-5 and IL-10) that induce näive B cells to produce antibodies. The shifting toward TH2 pattern is consistent with immune tolerance and an increased rate of tumor metastasis and decreased survival in many human and animal neoplasias (Lord, Frelinger 1998, Kidd 2003, Nishimura et al. 1999).

In the final phase of elimination, $CD8^+$ cytotoxic T lymphocytes (CTLs) are the major effectors of tumor regression; however $CD4^+$ T cells collaborate with CTL activation. Once activated CTLs do not need co-stimulation, since MHC class I bound antigen is sufficient. For eliminating target cells (neoplastic cells), CTLs use three effector molecules: perforins, granzyme and Fas ligand. Associated with these killing mechanisms, CTLs secrete specific cytokines, such as IFN-γ, TNF-α and TNF-β. This pattern of cytokines plays an important role in the activation of macrophages, which can exert direct cytotoxic effects or, conversely, stimulate tumor progression, depending on the tumor microenvironment (Dranoff 2004).

Second Phase of Equilibrium

Tumor cells surviving elimination phase enter the equilibrium phase. In this phase, lymphocytes and INF-γ exert a selection pressure on tumor cells that are genetically unstable and rapidly mutating. In this phase the immune system initially restrains cancer cell growth on the whole, but also selects out cancer cells with high mutation rates and less susceptibility to immunologic attack. This phase involves adaptive immunity – especially $CD8^+$ cells and NKs (Zitvogel et al. 2006).

Third Phase of Escape

Immunologically selected tumor cell variants that have acquired resistance to immunity enter the escape phase. In this phase, tumor cells expand in an uncontrolled manner and become clinically relevant. Tumor cells and stromal cells provide a favorable microenvironment and the inflammatory reaction that is still present suppresses anti-tumor immune activity of several immunocompetent cells such as macrophages, tumor infiltrating lymphocytes and DCs (Ben-Baruch 2006, Fricke, Gabrilovich 2006, Ganss, Hanahan 1998, Stewart et al. 2007). The tumor microenvironment has emerged as an important component contributing to the malfunction of many immune competent cells. Hypoxia in particular seems to play a crucial role in immunity beyond its effects on metabolism and angiogenesis (Baronzio et al. 2003, Baronzio, Freitas 2008). A brief overview of tumor microenvironment genesis and of hypoxia will be discussed.

Tumor Microenvironment, Hypoxia and Effects on Immunity

Tumors appear microscopically as an organoid or an ecosystem of nonuniform composition with regions well oxygenated and metabolically active near regions hypoxic and dormant. This chaotic 3D architecture is dictated by the need for nutritive substances and oxygen. Hypoxic and undernourished tumor cells exist within solid tumors when oxygen demand from rapid tumor growth outstrips blood supply. These undernourished tumor cells trigger signals for obtaining new blood vessels (neoangiogenesis and neovasculogenesis) to satisfy their demands. This neovasculature is, however, inefficient and does not take pace with tumor growth, so, new hypoxic areas are recreated. A vicious circle is triggered that leads to metabolically compromised microenvironments (Baronzio et al. 2003). Different metabolic and oxygenation states coexist together. These areas have contrasting metabolic activities and growth kinetics. Moreover, their rates of growth and progression change daily (Höckel, Vaupel 2001). Hypoxia, once thought merely as a consequence of tumor physiology appears to be a tumor adaptation to promote its own survival (Vaupel, Harrison 2004). In fact, hypoxic conditions generate local expression of a transcription factor, hypoxia inducible factor (HIF-1), which enhances angiogenesis and glycolysis, enabling tumor growth and progression (Shi, Fang 2004,

Vaupel, Harrison 2004). Although HIF-1 is recognized chiefly for stimulating angiogenesis and shifting cancer cell metabolism toward anaerobic glycolysis, recent evidence indicates that it also regulates host defenses (Zarember, Malech 2005, Lukashev et al. 2006). Hypoxic regions seem to alter the balance between Th1 cells and Th2 cells, and might alter the activities of cells of the innate immune system, thereby qualitatively and quantitatively affecting immune responses. Other studies indicate that hypoxia polarizes toward a Th2 cell host response, with concomitant increased production of Th2 anti-inflammatory cytokines (IL-4, IL-6; IL-10; TGF-β) (Lederer et al. 1999). Hypoxia may also induce a local adrenergic response that enables cancer to evade host immune surveillance with a shift toward Th2 cell response (Joon et al. 2004).

Hypoxia also affects intratumoral recruitment of macrophages and granulocytes. The tumor hypoxic core, rich in HIF-1, induces a series of cytokines and chemokines (VEGF, G-CSF, GM-CSF, SDF-1, MCP-1 CCL22, β–defensin, CXC4) which recruit circulating macrophages and bone marrow derived myeloid cells (BDMCs) (Pollard, Schmid, Burger, Kipps 2006). After recruitment, macrophages become the prominent part of the tumor stroma and exhibit a distinctive phenotype, termed tumor-associate macrophages (TAMs) (Pollard 2004). Two kinds of TAM phenotypes have been described: TAM-1 and TAM-2, each distinguished by their different production of cytokines and chemokines. TAM-2 are represented prominently in tumor masses, are associated with poor prognosis and are known to produce anti-inflammatory cytokines such as IL-10 (Sica, Bronte 2007, Sica et al. 2008, Lucas et al. 2008). BDMCs, in presence of VEGF, G-CSF, GM-CSF, SDF-1, MCP-1 CCL22, β–defensin and IL-10, incorporate within tumors as monocytes, macrophages or directly into blood vessels (unpublished data from Cogle et al.). In particular, IL-10 promotes BDMC differentiation into TAM-2 macrophages (Schmid, Varner 2007). In sum, tumor hypoxia exerts selection and polarization of recruited macrophages and myeloid cells to become permissive of tumor progression and growth (Yuan et al. 2008). Furthermore, macrophages in tumor hypoxic areas show decreased phagocytic activity, probably linked to a decreased production of free radicals in consequence of the shortage of oxygen (Sitkovsky, Lukashev 2005).

Moreover, leukocyte activiation is inhibited by low oxygen tension (Thiel et al. 2005). Macrophages and T cells recruited into hypoxic tumor regions transcriptionally upregulate TNF and IL-1, and downregulate IL-2, shifting further the Th1/Th2 toward the Th2 cell response (Ghezzi et al. 1991, Zuckerberg et al. 1994, Lucey et al. 1996).

HIF-1, as a consequence of low oxygen, induces the expression of VEGF (Forsythe et al. 1996), NF-kB (Taylor 2008), and growth factors such as galectin-1 (Le et al. 2005). Angiogenesis triggered by VEGF is one of the physiological responses to hypoxia (Kerbel 2008). However, VEGF has counter-productive effects by interfering with differentiation and maturation of antigen presenting cells, such as DCs (Oyama et al. 1998, Ohm et al. 1999, Ohm, Carbone 2001).

Stroma, around tumor hypoxic areas, also participate in production of these and other factors (Baronzio, Freitas 2008). Tumor stroma is composed by resident and

recruited cells. They form a complex of fibroblasts, neutrophils, macrophages, lymphocytes and mast cells working in concert with neoplastic cells to create distinctive hypoxic microenvironments. Fibroblasts provide a structural framework for many tissues and may promote tumor progression and metastasis (Silzle et al. 2004). In presence of TGF-β, PDGF, and GM-CSF, fibroblast progenitors are recruited into tumors and secrete elevated chemokine levels of stroma cell derived factor 1 (SDF-1) and monocyte chemotactice protein-1 (MCP-1) (Silzle et al. 2004). MCP-1 produced by fibroblasts recruit and induce the infiltration of tumors by macrophages and BDMCs (Silzle 2003); whereas SDF-1 recruits circulating CD4+ CD25+ regulatory T cells (CD4+ CD25+ FOXP3) (Treg) and plasmocytoid precursors of dendritic cells, contributing to tumor immune evasion and angiogenesis (Kryczek et al. 2007, Schmid, Varner 2007, Zou et al. 2001). Regulatory T cells possess suppressive activity and play a major role in maintaining the balance between immunity and tolerance (Beyer, Schultze 2006, Mottet, Golshayan 2007). In humans, Tregs account for 5% of total CD4+ T lymphocytes. These cells originate in the thymus and express a series of specific receptors (IL-2 receptor, CTLA-4 antigen, GITR and FOXP3) (Shubina et al. 2008). Tregs mediate immune suppressive activity on CD4 and CD8 cells by producing IL-10 and TGF-β (Bluestone, Abbas 2003). In clinical practice Tregs are main challenges to successful cancer immunotherapies and vaccination protocols (Curiel 2007, Juang et al. 2007). By depleting Tregs using antibodies (Zou et al. 2001), denileukin diftitox, fludarabine, and cyclophosphamide (Morse et al. 2008, Muranski), investigators and clinicians have achieved greater response rates and persistence of adoptively transferred T cells. Tregs seem more active at early stages of cancer; whereas at later stages, multiple immune evasion mechanisms are operating (Elpek et al. 2007).

Recently, new classes of regulatory cells have been recognized: immature myeloid-derived suppressor cells (MDSC), immature dendritic cells and IDO cells (Gabrilovich et al. 2001, Frey 2006, Ochoa et al. 2007, Villella et al. 2006). Increased arginase activity and production of reactive oxygen species (ROS) are among the main functional characteristics of MDSC cells (Nefedova). In mice, MDSC are characterized by Gr-1 and CD11b expression. In humans, no definitive markers on MDSC have been discovered due to the heterogeneity of this subpopulation (Movahedi et al. 2008). However, candidate phenotypes include cells expressing $CD33^{+}CD3^{-}CD14^{-}Cd19^{-}CD56^{-}$ or $CD33^{+}CD14^{-}$ $CD11b^{+}$ (Zea et al. 2005). Other authors have reported that MDSCs can be subdivided into two distinct cell populations. One is an immature monocytic fraction expressing high levels of CD11b, intermediate levels of Gr-1, and intermediate levels of the macrophage colony stimulating factor (M-CSF) receptor. These cells do not express Ly6G. The other fraction is a granulocytic fraction and consists of cells expressing high levels of CD11b, Gr-1, SSC, and Ly6G but do not express M-CSF receptors (Van Ginderachter et al. 2006). MDSCs utilize multiple biochemical pathways to elicit immune suppression. The major enzymatic mechanism involves the expression of arginase and nitric oxide synthase, two enzymes involved in L-arginine metabolism (Bronte et al. 2003). These enzymes are released by myeloid suppressor cells in response to exogenous INF-γ or autocrine production of INF-γ. The process also involves

autocrine production of IL13 and expression of the IL4 and IL13 receptors (Gallina et al. 2006). MDSCs also mediate Tregs (Huang et al. 2006). Crosstalk between MDSCs and Tregs impair tumor immunity by suppressing T cell activation and stimulating macrophages to increase IL-10 and decrease IL-12 production, thereby promoting a Th2 response (Sinha et al. 2007). MDSC are recruited into tumors via MCP-1 (Mailloux, Young 2008).

IDO Cells

Another class of immune regulatory cells includes a small subset of APCs which express indoleamine 2,3-dioxygenase (IDO) activity (Munn et al. 2002). IDO is an IFN-inducible enzyme that suppresses adaptive T-cell immunity by catabolizing tryptophan from the cellular microenvironment (Scheler et al. 2007). The tryptophan deprivation that follows and the generation of active metabolites of tryptophan cause T-cell apoptosis and inhibition of Th1-mediated immune responses (Scheler et al. 2007, Muller, Prendergast 2007). IDO expressing cells have been found in draining lymph nodes of patients with melanoma and breast cancer (Munn et al. 2002, Lee et al. 2003, von Bergwelt-Baildon et al. 2006). Accumulating evidence suggests that this discrete subset of APCs are influenced in their maturation by several factors produced both by tumor and stroma, such as VEGF and prostaglandin E2 (PGE2). A sequence of events initiated by HIF-1 and VEGF (itself induced by HIF) are generally induced and successively regulated by STAT3 (Jung et al. 2003, 2005). STAT 3 is overactivated in cancer and its excessive concentration in turn reduces secretion of TNF-α, IFN-β and other chemokines thereby inhibiting DC maturation (Yu et al. 2007). Three different subsets of human IDO-expressing APCs are identified in vivo: plasmacytoid IDO cells (CD 11c ^{low} CD123^{+} BDCA $-2/4$ $^{+}$), myeloid DC cells (Cd11c ^{high} BDCA $-1/3^{+}$ CD123^{+}), and Langerhans cells (CD11c+ CD1a+ CD207+) (Popov, Schultze 2008).

Other Immunosuppressive Factors

Several other factors, such as free radicals (ROS), nitric oxide (NO), prostaglandins, nuclear factor κB (NF-κB) and peroxisome proliferator – activated receptors (PPARs) are activated and coexist in hypoxic areas of cancers. A link between these factors and HIF-1 in stimulating innate immunity has been described (Taylor 2008, Rius et al. 2008). Hypoxia is a common feature of solid tumors and has been demonstrated to stimulate mitochondrial ROS formation (López-Lázaro 2006, Schumacker 2006). In fact, cancer cells generate more ROS than normal cells (Schumacker 2006, Szatrowski, Nathan 1991), which, in turn, activates HIF-1, leading to activation of NF-κB and upregulation of cyclo-oxygenase 2 (COX2) enzyme expression (Rius et al. 2008, Haddad 2002, Jung et al. 2003, Kaidi et al. 2006, Taylor 2008). The inter-relationship between PPARs and HIF-1 are less clear. Some studies indicate that HIF-1 decreases the expression of PPAR receptors (Narravula, Colgan 2001),

whereas a recent study purports a direct link between PPAR expression and HIF-1 in lung cancer (Zhang et al. 2003). In every case, however, PPAR is activated by excessive ROS, COX2 expression, and prostaglandins (PGs) such as PGE2 (Tachibana et al. 2008).

NF-κB

NF-κB plays a significant role in regulating both innate and adaptive immunity (Baldwin 2001, Karin, Greten 2005 Yamamoto, Gaynor 2001). Many aggressive cancers, express elevated NF-κB activity. Blocking NF-kB in cancer results in production of proinflammatory chemokines, recruitment of NK cells and T cells to the tumor microenvironment, polarization of immunity to a Th1 response, and enhanced expression of MHC class I antigens on tumor cells. These effects result in enhanced NK cell anti-cancer cytotoxicity (Jewett et al. 2006a, b).

Recent findings indicate that oxidative stress impairs NK and CD8 immune response to cancer (Czesnikiewicz-Guzik et al. 2008). New studies indicate that myeloid cells recruited in the tumor area are responsible for increased production of ROS, which compromise NK and T cell function as well as block differentiation of additional myeloid cells into mature macrophages or dendritic cells (Kusmartsev, Gabrilovitch 2006b).

Another molecule constitutively expressed in hypoxic areas of tumors is galectin-1, a family of animal lectins that play important functions in several aspects of cancer biology, including modulation of apoptosis, cell migration adhesion and immune response (Camby et al. 2006). Galectin-1 (Gal-1) is overexpressed by many different tumor types and is correlated with aggressive tumor growth and metastasis. In vivo, Gal-1 has powerful immunoregulatory effects through its ability to inhibit T-cell effector functions and may trigger the death of infiltrating T cells, resulting in immune evasion (Camby et al. 2006). When tumors are exposed to hypoxia, they increase production and secretion of Gal-1, which in turn promotes T-cell apoptosis and blocks T-cell activation. This allows tumor cells to escape from cellular immune surveillance and continue to survive and proliferate (Le et al. 2005).

Other mechanisms within the cancer microenivronment down-regulate immune competent cells, such as pools of cytokines and chemokines induced by peritumoural inflammatory reactions, acidic extracellular pH, decreased concentration of nitric oxide (NO), increased concentration of PGE-2, decreased expression of adhesion molecules on tumor endothelium and increase in interstitial fluid pressure (IFP).

Cytokines/Chemokines

Peritumoral chronic inflammation creates a favorable microenvironment for cancer growth and metastasis. This tumor-supporting microenvironment is formed by resident and recruited macrophages, DCs, T cells and NK cells that secrete

proinflammatory cytokines (i.e., VEGF, IL-6,IL-10, TGF-β) (Lin, Karin 2007). These immunosuppressive cytokines allow the cancer to evade the immune system, permitting tumor growth, invasion and spread (Lin et al. 2008). Some tumors such as oral squamous cell carcinomas, non-small cell lung carcinoma (NSCLC), breast cancer, renal cell carcinoma and glioma (Thomas et al. 2004, Yamaji et al. 2004, Conze et al. 2001, Graf, Merchant 1999) constitutively produce IL-6 in large quantities. In other cancers, tumor stroma produce large amounts of IL-10 and TGF-β, which inhibit DC stimulation of a Th1 cytotoxic immune response and instead activate regulatory T-cell population functions and tolerance (Yang, Lattime 2003, Seo et al. 2001).

VEGF is expressed by a large percentage of solid tumors and this over-expression is associated with a poor prognosis. VEGF is known to impair functional maturation of dendritic cells from CD34+ hematopoietic progenitors cells. An additinal consequence of this DC developmental defect is impaired activation of NF-κB (Ohm et al. 1999, Ohm, Carbone 2001).

Another inflammatory cytokine with dual effects in cancer is IL-18, which is overexpressed in several cancers (melanoma, breast, gastric cancer). IL-18 promotes inflammation in cancer, but when secreted in large quantities supports immune evasion and consequent tumor progression (Park et al. 2007, Dinarello 2006).

Adhesion Molecules

Alterations in leukocyte adhesion play a pivotal role in anti-tumor immunity (Carlos 2001, Wu 2007). Lymphocytes normally leave the blood stream and cross the endothelium into tumor microenvironments via ligand-receptor interactions involving L-selectin and alpha-4beta-7 integrin adhesion receptors expressed on venular endothelial cells. Interestingly, in tumors lacking tumor infiltrating lymphocytes, endothelial cells do not express L-selectin and alpha-4beta-7. It is the repressed expression of these and other ligands that prevent tumor infiltration by lymphocytes (Carlos 2001, Gao et al. 2008, Rüegg 2006), highlighting aberrant cancer vasculogenesis as abetting cancer immune evasion.

Nitric Oxide (NO)

TAM-1 cells exert their cytotoxic activity through two principal soluble mediators: NO and TNF-α. TNF-α activity is dependent on the generation of hydroxyl radicals generally diminished in hypoxic areas; whereas in hypoxic areas NO production is enhanced (Park et al. 2002). High levels of NO can inhibit tumor cell proliferation and cell death. In contrast, low NO levels protect tumor cells from apoptosis, suppress anti-tumor immunity, stimulate angiogenesis and increase tumor blood flow (Fukumura et al. 2006, Liao et al. 2007). The quantity of NO in hypoxic areas is regulated by the presence of the TAM types that regulate inducible nitric oxide

synthase (iNOS). TAM-1 cells, as previously described, are high producers of NO, but are less recruited in hypoxic areas; whereas TAM-2 cells that are preferentially recruited to hypoxic areas produce low levels of NO (Allavena et al. 2008, Liao et al. 2007). TAM-2 and MDSC cells, in fact, produce a large quantity of arginase, which is a negative regulator of nitric oxide synthase. The depletion of L-arginine can suppress macrophage cytotoxicity and T cell function (Bronte et al. 2003, Liao et al. 2007).

PGE-2

Many human cancers secrete elevated levels of prostaglandins of E2 type (PGE-2) (Ben-Baruch 2006, Wang, Dubois 2006). PGE-2 exerts anti–inflammatory activity by down-regulating production of Th1 cytokines (Il-2, INF-γ and TNF-α) and up-regulating Th2 cytokines (IL-4, IL-10 and IL-6) (Wang). In addition PGE-2 regulates DC maturation, differentiation and cytokine secretion through cross talk with IL-10. The net result is suppression of anti-tumor NK activity and enhance Treg tolerance activity (Harizi, Gualde 2006, Akasaki et al. 2004, Mottet, Golshayan 2007).

pH

Low tumor interstitial fluid pH is mainly attributable to the production of lactic acid. Lactic acid is another consequence of hypoxia. In fact, hypoxia increases many enzymes of the glycolytic pathway including lactate dehydrogenase (LDH) (Vaupel, Harrison 2004). The acidic interstitium inhibits lymphocyte proliferation and cytotoxic activity of lymphokine activated killer (LAK) cells (Lardner 2001). pH also affects release of perforins and DC activity (Gottfried et al. 2006).

IFP Elevation (interstitial fluid pressure)

Interstital fluid (IF) increase and elevation of interstitial fluid pressures (IFP) are common features of tumors (Baronzio et al. 2003). Reasons for liquid accumulation in tumors are several: (a) capillary morphology and physiology abnormalities; (b) hydrostatic and osmotic pressure differences; and (c) diminished lymphatic drainage. These elements are described in Chapters 4 and 6. Major factors leading to IFP elevation include VEGF and, indirectly, hypoxia (HIF-1). IFP elevation impairs anti-tumor immunity by inhibiting the release of tumor cells and antigens to secondary lymphoid organs, decreasing antigen concentration due to an increase in fluid, and binding tumor antigens to the extracellular matrix (Kim).

Therapeutic Methods to Overcome Immune Evasion Mechanisms

The dogma subverted. Radiotherapy and chemotherapy are rarely curative as single or associated methods of cancer treatment (Emens) and are generally regarded as unrelated with immunotherapy (Friedman, Motoyoshi, van der Most). However, this dogma has been recently challenged (Emens). In fact, several lines of research show vaccine immunotherapy in combination with either radiation or chemotherapeutic agents can alter the phenotype of tumor cells, rendering them more susceptible to T cell-mediated killing (Machiels et al. 2001). Furthermore, novel strategies are emerging from preclinical and clinical investigations for ablating the tumor immune suppressive circuitry, synergizing conventional anticancer therapies with active immunotherapy (Prendergast, Jaffee 2007). Moreover as outlined by Sharp, immunotherapy is most beneficial when used in early stage of disease process and in combination with standard therapies (Sharp et al. 2007).

The Importance of Cell Death

A common goal between chemotherapy and radiotherapy is cancer cell death (Fonseca, Dranoff 2008). Cell death in the course of every day homeostasis (apoptosis) normally does not trigger the immune system. But, when induced by radiation or chemotherapy, cancer cell death (necrosis) can trigger an inflammatory reaction (see Fig. 9.1, 9.2) (Fonseca, Dranoff 2008, Kono).

Apoptotic and necrotic cells are sequestered and cleared by macrophages and dendritic cells. In apoptosis, cellular presentation of phosphatidylserine, a cell membrane phospholipid normally kept on the cytosolic side, signals a dead cell and attracts phagocytes via binding to TIM-1 and TIM-4. Consequently, the dead cell is phagocytized and immunosuppressive cytokines such as IL-10 and TGF-β are liberated to quell a major inflammatory response.

In contrast, necrotic cells release mediators that sustain a robust inflammatory reaction. Necrosis consists of activation of a cascade of events that involve DNA fragmentation and disruption of plasma cell membranes. With breech of the cancer cell membrane, intracellar contents are liberated into the surrounding microenvironment and blood stream. Specific debris include uric acid, high mobility group box 1 (HMGB-1), heat shock proteins, and calreticulin (Fonseca, Dranoff 2008, Majno, Joris 1995). Release of intracellular contents promotes angiogenesis and tumor progression, but also stimulates adaptive immunity against the dead cancer cells (Rovere- Querini, Castiglioni 2008).

As we will describe later, some forms of cancer therapy, particularly radiation, platinum compounds and anthracyclines, release calreticulin and HMGB-1. These molecules activate DCs through Toll-like receptor-4 (Fonseca, Dranoff 2008). Other cancer therapies able to induce double-stranded DNA breaks upregulate a series of surface ligands on tumor cells. These ligands are principally human NKG2D ligands that include closely related major histocompatibility complex (MHC) class

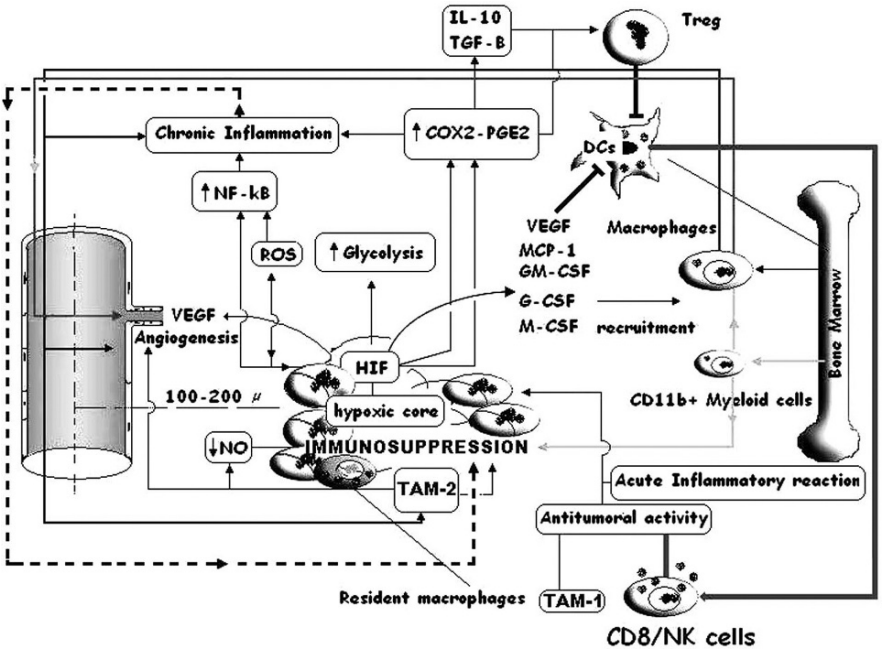

Fig. 9.1 The various factors that contribute tot tumor immunosuppression and their interaction is simply depicted

I chain-related proteins called MICA and MICB (Stephens 2001). These proteins are cell-surface molecules that activate NK and CD8 + CTL. However, some tumors shed MICA ligands that down-regulate NKG2D surface expression impairing NK cytotoxicity (Groh et al. 2002).

In necrosis, calreticulin, a calcium-binding protein that activates dendritic cells, induces Th1 immune response. On the contrary, phosphatidylserine released by apoptotic cells preferentially activate macrophages or neutrophils, which enhances the secretion of immunosuppressive cytokines such as TGF-β and IL-10.

Another important aspect in inducing an efficient T cell-dependent immune response is the presence of Toll-like receptors (TLR). It seems that the TLR-4 receptor is recognized as a danger signal provided by dying cells and is induced by some drugs such as oxaliplatin and doxorubicin (Ménard et al. 2008). However, for optimal antigen processing by dying tumor cells to DCs, an association between TLR-4 and HMGB-1 is necessary (Tesniere et al. 2008). Locally, dying tumor cells release HMGB-1, but this molecule (also known as "alarmin") is seldom neutralized by the excessive ROS present in the microenvironment (Kazama et al. 2008). Necrotic cells also release HSP, particularly HSP gp 96, HSP70 and HSP90 (Basu et al. 2000, Tsan, Gao 2004). When secreted in the extracellular microenvironment, HSPs elicit an immune response modulated either by the adaptive or innate immune system (Schmitt et al. 2007). As outlined by Basu, apoptotic cells, contrary to necrotic cells, are unable to release HSPs (Basu et al. 2000) (Fig. 9.2).

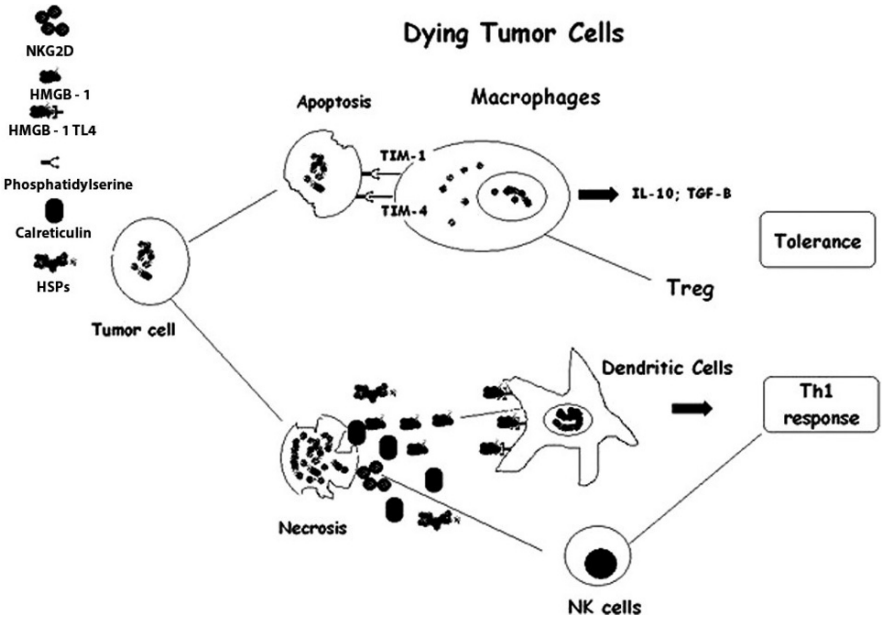

Fig. 9.2 The two ways by which cancer cells may die (apoptosis, necrosis) and effects on the immune system are illustrated

Chemotherapy and Immunotherapy

There is increasing evidence that innate cancer immunity and consequently adaptive immunity is dependent in part upon the method of cell death induced by therapies (Apetoh et al. 2008, Fonseca, Dranoff 2008, Ulrich et al. 2008a, b). Cancer chemotherapy is successful when it results in widespread cancer cell death and release of tumor-specific antigens (van der Most et al. 2005, 2006, 2007). Some classes of chemotherapeutic drugs induce immunogenic cell death and augment antigen cross-presentation, whereas others do not (Ullrich et al. 2008a, b). The mechanisms induced by the immunogenic chemotherapy are not completely known. Some mechanisms are intrinsic to the method of cancer cell death (apoptosis vs. necrosis). While others are indirectly due to the control exerted by some chemotherapeutic drugs on regulatory T cells (Apetoh et al. 2008, Fonseca, Dranoff 2008, van der Most et al. 2007). When chemotherapy kills tumor cells, these killed cells release antigens, eliciting and inducing maturation of DCs and of CD8+ CTLs (Van der Most et al. 2006). However, CTL activity is self-limited and quantitatively impaired due to common myelosuppressive side effects of chemotherapies. Another aspect is the debulking effect of chemotherapy that permits a favorable balance between tumor cells and immune effectors (Emens 2008). A synergy of effect has been found when comibining chemotherapy and cancer vaccination (Emens 2001). Many factors can influence the immunogenic activity of chemotherapeutic drugs and vaccination. Not all drugs have the same immune modulatory activity. Their activity is

dependent on different factors such as drug class, drug dosage, timing of drug and vaccination.

Cyclophosphamide. The alkylating agent cyclophospamide has direct cytotoxicity in rapidly proliferating cells and has been used to modulate host immune systems (Motoyoshi et al. 2006). The dose is critical for when aiming to induce either a cytotoxic or immunomodulatory effect. When used in low doses, cyclophospamide depletes Tregs while sparing other CD4+ and CD8+T cells. When used in high doses, cyclophosphamide exerts a cytotoxic effect on all lymphocytes. Together, these data indicate that the dose of cyclophosphamide should be chosen depending on intent to augment anti-tumor immunity (low dose) or generalized cytotoxicity (high dose) (Motoyoshi).

Cisplatin. Cisplatinum, like cyclophosphamide, augments immune response by depleting Treg cells (Tsuda et al. 1994). Cisplatin also enhances natural killer (NK) cell activity (Kleinerman et al. 1980), which stimulates lymphokine-activated killer (LAK) cells (Sodhi, Basu 1992, Buzaid 2000).

Other Cytotoxic Agents. In addition to cyclophosphamide and cisplatinum, other chemotherapies influencing Tregs include fludarabine and the combination of gemcitabine and FOLFOX 4 (Oxaliplatin, 5-fluoruracil + folinic acid) followed by IL-2 and s.c. GM-CSF (Beyer et al. 2005, Correale et al. 2005). Furthermore, gemcitabine promotes Th1 type response in cancer by reducing myeloid suppressor cells, and enhances CD8+CTL activity (Suzuki et al. 2005). Taxanes, placlitaxel, docetaxel and doxorubicin also exhibit immunomodulatory activity. Animal data indicate that docetaxel enhances efficacy of tumor vaccination in murine models of melanoma and lung carcinoma (Buzaid, 2000).

Metronomic Therapy and Treg Control

Recently, Ghiringhelli et al have demonstrated in animal and human studies that metronomic therapy (MC) regulates the presence of Treg cells (CD4+ CD25+ Foxp3). The clinical results were attributed to the antiangiogenic properties of MC (Ng, Figg 2004). Clearly low dosage of cyclophosphamide is an efficient regimen to blunt CD4+CD25+ regulatory T cells (Ghiringhelli et al. 2004, 2007, Ladoire et al. 2008).

Radiotherapy and Immunotherapy

Often radiation alone cannot eradicate cancer; however, combination immunotherapy and radiotherapy may permit local control and systemic control (Sharp et al. 2007). Radiation triggers several cellular and stroma reactions that can be exploited to alter the immunological environment of tumor. For example, irradiation induces intratumoural expression of IL-3, a cytokine that enhances the ability of DCs to present antigens. Another factor induced by radiation is the capacity to produce danger signals in the form of oligodeoxynucleotides (Demaria 2007). Furthermore, an

effective antitumor T cell response can be obtained if local tumor radiation is combined with antibody mediated CTLA-4 blockade (Demaria et al. 2005). Although there is much to learn on combining radiotherapy and immunotherapy, recent clinical applications support this effort (Harris et al. 2008, Lin et al. 2008, Mozaffari et al. 2008).

Conclusion

Intratumoural hypoxia is a consequence of a structurally and functionally disturbed microcirculation. Hypoxia activates nonspecific stress responses, such as anaerobic metabolism, angiogenesis and tissue remodeling. Associated with these events, stroma surrounding the tumor reacts by assisting in cancer immune evasion which permits tumor growth, invasion and spread. Several factors mediating cancer immune evasion have been described: prostaglandins (PGs), interleukin (IL)-10, vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-beta. Some of these factors, such as PGE2, VEGF, and Tregs can be controlled by appropriate chemotherapy. Moreover, the tumor microenvironment can be manipulated by radiation to increase antigen presentation. In the future, combination immunotherapy, chemotherapy and radiotherapy with the chief aim of modulating anti-cancer immunity will be exploited. On one extreme allogeneic hematopoietic cell transplantation represents an aggressive strategy and has been employed to treat some refractory and relapsing cancers. Another option with less intial morbidity is metronomic therapy. The ultimate goal will be to define the right treatment combination for each particular cancer with specific respect to the patient's unique immune response capabilities.

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Chapter 10
Effects of Tumor Microenvironment on Hyperthermia, Photodynamic and Nanotherapy

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Abstract A tumor mass is an association of normal cells and epigenetically modified cells in continuous evolution. Heterogeneous normal cell populations are forced to survive in a hostile environment in contact with cancer cells. Resident and recruited fibroblasts, and a complex infiltrate of neutrophils, macrophages, lymphocytes and mast cells work in concert with neoplastic cells to create a new, distinctive microenvironment that allows for the generation of a new interstitium and circulation (angioarchitecture). The tumor interstitium differs from normal interstitium in several ways (i.e., an elevated intracellular pH (pHi) and pressure (pi), a lowered extracellular pH (pHe), low oxygen concentrations and low glucose levels). These differences represent important characteristics that may be modulated positively or negatively by hyperthermia, photodynamic therapy and other treatment modalities. Furthermore, the tumor microcirculation creates barriers that hinder drug delivery to the tumor mass. Systemic chemotherapy often reduces tumor burden but rarely is effective in completely eliminating the tumor. This has created the need for the development of more effective cancer therapies. To this problem, a new class of drug delivery vehicles on the order of nanometer (nanocarriers, liposomes) has been developed to minimize side effects of chemotherapy and for directly targeting cancer cells. Notwithstanding their small dimensions, the distribution of these drugs is still influenced by tumor microenvironment. An overview of ways to overcome physiological barriers and exploit tumor pathogenesis for therapeutic gain is provided.

Keywords Drug delivery · Barriers to drug delivery · TIF · EPR phenomenon · Hyperthermia · Photodynamic therapy · Ultrasound drug delivery · Magnetic hyperthermia · Nanotherapy

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Barriers Limiting Drug Delivery

Following systemic administration, the efficacy of anticancer drugs depends on physicochemical properties of the drug itself and on biologic properties of the tumor, in particular the tumor angioarchitecture and blood flow (For a review see *Jang*).

Solid tumors are composed of highly heterogeneous populations of malignant, stromal and inflammatory cells in a continuously adapting extracellular matrix (Baronzio, Freitas 2008). All of the above components interact and regulate each other to produce distinct microenvironments within the tumor mass. This evolving structure, according to currents models, shows two main phases: an avascular and a vascular phase (Ribatti et al. 1999, Berges, Benjamin 2002). The growth of tumors beyond a critical mass >1–$2\,mm^3$ (10^6 cells) is dependent on adequate blood supply (Vaupel et al. 1989, Freitas, Baronzio 1991). Up to a distance from host vessel of 100–200 μm the initial foci of neoplastic cells (*avascular phase*) receive their nutrients and oxygen by diffusion. Beyond this distance, and beyond a critical mass of $2\,mm^3$, hypoxia occurs and the need of adequate blood supply is crucial (*vascular phase*). Hypoxia and several other mechanisms induce a variety of growth factors and cytokines able to switch angiogenesis that favor tumor growth and dissemination (Folkman et al. 1971, Ferrara, Davis-Smyth 1997, Vaupel 2004). Among various growth factors and cytokines, vascular endothelial growth factor (VEGF) appears to be the most specific inducer of angiogenesis (Adams, Alitala 2007, Hlatky et al. 1996, Dvorak et al. 1995). However, establishing neovascular supply in the attempt to overcome hypoxia in cancer is inefficient, irregular and may not keep the pace with the proliferation of the tumor (Vaupel et al. 1989, Freitas, Baronzio 1991). The result is persistence within the tumor mass of heterogeneous microregions of quiescent hypoxic cells, which are surrounded by vital, better nourished and proliferating cells. In fact, the continuously expanding tumor vascular network is disorganized and as a consequence a heterogeneity of oxygen supply and efficiency of waste product removal occurs. Scanning electron microscopy (SEM) of vascular corrosion casts studied by Konerding has shown that tumor neovasculature has a three-dimensional (3D) anatomical structure and microcirculatory flow different from the normal counterpart (Konerding et al. 1992, 1995). Furthermore, we have observed the existence of a tumor specific architecture irrespective of tumor localization and grading (Konerding et al. 1992, 1995). The casts revealed a chaotic network of tortuous and distended veins, venules and venous capillaries and intertwining capillaries branching from arterioles and veins. Changes in vessel diameter, lack of vascular hierarchy, blind ends and irregular sinusoidal systems originating from and draining to veins are common features of tumor angioarchitecture. Diameters of vessels with capillary wall structure ranged from 6 to 55 μm in human primary tumors (renal clear cell carcinoma, basalioma), and from 5 to 80 μm in xenografted tumors (sarcomas, colon carcinoma). Intervascular distances in human primary tumors ranged from 2 to 52 μm, and from 11 to 105 μm in xenografts. Interbranching distances ranged from 34 to 258 μm in the former, and from 11 to 160 μm in the latter (Konerding et al. 1992, 1995). Irregularities of vascular wall structure in tumors have also been described (McDonald, Foss 2000). To understand

these malformations it is however necessary to describe briefly the process of tumor neoangiogenesis. The process of new in-growing vessels may occur by three different processes. Endothelial cells (ECs) are normally quiescent and tightly regulated by a delicate balance between proangiogenic and antiangiogenic molecules (Berges, Benjamin 2002, Folkman et al. 1971, Papetti, Herman 2002). In presence of an excessive secretion of angiogenic molecules by tumors, ECs are stimulated and they organize themselves in a vessel structure through multistep sequential and distinct processes, depending on tumor type and anatomic localization (Griffioen, Molema 2000). These processes include vessel co-option, vasculogenesis and angiogenesis.

Vessel-co-option is the process in which tumors take up pre-existing normal blood vessels and use them for their initial growth. As just described, this is a limiting process and irrelevant in the great majority of solid tumors; in fact, cancer cells grow until oxygen demand exceeds supply and the distance from host vessels is lower than 100–200 μm. (Griffioen, Molema 2000, Papetti, Herman 2002).

Vasculogenesis is the mechanism in which precursors endothelial cells (ECs) from bone marrow are recruited by the tumor and aggregate to form new blood vessels. Recent studies have demonstrated this process in experimental animal tumors, but its relevance in human neoplasia is not fully elucidated (Abramsson et al. 2002, Lyden et al. 2001, Takakura et al. 2000).

Angiogenesis: Upon adequate stimulus, endothelial cells begin to sprout from pre-existing capillaries and after the degradation of the extracellular matrix (ECM) by matrix metalloproteases, and the expression of adhesion molecules such as αvβ3 integrin, they migrate and organize themselves in capillary tubes forming eventually a vascular network (Adams, Alitala 2007, Griffioen, Molema 2000, Papetti, Herman 2002, Folkman et al. 1971).

From this brief description, it appears that co-opted vessels keep the normal structure of host vessels, whereas vasculogenesis and neoformed vessels show a different structure and behaviour in presence of therapies such as hyperthermia and PDT.

Normal capillary wall is made by three basic structural constituents: the endothelium, the basal lamina and pericytes. Their inner diameter is 5–10 μm, their length is 20–100 mm and their density reflects the magnitude of metabolic rates. As revealed by electron microscopy, the endothelium, a sheet of thin squamous epithelium, can appear continuous, fenestrated or discontinuous, depending on the presence or absence of small transcellular openings (fenestrae) in its wall (Simionescu, Simionescu 1998). Continuous capillaries are present in skeletal, smooth, cardiac muscle, lung and central nervous system. Fenestrated capillaries are present in renal glomeruli, endocrine glands and gastrointestinal system. Discontinuous capillaries are found in spleen, bone marrow and liver. The normal endothelium rests on a basal lamina (basement membrane) that varies in thickness and continuity (Simionescu, Simionescu 1998). Tumor endothelium shows several differences compared to their normal counterpart. First, many endothelial cells originating from tumor vessels do not form a normal monolayer but are irregularly shaped and disorganized, and some of them overlap one another (McDonald, Foss 2000). These cells have loose

interconnections and focal intercellular openings. The size of the opening determined by electron microscope is generally less than 2 μm in diameter (McDonald, Baluk 2002). Pericytes, which play an important role in the regulation of vascular formation, stabilization, remodelling and function Armulik et al. 2005), have a different role in tumor vessels (Morikawa et al. 2002). They show diverse alterations, such as increased perivascular deposition of extracellular membrane (ECM) component in expression of marker proteins, loose association with endothelial cells and extension of their cytoplasmatic processes deep in the tumor tissue. They seem to play, also, a role in vessel sprout growth and metastasization (Morikawa et al. 2002, Xian et al. 2006). All these factors render the tumor endothelium generally more leaky, influencing the distribution of drugs, photosensitizers and tiny particles like nanoparticles. Furthermore, the blood flow that is dependent on 3-D architecture in tumor is anomalous and generally stagnant. The nonexistent arteriovenous pressure decrease, the osmotic pressure difference across the vascular wall and the poor lymphatic drainage (Heldin et al. 2004, Jain 1990, 1998) (For a discussion of the non-compliance with Starling Laws of fluid exchanges, see Chapters 3 and 6) produce an accumulation of liquid in the extra-vascular compartment (also known tumor interstitial fluid (TIF)). Accumulation of TIF determines an elevation in the interstitial fluid pressure (p_i) both in animal and human tumors (Gullino et al. 1964, 1965, Gullino 1966, Freitas, Sarntinoranont et al. 2003) forming barriers and gradients to the penetration of drugs and photosensitizers in tumor (Munn 2003, Jain 1990, 1998, Heldin et al. 2004). The TIF space may become very large and in the case of hepatoma may be twice that of host liver (Gullino, Grantham 1964). High interstitial pressures have been reported in several animal and human tumors as reported by Jain (1987).

Increased permeability of tumor blood vessels and formation of TIF is not only a consequence of abnormalities of endothelial tumor cells and forces governing blood flow, but also consequence of several other vasoactive factors (VEGF, bradykinin, nitric oxide (NO) and peroxynitrite ($ONOO^-$)) produced by the tumor itself (Maeda et al. (2000), Iyer et al. (2006)). Formation of pouches of extravascular liquid, or tumor interstitial fluid (TIF), and decreased lymphatic drainage leads to an increased deposition and retention of some classes of plasma components and drugs/photosensitizers, such as macromolecules, nanoparticles and lipid particles. The increased extravasation of these components is linked to poor lymphatic clearance, also known as EPR phenomenon (enhanced permeability and retention effect). For polymeric drugs, the EPR determines the local concentration of polymeric drugs in 1–2 days, which may be 10–50 fold higher than in normal tissue (Maeda et al. 2000).

From these observations it becomes evident that the transport of effective doses of anti-cancer drugs or photosensitizers to solid tumors is hindered by the barriers imposed by the tumor interstitium (Munn 2003, Jain 1987). In particular, antineoplastic drugs can be effective in solid tumors only if they can penetrate several cell layers and retain their activity in the tumor microenvironment (Durand 1989). When blood-borne molecules or particles enter the tumor vasculature, they must cross the vascular compartment and be transported across the microvascular wall and the

interstitial compartment. The transport mechanisms are altered by different mechanisms such as drug diffusivity and concentration, permeability of vessel geometry, and pressures differences in the TIF and in the tumor mass. The limited ability of drugs to penetrate tumor tissue and to reach all of the tumor cells in a potentially lethal concentration, associated to gene mutations, can produce gene amplifications, epigenetic changes or drug resistance and modify the uptake, metabolism, and export of drugs from single cells. A drug that consistently works in vitro can therefore be expected to only sometimes work in vivo (Minchinton, Tannock 2006).

Therefore, a human solid neoplasia should be regarded as an intricate, yet poorly organized "organoid", whose function is maintained by a dynamic interplay between neoplastic and host cells. Tumors develop their unique anatomical structure and build physiological barriers that reduce the penetration and transport of anti-cancer drugs (especially macromolecular agents). These barriers include poor blood flow in large tumors, tumor capillary wall permeability, elevated interstitial fluid pressure, stroma causing poor diffusion in the interstitium, modified fluidity of cancer cell membranes, and heterogeneous antigen expression (Jain 1987, 1990). Recent research has considerably improved our understanding and appreciation of these obstacles and the new wave of optimization strategies involves the use or association of drugs and other technical methods to modulate the drawback posed by solid tumors (Trédan et al. 2007). Several agents (*i.e.*, anti-VEGF antibodies, drug delivery methods (nanoparticles, liposomes)) and physical methods (hyperthermia (HT) and photodynamic therapy (PDT) are being used to improve the penetration and retention of drugs in desired tumor areas by modulating factors relating to the tumor microenvironment such as :tumor blood flow, vascular permeability, tumor interstitial fluid pressure, stromal cells and extracellular matrix components.

Effects of Tumor Microenvironment on Hyperthermia and PDT

Hyperthermia (HT)

Among the methods previously highlighted as modulators of tumor microenvironment, hyperthermia plays a major clinical role. In fact, the advent of new devices (microwave, radiofrequency and ultrasound-derived) has permitted HT, in combination with radiotherapy or chemotherapy, to become a promising method for cancer treatment. Several comprehensive reviews can be found in the literature that focus on recent clinical and technical aspects of hyperthermia (Dewhirst et al. 1997, Stauffer 2005, Habash et al. 2006).

Hyperthermia, a procedure for raising the temperature of a part of or the whole body above normal (41–44°C) for a defined period, is applied alone or as an adjunctive with various established cancer treatment modalities such as radiotherapy and chemotherapy (Habash et al. 2006). Different studies in vitro have demonstrated that hyperthermia is effective and cytotoxic against several tumor cell lines and

the most important parameter influencing tissue response to heat is blood flow (Kampinga 2006, Oleson et al. 1988). Heat can modify both normal and tumor blood flow (TBF) and modifications of tumor blood flow and its environment can influence heat response (Horsman, Murata 2002, Vaupel).

The effect of heat on TBF is temperature dependent. Mild HT increases TBF and tumor oxygenation, whereas at temperature above 40°C there is only a transient increase in TBF followed by damage to tumor vasculature and a rapid decrease in blood flow (Brizel et al. 1996, Dudar, Jain 1984, Horsman). At a temperature above and below 40°C, a saturation of oxyhemoglobin (O_2Hb) within tumor microvessels has been demonstrated by Vaupel. The increased saturation obtained at temperature near 40°C can be exploited when HT is used in associated with radiotherapy (RT). Generally, the increase in TBF and O_2Hb can last for up 24 h after heating, and this phenomenon has been clinically demonstrated by Brizel et al. (1996) and Jones et al. (2004). This aspect has a large clinic impact when heat is used in combination with RT. As universally known, RT is oxygen-dependent and temperatures inside tumor masses near 40°C are easily obtainable compared to temperature near 43°C. These drawbacks are linked to technical methods for delivering heat to tissue and to anatomic and biological factors (Dewhirst et al. 1997).

Normal vasculature responds differently than tumor vasculature to heat with flow increasing as temperature increases, even at temperatures that produce vascular occlusion in tumors (Song et al. 2001). After heat exposure macroscopic blood flow measurements in normal tissue such as skin or muscle indicates a rapid and a dynamic vasodilatation with increased permeability of the vascular wall. Song and Vaupel demonstrated that the degree of alteration was temperature and treatment duration dependent (Song 1984, Song et al. 1990, Vaupel et al. 1987, Li 1984). The heat changes in the tumor were slightly absent or increased at the beginning of treatment, whereas if the treatment time was prolonged a decrease with a stasis of blood flow took place (Song 1984, Song et al. 1990, Vaupel et al. 1987). Similar conclusions regarding blood flow behavior during hyperthermia have been reached by other investigators using microscopic measurements such as RBC velocity, laser Doppler flowmetry and hydrogen clearance methods (Reinhold, Endrich 1986). Dudar and Jain studied RBC velocity and vessel lumen diameter in mature granulation tissue and in neoplastic tissue (VX2 carcinoma); they found and confirmed the above-mentioned observations, but noted that stasis occurred both in normal and tumor tissue. The difference in stasis was dependent on temperature. In fact, the stasis in normal tissue occurred later and at higher temperature (47°C), whereas stasis in tumor tissue was reached before and at a lower temperature (41°C) (Dudar, Jain 1984). Aside from vasodilatation, complex further events follow heat application. They comprise different biochemical and microcirculatory changes, such as acidosis, RBC stiffening and aggregation, degenerative changes of endothelium, increased vascular permeability, platelet aggregation, leukocyte sticking and intravascular clotting. These phenomena worsen the tumor microenvironment further and explain why neoplastic cells are damaged more easily by temperature (42–45°C) than normal cells (Song 1984, Dudar, Jain 1998, Reinhold, Endrich 1986).

Other microenvironmental factors beyond blood flow, hypoxia and pH, play important effects in hyperthermia. Hypoxia, due to inadequate blood supply, occurs very early during tumor development (Harris 2002) and activates several cellular transcription factors such as hypoxia-inducible factor HIF-1α (HIF-1α), activator protein-1 (AP-1) and NF-κB, which play roles in malignant transformation and progression (Kunz, Ibrahim 1984). Hypoxia-inducible factor 1 (HIF-1) is a "master" gene in the hypoxic response and activates the transcription of genes involved in diverse aspects of cellular and integrative physiology, including energy metabolism, cell growth, survival, invasion, migration or angiogenesis (Luo et al. 2006, López-Lázaro 2006). Usually, cancer cells display many altered metabolic abnormalities, including an increased capacity to metabolize carbohydrates mainly by anaerobic glycolysis even under aerobic conditions. This metabolic behavior results from the induction of several enzymes involved in the intermediate reactions of glycolysis by HIF-1-activated genes. (Dang, Semenza 1999, Shapot 1980, Behrooz, Ismail-Beigi 1999). The relevance of these alterations is that oxidation of glucose stops at the stage of pyruvic acid and proceeds anaerobically, producing for the same ATP amount six times more lactic acid and H^+ than normal cells. The excessive lactic acid and H^+ accumulation in tumor milieu, matched with the compromised interstitial fluid transport, causes the decrease of extracellular tumor pH (pHe) (Asby, Cantab 1996. Gullino et al. 1965, Younes et al. 1996).

Gerweck et al have demonstrated that environmental parameters such as hypoxia and acidosis, modify the cellular hyperthermic response. Oxygenated and acutely hypoxic cells were equally sensitive to hyperthermia; however, sensitivity increased with the time of culturing under hypoxic conditions before treatment (Gerweck et al. 1979). These investigators have also studied the response of human glioblastoma cells at different pH. Glioma cells, which were 5 to 10 times less heat sensitive than Chinese hamster ovary cells, showed a temperature-dependent sensitizing effect more pronounced at acidic pH. The pH-sensitizing effect and its temperature dependency was more pronounced at pH 6.1. From these studies the investigators concluded that hypoxic and acidic cells appeared to be slightly more sensitive to hyperthermia than oxygenated cells (Gerweck, Richards 1981). Along with these sensitizing effects, HT damages tumor vasculature. This damage can be obtained by a direct action on the endothelium itself or by an indirect mechanism on angiogenesis process.

Different in vivo and in vitro studies have shown that endothelial cells (ECs) and, in particular, proliferating endothelial cells, can be damaged by heat. Histologic methods have revealed that after hyperthermia, a rapid reduction and rearrangement of F-actin stress fibers follows (Fajardo, Prionas 1994, Amatthew et al. 2000). These fibers maintain the junctional integrity among the cells. Their lack determines an increase in vascular permeability, a phenomenon usually observed after HT. However, the effects of hyperthermia are not similar in all tumor types. This difference has been demonstrated by Nishimura et al. (1988) and it is dependent on the amount of connective tissues present in the vascular architecture. Tumor blood vessels are more vulnerable to heat than normal surrounding blood vessels, probably because of their structurally immaturity (Li 1984).

Hyperthermia has an inhibitory effect on angiogenesis. In the 1960s the inhibition of angiogenesis was ascribed to ischemia with a consequent obstruction and destruction of tumor blood vessels followed by an inability to form new vessels (Fajardo, Prionas 1994, Amatthew et al. 2000). Recently, it was clearly demonstrated that heat application can inhibit angiogenesis through activation of a plasminogen activator inhibitor I-dependent mechanism (PAI-I) (Roca et al. 2003).

The modifications of TBF can have profound effects on HT. As a consequence there has been considerable interest in finding various drug-based approaches for modifying vascular supply and thereby improving efficacy of HT (Kampinga 2006, Oleson et al. 1988).

Among the various drug-based approaches, vascular targeting agents (VTAs) are expected to show the greatest therapeutic benefit as part of combined modality regimens. Preclinical studies have shown VTA-induced enhancement of the effects of conventional chemotherapeutic agents, radiation, hyperthermia, radioimmunotherapy, and antiangiogenic agents. Unlike antiangiogenic drugs that inhibit the formation of new vessels, VTAs occlude the pre-existing blood vessels of tumors and rapidly shut down tumor perfusion, leading to tumor cell starvation and death (Horsman, Siemann 2006).

The VTAs can kill indirectly tumor cells that are resistant to conventional antiproliferative cancer therapies, *i.e.*, cells in areas distant from blood vessels where drug penetration is poor, and hypoxia can lead to radiation and drug resistance. There are broadly two types of VTAs, small molecules and ligand-based, which are grouped together, because they both cause acute vascular shutdown in tumors leading to massive necrosis (Thorpe 2004). The small molecules include combretastatin A-4 disodium phosphate (CA4DP), and flavone acetic acid and its derivative DMXAA. Ligand-based VTAs use antibodies, peptides, or growth factors (*e.g.*, VEGF linked to the plant toxin gelonin, immunotoxins that bind selectively to tumor *versus* normal vessels) to target tumors with agents that occlude blood vessels. Combretastatin A-4 disodium phosphate, and DMXAA are undergoing clinical evaluation (Thorpe 2004). The VTAs are most effective against vessels in the inner tumor mass, possibly because the high interstitial pressure in these regions contributes to vascular collapse (Tozer et al. 2005), but the pharmacologic effect lasts for a short period. This short lapse of activity oblige their use with other modalities. Horsman et al. have experimentally demonstrated (C3H mammary carcinoma) a linear relationship between the time of heating and the tumor growth delay using VTAs (Horsman). They showed that DMXXA was more effective with HT than with CA4DP. The effect was maximal when heat was applied after drug administration and the optimal delay for combining VTAs + HT was 3 hours (Hokland, Horsman 2007, Horsman 2002). This result is in agreement with previous experiments of tumor vessels clamping or chemoembolization, two techniques able to render tumor totally or partially hypoxic (Song et al. 2001, Jirtle 1988). Hypoxia is an adverse condition that rend the tumor cells more sensitive to hyperthermia (Overgaard, Bichel 1977).

As described above, many barriers are formed in the tumor area that hinder drug delivery. HT $\leq 42^{\circ}$C (mild hyperthermia) exerts selective effects of on tumor vasculature (improvement in vascular perfusion, increase in tumor vascular permeability), that must be exploited for augmenting drug bioavailability to tumor mass. Elevated interstitial fluid pressure in the tumor mass may limit the delivery and distribution of therapeutic agents (Jain 1990). Leunig and coworkers (Leunig et al. 1992) have demonstrated that HT induces a significant decrease in IFP, improving in this sense the drug uptake. Furthermore, HT increases drug extravasation and uptake by altering tumor microvascular permeability (Nilsen 1984. Lefor et al. 1985). We report briefly some clinical applications with new carriers.

Liposomes

Liposomes are small unilamellar lipid vesicles designed to have specific temperature-dependent phase transitions point. Many drugs such as methotrexate and doxorubicin can be included in these lipid vesicles that are released at the point of phase transition temperature, avoiding systemic side effects and increasing tumor cell killing (Drummond et al. 1999). Maekawa et al. have demonstrated a survival prolongation in rats receiving temperature-sensitive liposomes containing bleomycin compared to groups receiving hyperthermia or bleomycin alone, or a combination of both (Maekawa et al. 1987).

Magnetic Drugs

Recently, a new class of liposomes (magnetic cationic liposomes (MCL)) has been developed. These liposomes consist of cationic liposomes containing 10 nm magnetite nanoparticles obtained by sonication. Preliminary experimental results on hamster osteosarcoma in association with hyperthermia (obtained by the application of a magnetic field with a frequency of 118 KHz), has shown complete regression in the group using this methodology compared to a control group (Matsuoka et al. 2004)

Nanoparticles

Nanoparticles are particulates with a size between 500 nm and 1 μm. They have been used since 1970 to carry vaccines or anticancer drugs (Moghini et al. 2001). Kong et al. have investigated their behavior during HT and have demonstrated that their extravasation was temperature dependent and lasting 6 h post heat application (Kong et al. 2001).

Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is a promising cancer treatment modality. The treatment consists of pre-administering parenterally or locally a photosensitizing agent (photosensitiser) and then illuminating with light of a specific wavelength (often in the red wavelength region) tumor. A photochemical reaction is induced as the photosensitiser absorbs the light, that determines an excitation of ground state of the oxygen present in the tumor tissue generating cytotoxic singlet oxygen. Singlet oxygen reacts with cellular targets such as plasma membranes, mitochondria and lysosomes, leading to damage of the biological tissue. The result is an efficient induction of cell death, primarily through apoptosis, microvascular damage, and an antitumor immune response (Dougherty et al. 1998, Harrod-Kim 2006, Castano et al. 2006). To produce the desired tissue destruction PDT requires the simultaneous presence of photosensitiser, tissue oxygen and light of a specific wavelength.

As already mentioned, most tumors harbor areas of severe hypoxia and develop highly variable tumor microenvironments, marked by gradients of nutrient (oxygen and glucose), with regions of hypoxia, acidity, and necrosis, and heterogeneous proliferation. The availability of oxygen is a critical feature for obtaining the desired photosensitization; cells distant from blood vessels are resistant to PDT since they receive low concentration of the phototsensitizer and their oxygenation is poor (Freitas 1985). Photoreaction is a localized reaction, as deduced by the short half-life (0.6×10^{-6}s) and diffusion distance (0.1 μm) of singlet oxygen and other free radicals produced (Henderson, Dougherty 1992). Besides oxygen concentration in that area, which is critical for generating the free radicals, other tumor environmental conditions (inflammation, hypoxia and tumor vascularization) are of outstanding importance (Verma et al. 2007). Different strategies acting on tumor microenvironment have been suggested for enhancing PDT therapy. In particular, combination with hyperthermia, regulation of inflammatory effects, action on tumor vasculature, different delivery strategies for carrying photosensitizers to tumor (Gomer et al. 2006, Verma et al. 2007). A synergistic interaction between PDT and hyperthermia has been described both in vitro and experimental mouse tumor systems (Rasch et al. 1996, Henderson et al. 1985). Hyperthermia must follow PDT to obtain maximum effect, as clearly demonstrated by Henderson et al. (1985) in animal experiments where the combination of these two modalities in the proper sequence potentiated cytocidal effects on the tumor cells in vivo. PDT alone followed by heat had a success rate of 45% whereas PDT alone and heat followed by PDT cured less than 10% of animals.

This study shows that these two modalities lead to tumor destruction by different but complementary mechanisms. Freitas et al. have analyzed some of these mechanisms and reached the conclusion that effects of both treatments on the tumor vasculature are responsible of this synergism (Freitas et al. 1990).

Gomer and Verma have analyzed other combined modalities able to modify tumor microenvironment and useful for enhancing PDT (Gomer et al. 2006, Verma et al. 2007). Different antiangiogenic factors such as inhibitors of VEGF like Avastin (bevacizumab) or the Cox2 inhibitors (Celecoxib) have shown an enhancement of

tumoricidal action of PDT (Gomer et al. 2006). A synergistic effect has been proven by Seshadri using a vascular targeting agent such as 5,6-Dimethylxantone-4-Acetic (DMXAA) (Seshadri et al. 2005).

The efficacy of PDT can be improved using two other modalities: (a) increase in the quantity of photosensitiser (PS) in the tumor area; and, (b) exploitation of the increase in antigen presentation produced by PDT treatment for enhancing antitumor immunity. The photosensitizer (PS) can be targeted to the tumor as a pro-drug or using a carrier. In the first case, *Krinik* used a polymer-bound PS that was susceptible to lysosomal degradation. However, the achieved selectivity was modest because the target enzyme is not specific to neuroblastoma cancer cells (Krinik et al. 1994). The second way is increase in uptake of tumor of macromolecules or nanoparticles bound with the photosensitiser. Polymers constituted by polylactic acid (PLA) or polylacti-co-glycolitic acid (PLGA) have been loaded with PS and studied for verifying their photoactivity. NuTu-19–cell ovarian cancer cells, derived from Fisher 344 rats, have been treated with PLA and PLGA and showed an higher photoactivity than animals treated with free PS (Zeisser-Labouèbe et al. 2006).

In advanced stages of disease, cancer patients show an impairment in mounting an effective anti-tumor immunity. This impaired anti-tumor immunity depends upon the lack of activated antigen presenting cells (APCs) (Baronzio, Freitas 2008). Gollnick demonstrated that the inflammatory reaction produced by PDT associated with components released by dying tumor cells results in the activation of antigen-presenting cells (APCs) with stimulation of effector T cells (Gollnick et al. 2006). This is another example of a clinical useful combination of therapies.

Nanomedicine: Nanoparticles-Mediated Drug Delivery to Solid Tumors

The ability to deliver highly efficient therapeutic compounds specifically to tumor mass is crucial for effectively treating it. Unfortunately, conventional chemotherapeutic strategies require systemic administration due to non-specific biodistribution and rapid metabolism of free drug molecules before reaching their targeted sites (Minchinton, Tannock 2006, Sanga et al. 2006). The first and most important biological barriers, which need to be addressed during drug delivery system development, include the rapid elimination of the drug from the body, its degradation, transport and sequestration within non target cells in the body. Nanotechnology can overcome some of these impairments. For example, nanodrugs with a size greater than 10 nm avoid kidney clearance, resulting in prolonged and elevated levels in the blood stream. Slightly larger particles, ranging from approximately 70–200 nm, demonstrate the most prolonged circulation times. In contrast, even larger particles, with diameters higher than 200 nm, are usually sequestered by the spleen because of mechanical filtration and are eventually removed by the cells of the phagocyte system, resulting in decreased blood circulation times (Kabanov, Gendelman 2007). The upper limit for particle size is approximately 100 nm to allow the particles to

leave the blood vessels and diffuse within the tumor tissue and be retained in the interstitium exploiting the "Enhanced Permeability and Retention" (EPR) effect. As previous pointed, the EPR effect can be attributed to two factors: long-circulating nano-particulate drugs are able to escape the vasculature through abnormally leaky tumor blood vessels and are subsequently retained in the tumor tissue due to a lack of effective tumor lymphatic drainage (Maeda et al. 2000). Factors that influence tissue distribution of such drugs include drug transport, receptor/drug binding, and cellular pharmacology, which is the processing and routing of the drug within cells (Sanga et al. 2006).

In order for an anticancer agent to work, it must extravasate, diffuse through the tumor mass, and then be transported into the cells, where it must bind to its target and express it inhibitory activity (Minchinton, Tannock 2006). The heterogeneity and three-dimensionality of the tumor environment impairs both the pharmacokinetics and pharmacodynamics of anticancer drugs and explain why a drug that works in vitro does not exert the same activity in vivo (Trédan et al. 2007). The effects of this last factor call for the development effective drug delivery mechanisms. The main objective of drug delivery systems is to deliver a drug effectively, specifically to the site of action and to achieve greater efficacy and minimize the toxic effects compared to conventional drugs (Kshirsagar et al. 2005).

Nanoparticle-based drug delivery systems consist in small colloidal particles that are made of non-biodegradable and biodegradable polymers, and have considerable potential for allowing controlled drug release to the desired tissue and for overcoming some these barriers. Their diameter is generally around 200 nm. Nanocarriers offer several important technological advantages compared to other delivering technologies; in particular: high stability, high carrier capacity, feasibility of incorporation of both hydrophilic and hydrophobic substances, and last but not least, favorable dimensions for targeting specific cells or cellular structures and because of its unique size (1–100 nm) a large surface/volume ratio (McNeil 2005, Ehdaie 2007). In fact, cells can absorb materials < 100 nm and nanocarriers can offer new delivery solutions in this sense (Jotterand 2007).

Nanoparticles used for drug delivering are defined as submicron ($< 1\,\mu$m) colloidal particles, composed of different biodegradable materials like natural or synthetic polymers, lipoids or phospholipids and even organometallic compounds (Rawat et al. 2006). Two types of nanoparticles can be distinguished: nanospheres, which are matrix systems; and nanocapsules, which are reservoir systems composed of a polymer membrane surrounding an oily or aqueous core (Cuenca et al. 2006, Fattal, Vauthier 2006). Amongst various nanocarrier systems, liposomes have generated a great interest as vehicle for different anticancer drugs (*i.e.* Doxorubicin, paclitaxel, irinotecan) (Drummond et al. 1999).

Liposomes are spherical vesicles composed of phospholipids and cholesterol that in aqueous medium form closed bilayer spheres. Within these spheres aqueous soluble drugs may be encapsulated, whereas lipid soluble drugs are complexed within the bilayer membrane.

Liposomes formed simply by the phospholipids are called naked liposomes and their use as anticancer drug delivery system was hampered by the rapid clearance from the circulation and entrapping by the reticuloendothelial system (RES)

(macrophages and liver) (Gregoriadis, Ryman 1972). The size of these liposomes, on the order of 400 nm was also an obstacle. In fact, these naked liposomes rapidly entered tumor sites from the blood, but were kept in the bloodstream by the normal vasculature, limiting so their efficacy. To overcome the disadvantage of rapid clearance and uptake in healthy tissues, hydrated polyethylene glycol (PEG) molecules were added at the exterior of the lipid bilayer (Drummond et al. 1999, Papahadjopoulos et al. 1991). These liposomes are called pegylated liposomes or "stealth" liposomes, commercially available as Doxil® or Caelyx®. They contain doxorubicin within the aqueous core and have a size < 200 nm. Stealth liposomes offer many advantages compared to naked ones: they have a long circulation time, are practically not recognized by the RES, target preferentially tumor tissue through the increased permeability of the capillaries (EPR phenomenon) that nourish these tissues and show a considerably reduced cardiotoxicity as demonstrated by clinical trials. In fact, as reported by Gabizon the long circulating time in blood was correlated with an increased uptake by tumor (Gabizon, Papahadjopoulos 1988).

Other types of stealth liposomes are the immunoliposomes and the cationic liposomes. For targeting tumor tissue, immunoliposomes are comprised of an antibody or a fragment of antibody for cell recognition, whereas cationic liposomes are stealth liposomes with surfaces that are positively charged to increase the loading efficiency of their engineered genetic material (DNA) for treating different kinds of genetic diseases.

Interrelationships Between Hyperthermia, Photodynamic Therapy and Nanotherapy

Several studies in this last decade have demonstrated an interrelationships between anticancer therapies and nanotechnology.

Magnetic Hyperthermia (MgHT). Several clinical trials have demonstrated that hyperthermia is an effective cancer therapy, however many disappointing aspects of this therapy were found. In hyperthermia the critical factor is to heat tumor cells while sparing surrounding normal tissues. The presence in the tumor area of hypoxic or not completely perfused regions due to the chaotic blood flow determines a non-homogeneous heat deposition. To overcome this problem, alternative approaches to the classical methods (radiofrequency, ultrasound, microwave) for delivering heat to desired tissues are being studied: **intracellular hyperthermia** and **magnetic fluid** hyperthermia (MFH).

In **intracellular hyperthermia,** biocompatible nano particles (Superparamagnetic and fine ferrimagnetic particles such as Fe_3O_4) can be used as heat mediators, after their incorporation into tumor cells they are selectively heated up by coupling alternating coupled magnetic field. As a result, the whole tumor is heated uniformly, and a more concentrated and localized heating is obtained (Jyotsnendu et al. 2003).

Magnetic fluid hyperthermia (MFH) consists of fluids of ultramicroscopic particles ($\sim$100Å) of magnetic oxide (Micron-sized magnetic particles (Fe_3O_4)). When these magnetic nanoparticles are placed in an external alternate current magnetic

field of appropriate frequencies they produce heat through various kinds of energy losses. The frequencies must be comprised between 50 KHz and 10–100 MHz. Current > of 50 kHZ are used to avoid neuromuscular electro-stimulation and currents of 10–100 MHz are used to reach tissue deeply situated in the human cavity (i.e liver, kidney). The special feature of ferrofluids is the combination of normal liquid behavior with superparamagnetic properties.The iron oxide nanoparticles generate heat through two phenomenon. Size seems to be determinant, in fact for nanoparticles with size $\approx$ 10 nm heat is generated by hysteresis loss, whereas for size > 10 nm heat is generated by the relaxation phenomenon of Nèel (Duguet et al. 2007). In both methods, nanoparticles can be targeted to tumor area by focused external magnetic fields or by intratumoral injection (Johannsen et al. 2005, Besic 2007).

Physically Controlled Drug Release (Hyperthermia, Ultrasound Therapy)

Most anticancer therapies in use today are not targeted to tumor sites. Therefore, targeted cancer treatment can potentially maximize cancer cure and minimize normal tissue toxicity. Furthermore, release of anticancer drugs on demand at the tumor site is important. Both of these aims can be accomplished by methods that generate physical energy such as heat or ultrasound (US) combined with nanotechnology.

Acoustic controlled Drug Delivery (**ATDD)** is a method that uses ultrasound (US) energy to enhance the transport of molecules into or across specific tissues (Pitt 2003). Ultrasound is a very attractive modality for drug delivery and show two important advantages compared to other delivery methods. The primary advantage of ultrasound is that it is as a physical, rather than a chemical approach, and applicable to a variety of drugs and cell types (Sundaram et al. 2003). The second advantage is that energy that is noninvasively transmitted through the skin can be focused on a specific location and employed to release drug at that site (Pitt 2003). Ultrasound has been shown to facilitate the delivery of chemotherapeutic drugs into tumors through the cavitation effect. Cavitation is a physical effects that perturbs cell membrane structures via several modes and increases the permeability to drugs or other solutes (Pitt 2003).

Another potential of using ATDD is the transport of drugs by ultrasound contrast agents (microbubbles) (Besic 2007). Microbubbles are very small encapsulated gas bubbles (diameters of micrometers) that can be used as contrast media and upon exposure to sufficiently intense ultrasound, they will cavitate, rupture and release gas content. Such characteristics are used to carry a drug or a gene until a specific area of interest is reached, and then ultrasound is used to burst the microbubbles, causing site-specific delivery of the bioactive materials (Tsutsui et al. 2004). Ultrasound not only enhances the drug release but also enhances the intracellular uptake of microbubbles through the formation of cavities along the cellular membranes (Besic 2007). As reported by *Ka-Yun,* the ultrasounds enhance chemotoxicity not

only through cavitation but also through thermal mechanisms. Not all drugs appear to work through such approach. In fact, cytotoxicity is enhanced in presence of ultrasound for daunorubicin, doxorubicin, nitrogen mustards (BCNU), and diaziquone. Whereas, for cisplatin and mitomycin–C, cytotoxicity is not enhanced, (Ka-Yun, Matsunaga 2005).

Hyperthermia Controlled Drug Delivery

The phospholipid composition of liposomes can be manipulated to obtain temperature sensitive liposomes. The idea of using temperature-sensitive liposomes was advocated firstly by Yatvin but this method for drug delivery was developed later by Dewhirst and his group (Needham, Dewhirst 2001). This group developed liposomes made of 1,2-di-palimitoyl-sn-glycero-3-phosphocoline (DPPC) with a transition temperature at 41.5°C that have doxorubicin (DX) incorporated inside. This new type of liposome in combination with mild hyperthermia, was significantly more effective than free drug or current liposome formulations at reducing tumor growth in a human squamous cell carcinoma xenograft line (FaDu), producing 11 of 11 complete regressions lasting up to 60 days post-treatment (Needham et al. 2000). These studies have established that thermosensitive liposomes plus hyperthermia release more drug than conventional therapies at tumor sites. The mechanisms responsible for this improvement were increased vascular perfusion and increased extravasation of liposomes induced by heat. A ∼2–4 fold increase in drug uptake and a 2–16 fold increase in delivery has been demonstrated in heated tumor compared to non-heated tumors (Ponce et al. 2006). In conclusion, thermosensitive liposomes successfully exploit the EPR phenomenon and the effects on tumor vasculature induced by hyperthermia.

Conclusion

Development of effective drug delivery mechanisms depends on a thorough understanding of tumor physiology. The vasculature structure is one of the most important aspects of tumor physiology, affecting nutrient delivery, cell growth and patterning of the microenvironment (e.g. regions of hypoxia, acidosis, and necrosis). Therefore, it should be the first structure to modify or exploit.

Because tumor survival is critically dependent on a functional vasculature, selective damaging of the vasculature of tumors has been proposed as an attractive strategy against cancer. The unique environment that ensues does not simply produce a hostile microenvironment but offers an opportunity for it modification. In this overview we have demonstrated some applications of new technologies used to modify the cancer microenvironment, thereby resulting in more efficient therapies for treating cancer patients.

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Chapter 11
Targeting Tumour Vascularization from Bench to Bedside: Suggestions for Combination with Hyperthermia

Girolamo Ranieri, Annamaria Catino, Vittorio Mattioli, Vito Fazio, Gennaro Gadaleta-Caldarola and Cosmo Damiano Gadaleta

Abstract Angiogenesis is an important pathway in tumour growth and progression. Overexpression of pro-angiogenic factor or down regulation of physiologic angiogenic inhibitors are the stimuli that induce new blood vessel formation from a pre-existing vascular bed. On the other hand tumour vasculature is a major important factor influencing the therapeutic application of hyperthermia used as anticancer therapy. Both endothelial cells and microvessels can be lethally damaged by the hyperthermia. Because tumour vasculature is a target of hyperthermia combined treatments with angiogenesis inhibiting agents or vascular disrupting agents and hyperthermia may lead to synergetic effects or potentiation of the combined therapy over each modality alone. In this chapter we summarize the state of the art regarding the combination between drugs that targeting tumour vasculature and hyperthermia, furthermore the pre-clinical rationale for future clinical trials is suggested.

Keywords Angiogenesis · Hyperthermia · Angiogenesis inhibiting agents · Vascular disrupting agents · Clinical trials

The Central Roles of Angiogenesis in Tumour Growth and Progression

The term angiogenesis was first proposed by Judah Folkman in 1971 to define the process by which tumour growth depended on new blood vessels formed from the pre-existing vascular bed (Folkman, 1971).

In the adult life, turnover of endothelial cells is very slow and angiogenesis usually occurs during wound healing and menstrual cycle in ovaries and endometrium. Tumor endothelial cells may divide up to 50 times more frequently than endothelial

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cells of normal tissue. Acquisition of the angiogenic phenotype is of pivotal importance during tumour growth and progression. It has been demonstrated that growth of tumours beyond a limited size of 0.2–2 mm in experimental models depends on angiogenesis. In its absence, tumours are unable to grow further although active cell proliferation, counter-balanced by apoptosis, is seen in so-called dormant tumour areas (Holmgren et al. 1995, Bergers et al. 1998). The passage from pre-angiogenic phenotype to angiogenic phenotype, often referred as the "angiogenic switch", permits the formation of new capillary blood vessels from existing vasculature that indispensable for further tumour growth. The same process also favours metastatic dissemination (Folkman 2002, Gasparini et al. 1998, Ranieri et al. 2004). The angiogenic process is regulated by a balance of pro-angiogenic factors and naturally occurring angiogenic inhibitors (Distler et al. 2002, Ranieri and Gasparini 2001, Kumar-Singh et al. 1999). Pro-angiogenic factors include vascular endothelial growth factor (VEGF), thymidine phosphorilase (TP) and basic - fibroblast growth factor (b-FGF) (Distler et al. 2002, Ranieri and Gasparini 2001, Kumar-Singh et al. 1999). VEGF is presently the most extensively characterized endothelial growth factor and it is secreted by tumour cells in response to environmental stimuli, mainly hypoxia and oncogenic mutations. Soluble isoforms of VEGF protein bind to two specific tyrosine-khynase receptors: VEGF-1 (flt-1) and VEGF-2 (KDR/flk-1), which are up-regulated almost exclusively in migrating/proliferating blood endothelial cells (Fig. 11.1). In vivo, VEGF induces agiogenesis and also prolongs survival of newly formed vessels by inducing expression of Bcl-2 (Ranieri et al. 2004, Byrne et al. 2005). Among angiogenic inhibitors angiostatin and endostatin are most important (Folkman 2004, Vailhe and Feige 2003). Purified human angiostatin is an internal fragment of plasminogen and it contains three molecular weight species of 40, 42 and 45 kDa. Each of the three inhibit endothelial cell proliferation. Similarly endostatin is a 20 kDa terminal

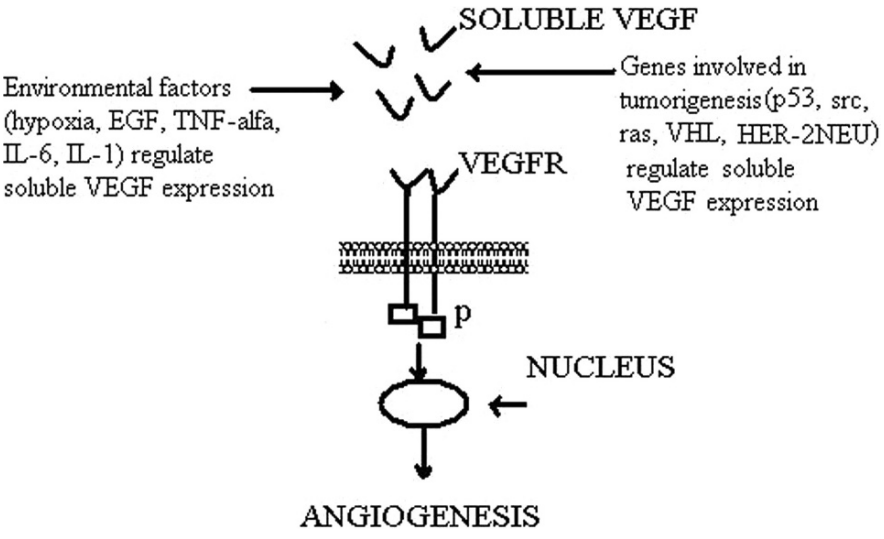

Fig. 11.1 VEGF signalling in angiogenesis

fragment of collagen XVIII, which is able to inhibit both proliferation and migration of endothelial cells. In in vitro studies, endostatin was shown to induce a block in cell cycle progression of endothelial cells.

Several mechanisms are responsible for initiating the switch to tumour angiogenesis. In particular, hypoxia and factors related to hypoxia increase expression of VEGF and VEGF receptors (Forsythe et al. 1996). Oncogenes, including H-ras, K-ras, fos, HER-2/neu, src and v-raf stimulate angiogenesis by up-regulating VEGF (Fig. 11.1) (Rak et al. 1995, Okada et al. 1998). Other oncogenes such as proto-c-met, p53, RB, VHL and thrombospondin-1 control the expression of VEGF (Dameron et al. 1994, Griffioen and Molema 2000, Rak et al. 2000).

Indeed, common pathways link tumour and endothelial cell invasiveness. VEGF stimulates integrin $\alpha\upsilon\beta3$ which plays a pivotal role in promoting both neovascularization and tumour cell invasiveness through pathways of degradation of plasminogen and modulation of matrix metalloproteases activity (Brooks et al. 1995). In the same manner, b-FGF is associated with increased levels of plasminogen activity. BRAF is also an important signalling factor for both vasculogenesis and angiogenesis. It acts as a natural inhibitor of angiogenesis and induces apoptosis in both endothelial and tumour cells (Gasparini 1999). Angiogenesis is also connected with the immune system. VEGF facilitates the expression of several adhesion molecule such as vascular cell adhesion molecule-1 and E-selectin which are involved in cell-cell contact with circulating immune cells (Melder et al. 1996).

Compelling studies have suggested that the assessment of microvessel density (MVD) or endothelial area (EA) can be considered both surrogate angiogenic markers and prognostic factors in cancer (Ranieri et al. 2002, Vermeulen et al. 2002). MVD assessment is the most commonly used technique to quantify intratumoral angiogenesis in cancer and it was first developed by Weidner et al. (1991). More recently, some studies suggested that also EA is a suitable angiogenic marker in tumours (Ranieri et al. 2005, Patruno et al. 2006).

In other studies both tissue expression and serum levels of VEGF correlate with prognosis and responsiveness to antiangiogenic agents (Gadaleta et al. 2004, Brattstrom et al. 2004). Moreover, recent investigations suggest that plasma VEGF levels may be a suitable angiogenic marker of tumor aggressiveness and clinical outcome (Patruno et al. 2008). In general, highly vascularized tumors are biologically more aggressive and are associated with shorter survival.

Microenvironmental Factors and Oncogenes that Up-Regulate VEGF

Main Steps of Angiogenesis Leading to Tumour Vascular Network

Overexpression of angiogenic factors, in particular VEGF, or down-regulation of angiogenesis inhibitors induce endothelial cells to proliferate (Folkman 2002). On tumour endothelial cells, expression of membrane receptors for angiogenic

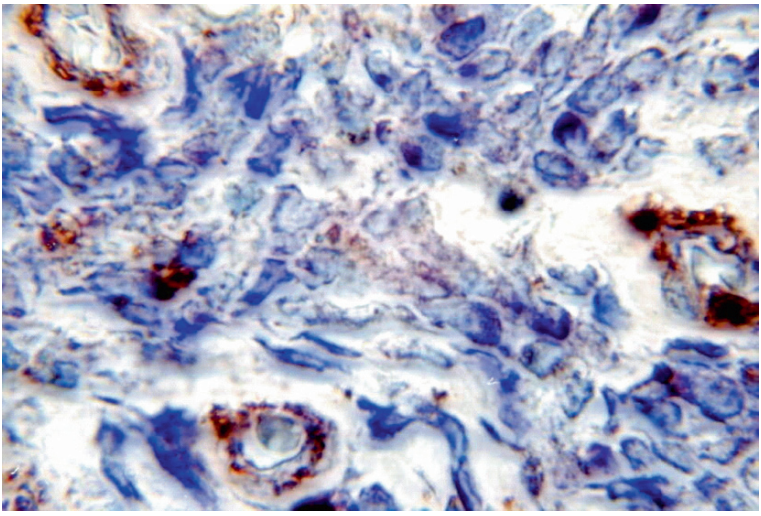

Fig. 11.2 Double staining of a dog mast cell tumor with immunohistochemistry specific for vessel identification (anti FVIII-RA antibody) and with toluidine blue for mast cells. Degranulated mast cells stimulate angiogenesis by the release of pro-angiogenic factors (magnification 1000x in oil)

factors (e.g., E-selectin) are often up-regulated in concomitance with increased expression of angiogenic ligands. Subsequently, proteolytic enzymes (collagenase, plasminogen activator and metallopreteinases) are released with consequent degradation of underlying basement membrane and extracellular matrix (Ranieri and Gasparini 2001). Endothelial cells tumour cells and host inflammatory cells (macrophages, mast cells) take part in the release of proteolytic enzymes (Fig. 11.2). In addition, macrophages and mast cells carry proangiogenic factors and proteolytic enzymes (Patruno et al. 2008). Endothelial cells start to migrate across the break of the wall vessel into stromal matrix producing a "sprout". Sprout cells begin to proliferate and elongate with consequent canalization. The newly migrating and proliferating endothelial cells induce specific integrins ($\alpha\beta3$ and $\alpha\beta5$) which are essential for their viability while growing (Fig. 11.3) (Gasparini et al. 1998). Subsequently, the new capillary-tubes interface to produce the intratumoral vascular network. In the end, the sub-endothelial basement membrane is apposed and pericytes finish the capillary wall (Fig. 11.4). These new blood vessels are characterized by altered morphology with loops, arteriovenous anastomoses and a disorganized network pattern. Lack of innervation impairs their capacity of autoregulation and of response to physiological neurovegetative stimuli (Vermeulen et al. 2002). The altered morphogenesis of tumour neovascularization is related also to the up-regulation of the E-selectin adhesion molecules.

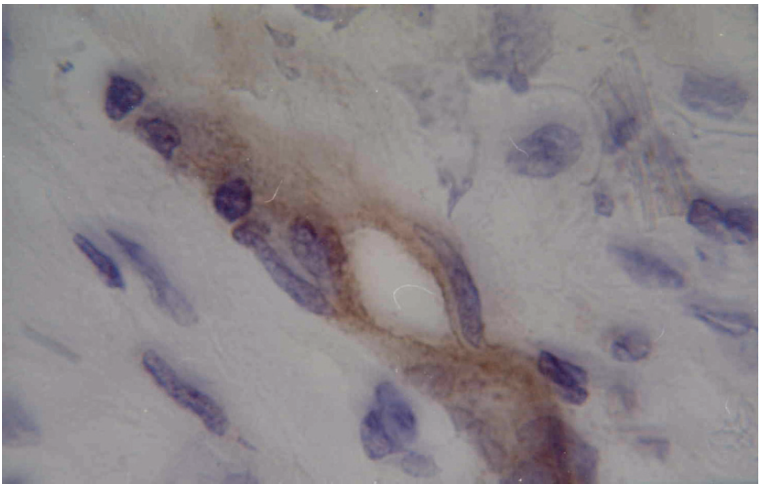

Fig. 11.3 Immunostaining specific for a single neoformed microvessel from a section of primary human breast cancer tissue. Primary antibody is an anti αβ3 expressed preferentially on proliferating endothelial cell. Microvessel is red stained and nucles of endothelial cells are evident near the lumen (magnification 1000x in oil)

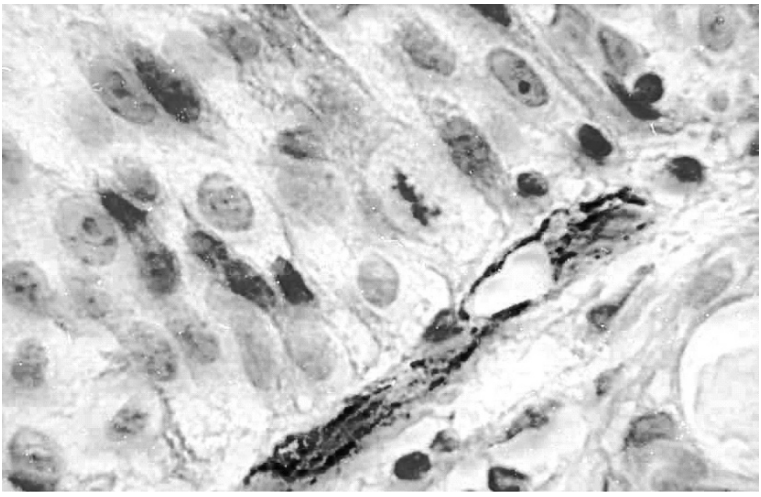

Fig. 11.4 Immunostaining for a single neoformed microvessels from a section of primary human squamous cell cancer tissue. Primary antibody is an anti CD-34 antibody. An immature microvessel is red stained with an evident lumen (magnification 1000x in oil)

Hyperthermia and Angiogenesis

Rilevance of Angiogenesis for Hyperthermia

As previously described, the tumour vasculature plays an important role in tumour growth and progression due to it importance for survival of tumour cells (Reinhold and Endrich 1986). On the other hand tumour vasculature is an important factor influencing the therapeutic applications of hyperthermia. The vascular network is known to be a target for hyperthermia damage (Kampinga 2006, Dewhirst et al. 2005). Both endothelial cells (EC) and microvessels can be lethally damaged by the hyperthermia doses used as antineoplastic therapy. In vitro data indicate that capillary EC are sensitive to hyperthermia. Proliferating EC are more thermosensitive suggesting that microvessels of malignant neoplasms (which contain many proliferating EC) are more affected than microvessels of normal tissues. This differential sensitivity of microvessels has also been observed in blood flow studies. Furthermore, hyperthermia inhibits angiogenesis (Horsman 2008). Thus, some of the antineoplastic effects of heat are caused by ischaemia due to obstruction or destruction of the tumour vessels or to inability to form new vessels. Sublethal EC damage can also be demonstrated, resulting in decreased synthesis of most proteins including adhesion molecules (as well as increased expression of a few such as heat shock proteins) and producing reversible loss of cytoskeletal elements (Calderwood et al. 2005). Accordingly heat treatment of EC strongly inhibits their differentiation into vascular structures both in matrigel assay, which recapitulates numerous aspects of the sequential steps of angiogenesis, including cell migration, differentiation, and metalloproteinase activation, and in spheroid assay, which mimics vessel sprouting from a preexisting one (Roca et al. 2003). Hyperthermia also inhibits angiogenesis in aortic ring and in chick embryo chorioallantoic membrane assays. These results parallel those obtained in mice where hyperthermia reduces tumor vascularization (Uchibayashi et al. 1992). Gene profile analysis performed on heated EC clearly show that hyperthermia activates a specific gene response, involving the transcription of plasminogen activator inhibitor-1 (Roca et al. 2003). Consequently, a proteolytic cascade ensues, including vascular thrombosis, metastasis diffusion, inflammation, and angiogenesis. During neo-vascularization, this system is pivotal in remodeling extracellular matrix and allows EC to find a more favorable microenvironment for their proliferation and differentiation. On the other hand tumor neovasculature is very different from normal vessels from which it arises, being both structurally and functionally abnormal (Ranieri and Gasparini 2001). As a result, tumour cells that do not have an adeguate blood supply become oxygen starved (hypoxic). In addition blood flow is a major means by which heat is dissipated from tissues, and thus influences the ability to heat tumours.

Targeting Tumour Vascularization

The importance of tumour vasculature makes it an intriguing target for therapy and two major strategies have been developed. The first is based on agents that inhibit

one or several steps of angiogenesis blocking blood vessel development (angiogenesis inhibiting agents, AIA) (Ranieri and Gasparini 2001). The second, vascular targeting, aims to destroy the existing vasculature acutely inducing tumour necrosis. The second is performed by so called vascular targeting performed by mean of Vascular Distrupting Agents (VDA) (Denekamp 1982).

Combination Between AIA and Hyperthermia

Biological Rationale of Antiangiogenic Therapy

Activated endothelium is the primary target for inhibition of angiogenesis and this therapeutic targeting presents several advantages: (i) tumour endothelial cells are diploid cells with a stable genome. Consequently, these cells represent a uniform target with less probability to develop resistance to angiosuppressive agents; (ii) under physiological conditions endothelial cells are quiescent in the adult life, whilst tumour endothelium is activated and proliferating. This is a very important point that suggests a selective target with few effects on normal vasculature and few systemic adverse effects; (iii) easy accessibility of therapeutic agent to endothelial cells through the blood. Because the tumour vasculature is a target of hyperthermia, combined treatments with AIA and hyperthermia may lead to synergetic effects.

TNP-470

TNP-470 is a synthetic analogue of fumagillin isolated from the fungus *Aspergillus fumigatus fresienus.* TNP-470 is angiostatic in vitro and acts by preventing the entry of endothelial cells into G1 phase of the cell cycle. The effect of a combining TNP-470 and hyperthermia on tumour growth was examined using human esophageal and gastric cancers transplantable to nude mice. TNP-470 alone at a dose of 30 mg/kg three times a week for 2 weeks was sufficient to obtain an antitumour effect. A combination of this dose of TNP-470 and 43°C hyperthermia for 30 min inhibited tumour growth markedly in comparison with either treatment alone. It was considered that angiogenesis after hyperthermia was inhibited by TNP-470, and then regrowth of the tumour cells was potently suppressed by reduction of O2 pressure, pH and nutrient supply in the tumour tissue (Yano et al. 1995).

In another study significant delay of tumour growth was observed when TNP-470 (100 mg kg-1 × 2 or × 4) was administered after hyperthermia at 44°C. The tumour growth times of the combined treatment were significantly longer than those of heat alone (44°C) or TNP-470 (100 mg kg-1 × 2 or × 4) alone. However, the tumour growth time of combined treatment with TNP-470 and hyperthermia at 42°C was quite similar to that of TNP-470 alone. This conflicting result on the combined effect of TNP-470 and hyperthermia may be related to the temperature-dependent vascular damage by hyperthermia. Dose-dependent inhibition of angiogenesis by TNP-470 was demonstrated in microangiograms obtained 4 days and 7 days after hyperthermia (44°C for 30 min). It is, thus, suggested that the combined effect of

TNP-470 and hyperthermia is attributable to the inhibition of angiogenesis by TNP-470 following heat-induced vascular damage (Nishimura et al. 1996).

Ikeda and co-workers investigated the effect of angiogenesis inhibitors (FR118487 and TNP-470) on two murine tumors (FM3A and SCC-VII) with and without hyperthermia. When angiogenesis inhibitors were administered immediately after tumour implantations there was no tumor growth. As the tumor diameter increased, the antitumour effect of the inhibitors decreased. When FR118487 was combined with hyperthermia, the antitumour effect was potentiated. This study suggested that angiogenesis inhibitors show their maximal effect against small tumors and have increased activity when combined with hyperthermia (Ikeda et al. 1998).

Finally intratumoral localization of VEGF following administration of hyperthermia and/or TNP-470 was evaluated using SCC VII tumours in C3H/He mice. Hyperthermia at 44°C for 30 min was given with a water bath on day 0. TNP-470 (100 mg/kg) was administered alone or after hyperthermia on day 0 and day 3. The average percentage of necrotic area of the untreated SCC VII tumours was 7%, while those of tumours treated with TNP-470 alone and hyperthermia alone were 27 or 65%, respectively. When HT and TNP-470 were combined, the percentage of necrotic area was 82%, which was significantly higher than that caused by HT alone suggesting an additive effects (Masunaga et al. 2000, Kanamori et al. 1999).

Batimastat (BB-94)

Batimastat (BB-94), a pseudopeptide hydroxamic acid, showed antitumour, antimetastatic and antiangiogenic activity in several tumor models (Wylie et al. 1999). BB-94 was the first syntetic metalloproteinases inhibitor tested on a human tumour (Zhang 1998). The anti-vascular activity of local hyperthermia (44°C, 60 min) in s.c. BT4An rat gliomas, and the influence on tumour growth of hyperthermia and the anti-angiogenic compound batimastat (30 mg/kg i.p.) was investigated. Heat-induced vascular damage was assessed in small (82 mm3) and large (171 mm3) tumours using confocal microscopy and immunostaining for von Willebrand factor. Hyperthermia disrupted 25–50% of the vasculature in the BT4An tumours, and the vascular damage was most extensive in the central part of the large tumours. The heated tumours exhibited a 40–60% blood flow reduction, which persisted until the last measurement after 24 h. One fraction of hyperthermia caused a significant growth delay of 4 days, compared with the control group, but no additional tumour response was produced by a second heating session. Batimastat had no influence on tumour growth and the combination of drug and local heating did not enhance the tumour response, compared with heating alone. It was concluded that hyperthermia at 44°C for 60 min exhibits anti-vascular activity and inhibits tumour growth in the BT4An tumour model, but Batimastat had no effect on tumour growth (Eikesdal et al. 2002). Further clinical trials based on the combination with hyperthermia plus other metalloproteinases inhibitors such us Marimastat are awaited (Ramnath and Creaven 2004).

Bevacizumab

Due to the central role of VEGF in tumour angiogenesis there is a strong preclinical biological rationale to utilize the combination of bevacizumab plus hyperthemia in clinical trials. Bevacizumab is recombinant humanized monoclonal antibody (RhuMAb) anti-VEGF, (Avastin^{TM}; Genentech, Inc.; South San Francisco, CA) composed of a human IgG_1 framework and antigen-binding regions (93%) and compliment-determining regions from the murine mAb A.4.6.1 (7%) (Fig.11.5) (Ranieri et al. 2006). Bevacizumab neutralizes all isoforms of human VEGF with a dissociation constant (k_d) of 1.1 and 0.8 nmol/L, respectively. In addition, it inhibits VEGF-induced proliferation of endothelial cells in vitro with an ED_{50} of 50 ± 5 and 48 ± 8 ng/ml, respectively. Bevacizumab is administered intravenously at doses ranging from 3 to 15 mg/kg every two/three weeks. Preclinical data suggest that bevacizumab is cleared from the circulation in a manner similar to that of endogenous antibody. The terminal half-life of Bevacizumab is long (1–2 weeks). To date, bevacizunab has not been used cinically in combination with hyperthermia. Because bevacizumab is an immunoglobulin, heat could hamper its proteic structure so in clinical trials hyperthermia should be administered first in sequence. In this way hyperthermia may play a role in targeting established vasculature and killing tumour cells while bevacizumab may inhibit neo-blood vessel formation and tumour regrowth. Bevacizumab is already approved in combination with chemotherapy for treatment of colorectal, lung and breast cancer and many clinical trials are ongoing in other tumour types.

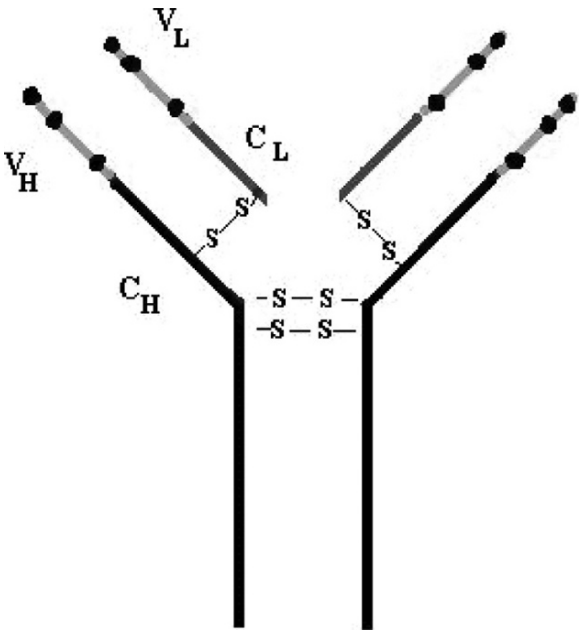

Fig. 11.5 Structure of bevacizumab. The humanised bevacizumab antibody consists of six murine specificity sequences (*black dots*) grafted onto a backbone of disulfide linked heavy and light chains containing variable (V_H and V_L) and constant (C_H and C_L) regions

Sorafenib (BAY 43-9006)

Sorafenib is a novel bi-aryl urea, initially developed as a specific inhibitor of Raf signalling pathway in tumour cells. The serine/threonine kinase Raf is a downstream effector enzyme of Ras and is involved in the transmission of proliferation signals from the cell surface to the nucleus. Subsequent studies have shown this compound to also inhibit several other tyrosine kinases involved in tumour progression, including VEGFRs (Ng and Chen 2006). Xenograft models of colon, breast, and non-small cell lung cancer (NSCLC) treated with sorafenib demonstrated significant inhibition of tumor angiogenesis, as measured by anti-CD31 immunostaining. Based on these results, the FDA announced in December 2005 the approval of sorafenib for patients with advanced renal cancer and subsequently for hepatocellular cancer. The recommended doses of Sorafenib is 400 mg orally twice daily. Because Sorafenib inhibit angiogenesis and tumour cell proliferation there is a strong biological rationale to associate it with hyperthermia in cancer patients. Clinical trials are pending.

Sunitinib (SU11248)

Sunitinib is a novel, oral, multitargeted receptor tyrosine kinase inhibitor, with both direct antiproliferative effects and antiangiogenic properties, targeting the VEGFRs, PDGFR-β, and c-Kit. In mouse xenograft models, sunitinib exhibited potent antitumor activity causing regression, growth arrest, or reduced growth of various established xenografts derived from human or rat tumor cell lines. In phase I clinical studies, the recommended dose of sunitinib was 50 mg orally, once daily for 4 weeks, followed by 2 weeks off, in repeated 6-week cycles. In addition to targeting VEGFRs, sunitinib targets c-Kit, often expressed in gastrointestinal stromal tumours, and it is thus a good candidate for the treatment of this disease. In January 2006, the FDA announced approval of sunitinib for patients with advanced renal cancer and gastrointestinal stromal tumors after disease progression on or intolerance to imatinib mesylate (Polyzos 2008). Currently, sunitinib is being evaluated in other tumour types. Due to antiangiogenic and antiproliferative properties of sunitinib there is a strong biological rationale to associate it with hyperthermia and clinical trials are pending.

Combination of Vascular Targeting and Hyperthermia

Biological Rationale of Vascular Targeting

The pivotal role of tumor vasculature and the effects of its selective destruction were highlighted as early as 1923, when Woglom (1923) suggested that damage to the capillary system might be the most effective way to inhibit tumor growth. More recently, it has been demonstrated in preclinical tumor models that clamping off the tumor-feeding blood supply to artificially induce ischemia results in extensive tumor cell death (Denekamp and Hobson 1983). On this basis Denekamp suggested the exploration of therapeutic strategies which would selectively compromise the

function of the tumor vasculature. The proposed principle of action for vascular-disrupting agents is based on the concept that a single neovessel provides nutritional support to large numbers of tumor cells. As a consequence, treatment with a VDA induces endothelial cell dysfunction, which leads to partial occlusion of the vessel (Denekamp 1990, Ranieri et al. 2005). The diminished blood flow that results causes tumor cells farthest from the vessel to become increasingly hypoxic. As damage to the endothelium progresses, coagulation events are initiated, and blockage of the vessel ultimately occurs. This loss of vessel potency results in widespread tumour cell necrosis. Due to the role of vasculature to dissipate heat away from tissues, combined treatment with VDA plus hyperthermia may lead to synergetic effects and improve tumour response (Horsman 2008). VDAs can be divided into two categories: biologics such as tumour necrosis factor (TNF) and small-molecule agents (Thorpe et al. 2003). Because small molecule agents have progressed from preclinical to clinical development here we focused our attention on small-molecule agents.

Combretastatin A-4 Disodium Phosphate

Combretastatin A-4, originally isolated from the South African *Combretum caffrum* tree, is a tubulin-binding agent that resembles colchicine in structure. It inhibits tubulin polymerization by binding the tubulin molecule (Griggs et al. 2001). The limited water solubility of combretastatin A-4 and complicated drug formation led to the synthesis of water-soluble prodrugs. The CA4P prodrug (Oxigene, Boston, MA) has subsequently undergone extensive preclinical evaluation (Malcontenti-Wilson et al. 2001). The soluble prodrug is cleaved to its natural form by endogenous phosphatases. In experimental tumors CA4P causes rapid, selective, and extensive vascular damage resulting in hemorrhagic necrosis within 1 h of treatment and subsequent tumor growth delay. Tumor blood flow reduction is rapid, can drop to $<5\%$ of the starting value 1 h after drug administration, and is accompanied by an increase in vascular permeability. In experimental animals, effects on tumors are greater than effects on normal tissues (Tozer et al. 1999). Noncytotoxic concentrations result in microtubule depolymerization and the disorganization of F-actin and ß-tubulin in endothelial cells, and changes in endothelial cell shape (Galbraith et al. 2001). Cytoskeletal alterations in endothelial cells have been attributed to Rho/Rho-kinase activation, which leads to phosphorylation of myosin light chain, actin-myosin contractility, assembly of stress fibers, and formation of focal adhesions. Endothelial contraction and retraction may cause an increase in vascular resistance and obstruction of tumor blood flow (Kanthou and Tozer 2002). CA4P-induced reductions in vascular volume are augmented by nitric oxide synthase inhibitors, suggesting that nitric oxide is involved in the mechanism of action of the drug (Davis et al. 2002). Several clinical trials have been reported with CA4P and other studies are ongoing (Scot). In the tumour models of BTT4An glioma, CA-4 (50 mg/kg intra peritoneally) was combined with hyperthermia (44°C, 60 min) at different time intervals (Eikesdal et al. 2000). It was found that CA-4 induced a

profound, but transient reduction in tumor perfusion 3–6 h postinjection. If hyperthermia was administered 3–6 h after injecting CA-4, massive hemorrhagic necrosis developed, and tumor response was significantly enhanced compared to simultaneous administration of the two treatment modalities. Similar effects was also demonstrated in the C3H mammary carcinoma tumour model (Murata et al. 2001, Eikesdal et al. 2001, b). Up to now no clinical trials are reported regarding the combination between CA4P and hyperthermia.

Combretastatin-A1-Disodium-Phosphate (OXi4503)

Hokland SL and co-worker studied a novel VDA, combretastatin-A1-disodium-phosphate (OXi4503), when combined with mild hyperthermia. A C3H mammary carcinoma was grown subcutaneously in the rear right foot of female CDF1 mice, and treated when a volume of 200 mm(3) was reached. OXi4503 was administered intra-peritoneally at varying doses. Hyperthermia was administered locally to the tumour-bearing foot using a thermostat-controlled water bath. The optimal delay between administration of 50 mg/kg of OXi4503 and hyperthermia was found to be 3 h. The linear relationship between tumour growth time (TGT) and heating time at a specific temperature resulted in slope values between −0.003 days/min and 0.09 days/min at temperatures between 40 and 42.5°C. When combined with OXi4503 TGT was significantly increased to 0.008 days/min and 0.03 days/min at temperatures between 39.5 and 41°C, respectively (Hokland and Horsman 2007). Phase 1 studies are pending.

Other Combretastatin Derivates: AVE8062 and TZT-1027

Other combretastatin derivatives are being synthesized and evaluated as potential antivascular agents. AVE8062 (formerly AC-7700) is a synthetic water-soluble combretastatin A4 derivative that causes shape changes in proliferating endothelial cells, resulting in rapid shutdown of tumor blood flow and extensive necrosis in experimental tumor models (Ohno et al. 2002). Dolastatin 10 is a natural product isolated from the marine mollusk *Dolabella auricularia* and a tubulin-binding agent that has antivascular activity. Like the combretastatins, dolastatins differ from *Vinca* alkaloids in their site of interaction with tubulin. A synthetic derivative of dolastatin 10, designated TZT-1027/Sonidotin, has potent antitumour activity and is being developed in Japan (Otani et al. 2000). No published clinical trials regarding the combination between these drugs and hyperthermia have be found.

ZD6126

ZD6126 is a phosphate prodrug of the tubulin-binding agent *N*-acetylcolchinol that inhibits microtubule polymerization. ZD6126 disrupts the tubulin cytoskeleton of endothelial cells leading to endothelial cell detachment at noncytotoxic concentrations. In vivo, a well-tolerated dose of ZD6126 was shown to cause tumor endothelial cell retraction, exposure of basal lamina in endothelia, and extensive

endothelial cell loss. Rapid reductions in tumor blood flow and vascular volume are seen. ZD6126 causes massive necrosis in experimental tumor models, has activity in a range of tumor xenograft models (Blakey et al. 2002). Phase 1 studies on the combination between ZD6126 and hyperthermia are awaited.

Small Molecule Cytokine Inducers: FAA and DMXAA

FAA is a synthetic flavonoid with impressive activity against experimental tumors but was toxic in cancer patients. The antivascular effects of FAA in experimental tumors are mediated via the release of TNF-α from activated mouse macrophages (Philpott et al. 1995). Lack of clinical activity of FAA led to the development of the FAA analogue DMXAA. DMXAA has shown activity in experimental tumors resulting in necrosis. Preclinical studies have shown a selective and significant dose-dependent reduction in tumor perfusion in mice. The production of TNF-α is important for the mechanism of action of DMXAA, but it can induce endothelial cell apoptosis in tumors, independent of TNF-α induction. The recent observation that DMXAA has activity in tumors growing in tumor necrosis factor receptor-1 knockout mice suggests that the antitumour effects of DMXAA can be mediated via other cytokines or vasoactive factors. Other studies have implicated the induction of IFN-inducible protein 10, serotonin, and nitric oxide in the antitumour effects of DMXAA (Baguley 2003). Interestingly DMXAA has also been shown to augment the antitumour effects of hyperthermia in a transplanted mice experimental model, but no clinical studies have been published.

Conclusion

The central role of tumour vascularization makes it an important target for therapy. AIAs represent a milestone in the tumour therapy due to the recent approvation by FDA of bevacizumab, sorafenib and sunitinib. In regards to combining with hyperthermia, pre-clinical models suggest that the best effect is generally seen if the AIA follows heat. With VDAs the main anti-tumour activity is seen when heat is administered at the time of maximal vascular shutdown by VDA, which is after VDA administration. Based on positive pre-clinical results, a strong rationale exists for the combination of AIAs and VDAs with hyperthermia in future clinical trials.

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Chapter 12
New Indications for Established Drugs Able to Modify Tumour-Host Interactions

Annika Bundscherer and Christian Hafner

Abstract Stroma-targeted and antiangiogenetic strategies emerge as a powerful alternative in the treatment of chemorefractory and metastatic cancer. Several well established drugs such as PPARγ agonists, COX 2 inhibitors, mTOR antagonists and thalidomide, which initially have been developed for non-oncological indications, have demonstrated broad antitumour activities. In addition to direct antineoplastic effects like induction of apoptosis and inhibition of cell proliferation these agents are able to exert antiangiogenetic and immuno-modulating activities by interruption of the tumour-stroma interaction. Compared with conventional high dose chemotherapy, stroma-targeted approaches are thought to cause less toxicity and to diminish the risk of development of drug-resistant tumour cell clones. Because combinatorial use of biomodulating agents might lead to super additive antineoplastic effects without causing severe toxicities, stroma-targeted therapy might represent a promising therapeutic approach in tumour palliation.

Keywords PPARγ agonists · COX 2 inhibitors · mTOR antagonists · Thalidomide · Stroma-targeted therapy

Introduction

Conventional cancer therapy consists of surgery, radiotherapy and cyclic high dose chemotherapy. For many years the fundamental principle in oncology has been to kill as many malignant cells as possible by maximum tolerated doses of chemotherapeutic drugs. These agents cause DNA damage and disrupt DNA replication in rapidly dividing cells. As these treatment strategies affect all tissues showing a high proliferation rate, they are accompanied by severe and dose limiting toxicities like myelosuppression and damage of the intestinal mucosa. To allow recovery of

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the normal tissue, cycles of therapy have to be interrupted by drug-free rest periods. Although the initial effects of chemotherapy often are quite impressive, this response mostly is short lived (Emmenegger et al. 2004). Due to the selection of chemoresistent tumour clones after repeated cycles of therapy, patients experience a relapse. To overcome this drug resistance, maximum tolerated doses are steadily increased and chemotherapeutic drugs are often used in combination. However, these strategies also lead to a potentiation of toxicities and diminish quality of life of the patients.

In search for alternatives to improve efficiency of cancer treatment, stroma targeting therapies have come into the picture. The concept emerges that, by modulation of the surrounding stroma, tumour cells are able to create a favourable environment for survival and cell growth and to foster invasion and metastasis. The major stromal components in this crosstalk are fibroblasts, endothelial and inflammatory cells. Tumour-stroma interaction is mediated by a variety of soluble agents such as cytokines, chemokines and growth factors (Hafner et al. 2005). The aim of stroma-targeted therapy is the disruption of the tumour-stroma interaction. This strategy seems to have some major advantages: As tumour associated stroma cells differ from healthy tissue in the expression of surface molecules, a specific targeting of these cells appears to be possible. Moreover, stroma cells are genetically more stable than cancer cells. Thus, the development of drug resistance could be delayed or even circumvented. The toxicity of stroma-targeting drugs is often lower than of conventional chemotherapy. Thus, quality of life could be improved for the patients during palliative therapy. For this reason stroma-targeted therapy is a promising approach in tumour palliation.

In recent years some well established drugs, which initially have been developed for non-oncological indications, have been shown to bring about direct anticancer effects as well as indirect stroma-targeted antineoplastic activities. This chapter focuses on the mechanisms underlying the antitumour activities of PPARγ agonists, COX 2 inhibitors, mTOR antagonists and thalidomide and summarizes previous results of clinical and preclinical studies using these biomodulators for cancer therapy.

PPARγ Agonists

The PPARγ Receptor

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors playing a key role in the regulation of several metabolic pathways including lipid homeostasis and glucose metabolism (Grommes et al. 2004). Three isoforms have been identified, PPAR α, PPAR β/δ and PPARγ, which are coded by different genes and vary in their tissue distribution. PPARγ is mainly expressed in adipocytes and cells of the immune system. The natural ligands of PPARγ are several lipophilic agents such as long-chain polysatturated fatty acids and eicosanoid derivates. After

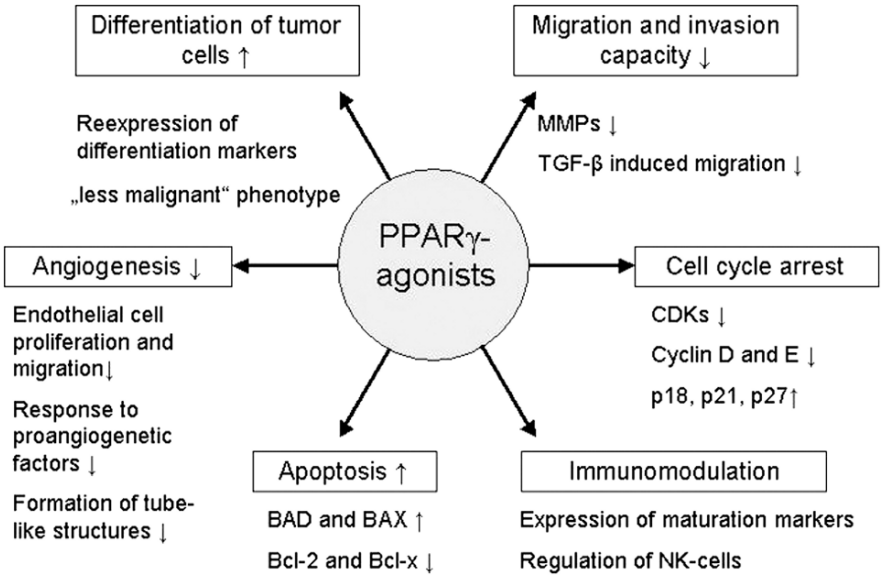

Fig. 12.1 Antitumoral and stroma-targeted effects of PPARγ agonists

ligand-activation, PPARs heterodimerize with the 9-cis retinoic acid receptor RXR and regulate the transcription of specific genes (Wang et al. 2006). As activation of PPARγ decreases concentration of serum glucose, PPARγ agonists like pioglitazone, rosiglitazone and ciglitazone are in clinical use in treatment of type 2 diabetes. Beside their insulin-sensitizing effects, these thiazolidinediones (TZD's) have been shown to exert direct and indirect antineoplastic effects (Fig. 12.1).

Direct Anticancer Activities of PPARγ Agonists

PPARγ protein can be detected in a variety of human malignancies. In some tumour entities such as glioblastoma, PPARγ expression level was even higher than in healthy tissue (Zang et al. 2003). In many tumour cell lines PPARγ activation cause inhibition of cell proliferation (Kawa et al. 2002. Rumi et al. 2002, Zan et al. 2003).

Treatment with troglitazone blocked cell cycle progression by inhibition of cyclin depending kinases (CDK) and upregulation of CDK inhibitors such as p18, p21 and p27 (Chen and Harrison 2005, Kawa et al. 2002, Rumi et al. 2002). In non small cell lung cancer (NSCLC) induction of cell cycle arrest was accompanied by down-regulation of cyclin D and E levels (Keshamouni et al. 2004). In addition, PPARγ agonists induce apoptosis via increased levels of proapoptotic proteins like BAD and BAX (Zander et al. 2002), which results in release of cytochrome c and activation of several effector caspases. Furthermore, reduction of antiapoptotic factors such as Bcl-2 and Bcl-xl could be detected (Zang et al. 2003). Interestingly, TZD's have

been shown to induce terminal differentiation in cancer cells in vitro as well as in patients with liposarcoma, which may be associated with a better clinical prognosis (Demetri et al. 1999, Frohlich et al. 2005). As antiproliferative and proapoptotic effects of TZD's could be observed in cancer cell lines lacking PPARγ expression, some of the antineoplastic effects appear to be mediated via PPARγ independent mechanisms (Abe et al. 2002, Shiau et al. 2005).

Stroma Mediated Anticancer Activities of PPARγ Agonists

PPARγ ligands are able to disturb several pathways in the tumour-stroma interaction. The tumour-mediated neoangiogenesis can be affected by targeting PPARγ. Activation of PPARγ inhibits the proliferative response of human umbilical vein endothelial cells (HUVEC) to vascular endothelial growth factor (VEGF) and other growth factors in vitro and in vivo. Furthermore, the differentiation into tube like structures can be suppressed (Xin et al. 1999). Low doses of rosiglitazone inhibited proliferation of bovine capillary endothelial cells but not of cancer cells (Panigrahy et al. 2002). In NSCLC bearing SCID mice, PPARγ ligands inhibited tumour associated angiogenesis by blocking the production of chemokines (Keshamouni et al. 2005). In addition, PPARγ activation affect leptin induced migration of endothelial cells (Goetze et al. 2002) and diminish VEGF secretion of tumour cells (Panigrahy et al. 2002).

One important mechanism in tumour cell invasion and metastasis is the proteolytic matrix degradation. Treatment with TZD's inhibits the activity of matrix metalloproteinases (MMP's) and reduce matrix invasiveness in vitro and in vivo (Ferruzzi et al. 2005, Galli et al. 2004, Grommes et al. 2006, Shen et al. 2007). In addition, troglitazone was shown to be a potent inhibitor of transforming growth factor β (TGF-β) mediated glioma cell migration and brain invasion (Coras et al. 2007).

PPARγ ligands also improve antitumour immunity via recruitment of natural-killer (NK) T cells and maturation of dendritic cells. Activation of PPARγ can further the expression of maturation markers like CD14 and CD36 and the PPARγ expression level was shown to increase with the degree of monocyte and macrophage development (Szanto and Nagy 2005). Furthermore, PPARγ stimulation was shown to subvert macrophage-mediated suppression of cytotoxic T lymphocytes in cancer (Van Ginderachter et al. 2006).

COX 2 Inhibitors

Cyclooxygenase and Its Physiological Functions

COX 1 and 2 are isoforms of the cyclooxygenase (COX), a key enzyme catalyzing the conversion from arachidonic acid to prostaglandins. COX 1 is constitutively expressed in many tissues and regulates housekeeping effects such as intestinal

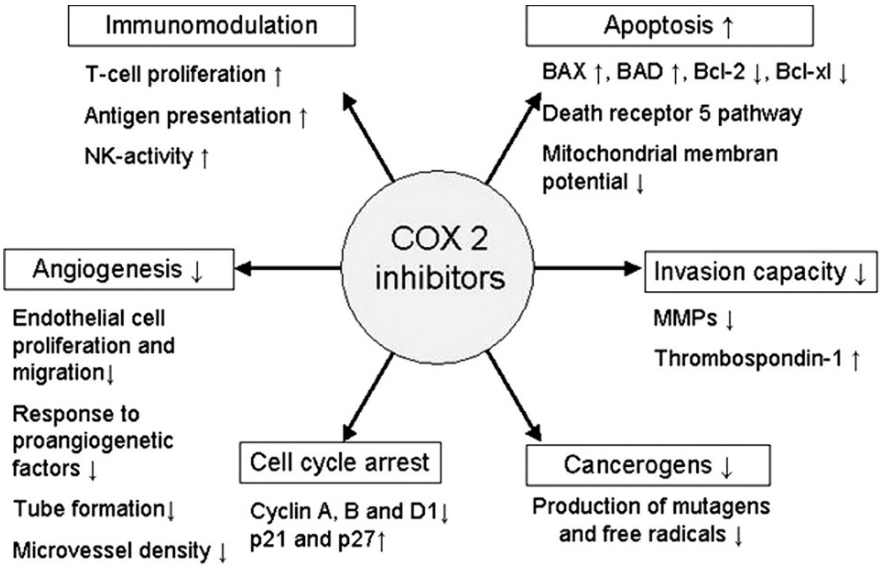

Fig. 12.2 Antitumoral and stroma-targeted effects of COX 2 inhibitors

cytoprotection and platelet aggregation. Under normal conditions, COX 2 expression is absent or very low in most tissues, but some exceptions are known including CNS and kidney. Being an immediate early response gene, COX 2 expression can be rapidly induced by hypoxia, growth factors and cytokines. COX 2 is involved in pathological processes like inflammation and pain sensation. In a variety of human malignancies including breast, colorectal and pancreatic cancer, COX 2 overexpression was detected (Sanborn and Blanke 2005). COX 2 foster cancer development via different mechanisms: COX 2 activity may be associated with the generation of free radicals and the conversion of pro-carcinogens to carcinogens. In addition, via immune modulating properties of prostaglandin E_2, COX 2 may facilitate the escape from host surveillance mechanisms. For this reason, the blockade of COX 2 by selective COX 2 inhibitors could be a promising approach in tumour therapy (Fig. 12.2).

Direct Anticancer Activities of COX 2 Inhibitors

In many tumours, there seems to be a positive correlation between COX 2 overexpression and resistance to apoptotic cell death. COX 2 inhibitors are able to overcome this resistance and induce apoptosis in cancer via different mechanisms such as increasing activity of caspase 3, 7 and 9 (Bundscherer et al., Dandekar et al. 2005). In addition, increased level of proapoptotic BAD and decreased Bcl-xl concentration as well as reduction of the Bcl-2/BAX ratio could be detected in cancer cells treated with COX 2 inhibitors (Chen et al. 2007, Dandekar et al. 2005).

Activation of the mitochondrial pathway of apoptosis by decrease in mitochondrial membrane potential (Yoshinaka et al. 2006) as well as activation of death receptor 5 (Liu et al. 2004) seems to be involved in COX 2 inhibition triggered programmed cell death. Another mechanism underlying the antiproliferative effects of COX 2 inhibitors is the induction of cell cycle arrest by upregulation of CDK inhibitors p21 and p27 (Han et al. 2004) as well as downregulation of cyclin A, cyclin B and cyclin D1 expression (Bock et al. 2007, Kardosh et al. 2004). Interestingly, some of the anticancer effects were judged to follow COX 2 independent pathways. Waskewich et al. observed no difference between antiproliferative effects of celecoxib in COX 2 positive and negative endothelial cell lines (Waskewich et al. 2002). Similar results were obtained in several other tumour entities by investigating either COX 2 deficient cell lines (Patel et al. 2005, Vogt et al. 2001, Waskewich et al. 2002), cell lines with a low baseline COX 2 expression or silencing COX 2 activity by antisense depletion (Han et al. 2004).

Stroma-Mediated Anticancer Activities of COX 2 Inhibitors

COX 2 promotes tumour cell invasion, metastasis and angiogenesis via different mechanisms: Prostacyclin (PGI_2) stimulates VEGF-induced vascular permeability and sprouting (Gately and Li 2004). Thromboxan A_2 (TXA_2) enhances endothelial cell motility and the formation of capillary like structures (Tsujii and DuBois 1995). Furthermore, integrin $\alpha_5\beta_3$-dependent cell migration, induction of endothelial cell invasion by matrix metalloproteinases and their resistance to apoptosis via Bcl-2 and AKT signalling are regulated by COX 2. Treatment with selective COX 2 inhibitors may diminish these effects. In preclinical studies inhibition of basic fibroblast growth factor (bFGF)-induced invasion and tube formation of bovine aortic endothelial cells as well as decreased levels of VEGF could be detected (Jung et al. 2007, Ragel et al. 2007). In an orthotopic xenograft model of human Wilms' tumour the specific COX 2 inhibitor SC-236 caused defective vascular assembly by attenuating incorporation of vascular mural cells into tumour vessels and impaired endothelial survival (Lee et al. 2006). Treatment with celecoxib for 14 days reduced microvessel density in patients with nasopharyngeal carcinoma (Soo et al. 2006). Healthy endothelial cells only express COX 1, while in cancer-associated endothelial cells often both isoforms can be found (Masferrer et al. 2000). This may open the door for a specific antiangiogenetic cancer therapy by COX 2 inhibition. In addition to antiangiogenetic effects, COX 2 inhibitors reduce tumour cell invasion via downregulation of matrix-metalloproteinases (Jung et al. 2007) and increased levels of thrombospondin-1 (Moon et al. 2005).

Blocking COX 2 function also exerts immunomodulatory properties in cancer. COX 2 expression in tumours suppresses T-cell proliferation and diminishes antigen presentation by dendritic cells (Evans and Kargman 2004). Furthermore, enhanced IL-10 production and activation of T-regulatory cells attenuate antitumour immune response (Harizi et al. 2002, Sharma et al. 2005) and allows the tumour to escape

host surveillance mechanism. Specific blockade of the COX 2 by coxibs can alter the balance of IL-10 and IL-12 (Stolina et al. 2000) and modulate NK activities leading to recognition and lysis of metastatic tumour cells (Kundu et al. 2005).

COX Inhibitors in Clinical Trials

In several epidemiologic studies unselective non-steroidal anti inflammatory drugs (NSAID's) as well as COX 2 inhibitors were shown to play a beneficial role in chemoprevention of cancer. Specifically in patients with familial adenomatous polyposis the daily use of 400 mg of celecoxib reduced the occurrence of colorectal adenomas within three years after polypectomy (Arber et al. 2006). Harris et al. observed a 68% reduction in the relative risk of lung cancer after daily application of NSAID's for at least 2 years (Harris et al. 2002). There are clinical trials ongoing using COX 2 inhibitors as single agents in the treatment of cancer. In a randomized phase II trial for treatment of cervical dysplasia, 75% of the celecoxib treated patients but only 31% of the placebo patients revealed a clinical response (Farley et al. 2006). In addition, results of clinical studies indicate that treatment with COX inhibitors improves energy homeostasis and body composition in patients with progressive cachexia. Decreasing systemic inflammation by blocking COX function attenuates the resting metabolism and improves appetite. Significant increase in weight and body mass index as well as improved quality of life was demonstrated (Lai et al. 2008, Lundholm et al. 2004).

Taking in consideration the mechanisms described above and the results of clinical studies using COX 2 inhibitors as single agents or in combination with other biomodulatoring or cytotoxic agents (Table 12.1), COX 2 inhibitors seem to be a promising tool in cancer therapy.

mTOR Antagonists

The mTOR Receptor and Its Antagonists

In 1975, the first mTOR antagonist rapamycin (sirolimus) was isolated from the soil bacteria streptomyces hygroscopius (Vezina et al. 1975). Due to its profound immunosuppressive activity, rapamycin is used to prevent allograft rejection in organ transplant patients. Although rapamycin revealed potent activity in malignancies during its preclinical evaluation (Douros and Suffness 1981, Eng et al. 1984), the poor aqueous solubility and chemical instability limited its clinical use. The development of rapamycin analogues with improved pharmacologic properties such as CCI-779, RAD-001 and AP-23573 opened the door for clinical trials (Hidalgo and Rowinsky 2000). Rapamycin and analogues bind to the immunophilin FK 506 binding protein (FKBP 12) and this newly formed complex interacts with the mammalian target of rapamycin (mTOR), resulting in cell cycle arrest and inhibition of proliferation in many cancer cells.

Table 12.1 Combinatorial treatment in clinical trials

Cancer	Drugs	Clinical trial	Result	Reference
Angiosarcoma	Pioglitazone, Rofecoxib Trofosfamide (metronomic)	pilot study (n=6)	2× CR 1× PR 3× SD	Vogt et al. (2003)
Breast cancer	Trastuzumab, Celecoxib	Phase II (n=12)	No effect	Dang et al. (2004)
Colorectal carcinoma	5-FU, Leucovorin, Rofecoxib	Phase II (n=10)	No effect	Becerra et al. (2003)
Glioblastoma multiforme	13 cis retinoic acid, Celecoxib	Phase II (n= 25)	44% SD	Levin et al. (2005)
Histiocytosis	Pioglitazone, Rofecoxib, Trofosfamide (metronomic)	Case report	Tumor regression	Reichle et al. (2005)
Kaposi-sarcoma	Pioglitazon, Rofecoxib Trofosfamid (metronomic)	Case report (n=1)	SD > 1 year	Coras et al. (2004)
Lung cancer	Celexocib, Paclitaxel, Carboplatin	Phase II (n=29)	17% CR 48% PR	Altorki et al. (2003)
Lung cancer	everolimus gefitinib	Phase I (n=10)	2× PR	Milton et al. (2007)
Lung cancer	Rofecoxib Gefitinib	Phase I/II (n=42)	1× CR, 2× PR 12× SD Similar to gefitinib alone	O'Byrne et al. (2007)
Lung cancer	Thalidomide Carboplatin Etoposide	Phase II (n=25)	4× CR 13× PR	Lee et al. (2008)
Melanoma	Pioglitazone, Rofecoxib Trofosfamid (metronomic)	Phase II (n=19)	19% OR 14% SD	Reichle et al. (2004)
Melanoma	Temozolomide Celecoxib	Phase II (n=50)	5× CR, 6× PR 20× SD 19× progession	Gogas et al. (2006)
Multiple myeloma	Thalidomide, Celecoxib	Phase II (n= 66)	42% response rate	Prince et al. (2005)
Non-Hodgkin's lymphoma	Cyclophosphamide (metronomic) Celecoxib	Phase II (n= 32)	2× CR 9× PR	Buckstein et al. (2006)
Pancreatic cancer	Gemcitabine, Celecoxib	PhaseII (n= 42)	4× PR 26× SD	Ferrari et al. (2006)
Renal cell carcinoma	Temsirolimus Interferon α	Phase I/II (n=71)	8% PR 36% SD >24 month	Motzer et al. (2007)
Soft tissue sarcoma	Pioglitazone, Rofecoxib Trofosfamid (metronomic)	Phase II (n= 21)	11% OR 11% SD	Reichle et al. (2004)
Squamous cell carcinoma	Gefitinib, Celecoxib	Phase I (n= 19)	22% PR	Wirth et al. (2005)

Combinatorial treatment of various cancers with biomodulators and chemotherapy, radiation or biologicals; CR = complete response, PR = partial resonse, SD = stable disease, OR = objective response.

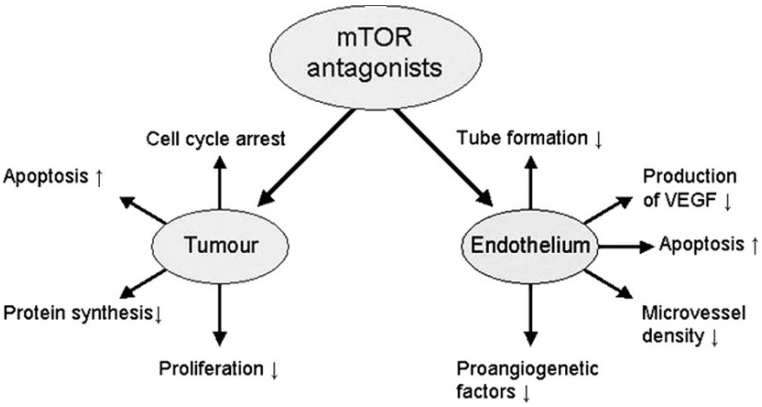

Fig. 12.3 Antitumoral and stroma-targeted effects of mTOR antagonists

The atypical serine/threonine kinase mTOR is a master switch between catabolic and anabolic metabolism and regulates multiple functions such as transcription and translation. Thus, mTOR signalling is involved in the control of cell proliferation, differentiation, migration and survival. The activity of mTOR is regulated by the concentration of amino-acids, ATP and glucose as well as by growth factors and their receptors. Growth factor signalling is transmitted by the IGFR-PI3K-AKT-mTOR pathway, a cascade of protein kinases stimulating mTOR function (Fig. 12.3). TSC1/2 and PTEN, are tumour suppressor genes and important negative regulators of mTOR. PTEN is often mutated in cancer. Therefore, mTOR function is enhanced and tumour cells are hypersensitized to mTOR antagonists (DeGraffenried et al. 2004). For this reason, targeting mTOR could be a promising strategy in cancer therapy.

Direct Anticancer Activities of mTOR Antagonists

By phosphorylation of the 40 S ribosomal protein p70 S6 Kinase (S6K1) and the eukaryotic translation initiation factor, 4E binding protein 1 (4E-BP1), mTOR regulates protein translation. Thus, inhibition of mTOR activity results in a 5–20% reduction of total protein synthesis. In many cancer cell lines, blocking of the mTOR signalling pathway leads to a dose dependent growth inhibition (Bundscherer et al., Calastretti et al. 2001, Haritunians et al. 2007, Mabuchi et al. 2007, Zitzmann et al. 2007). By altering the balance between cyclins, CDKs and CDK-inhibitors, these antiproliferative effects often are associated with arrest in G_1-phase of cell cycle. Upregulation of CDK-inhibitors p21 and p27 as well as reduced expression of cyclin A, cyclin D3, cyclin E and survivin are described (Decker et al. 2003, Vega et al. 2006). The question whether inhibition of mTOR also enhances apoptosis in tumour cells is still discussed controversially. While treatment with rapamycin caused apoptosis in some cancer cell lines, in other studies no effects or even

protection against apoptosis could be detected (Hosoi et al. 1999, Luan et al. 2003, Schumacher et al. 2005, Shi et al. 2005).

Stroma-Mediated Anticancer Activities of mTOR Antagonists

Treatment with rapamycin reduced the number of pulmonary metastasis and prolonged survival in SCID mice bearing human renal cancer (Luan et al. 2003) and diminished extrahepatic metastases and ascites in a rat model of hepatocellular cancer (Semela et al. 2007). However, the major stroma-mediated anticancer activity caused by mTOR antagonists is the inhibition of tumour angiogenesis. Hypoxia triggers the expression of proangiogenetic factors like VEGF and platelet-derived growth factor (PDGF) by accumulation of hypoxia inducible factors (HIFs). In some cancers it has been demonstrated that mutations in these pathways further the increased production of such growth factors. As mTOR is an upstream activator of HIF-1 function, mTOR antagonists can specifically counteract angiogenesis mediated by mutations in this pathway (Hudson et al. 2002). In addition, mTOR antagonists were shown to attenuate the expression of VEGF, another central regulator in vessel development (Luan et al. 2003, Mabuchi et al. 2007). VEGF-driven endothelial cell proliferation and morphogenesis could be decreased as well (Del Bufalo et al. 2006, Guba et al. 2002). In a rat model of hepatocellular carcinoma rapamycin prolonged survival by decreasing intratumoural microvessel density. Interestingly, lower drug concentrations were needed to inhibit endothelial cell proliferation compared with tumour cells. Furthermore, tube formation and vascular sprouting of aortic rings were attenuated by blocking mTOR (Semela et al. 2007).

mTOR Antagonists in Clinical Trials

The knowledge about antitumour effects of mTOR antagonists is not limited to the results of preclinical studies. Currently, several clinical trials using rapamycin or analogues are ongoing or already finished, showing encouraging antineoplastic effects and low toxicity profiles. Therapy with temsirolimus (CCI-779) in patients with recurrent glioblastoma multiforme was associated with significantly longer time to progression and radiographic improvement was observed in 36% of the patients (Galanis et al. 2005). In another study, 15 kidney transplanted patients who had developed a Kaposi sarcoma were treated with sirolimus. After 3 month of treatment time, all cutaneous Kaposi sarcomas had disappeared (Stallone et al. 2005). In a phase III trial temsirolimus improved overall survival among patients with metastatic renal cell carcinoma compared with interferon α monotherapy or even combination of both agents (Hudes et al. 2007). However, monotherapy with mTOR antagonists lacked to be efficient in some tumour entities such as metastatic melanoma (Margolin et al. 2005). As treatment with these agents cause low toxicity, mTOR antagonists may merit further investigation in combination with other biomodulators.

Thalidomide

Thalidomide and Its History

Thalidomide is a derivative of glutamic acid which exists as a racemic mixture of R(+) and S(−) enantiomers. It was introduced as a sedative and sleep inducing agent and was also used for treatment of morning sickness in pregnant women. Due to severe congenital limb defects, which were associated with the use of thalidomide during pregnancy, the agent had to be withdrawn form the market in 1961. However, thalidomide revealed to be an effective therapeutic agent in the treatment of various dermatologic and inflammatory conditions. In 1998, thalidomide was approved for sale in the United States and was recommended for the treatment of lepromatous leprosies by the World Health Organisation (Teo 2005). To reduce its teratogenic potential, all patients have to join to the System for Thalidomide Education and Prescribing Safety program. For this reason, today thalidomide is the most restrictively prescribed agent ever approved (Teo 2005). In addition to its anti-inflammatory properties, thalidomide was assessed for its benefit in various disease states including cancer. With the intent of minimizing the teratogenic risk while enhancing the immunomodulatory effects, synthetic thalidomide analogues such as CC-4047 (actimide) and CC-5013 (lenalidomide), referred to as immunomodulatory drugs (IMiD's) have been developed (Fig. 12.4).

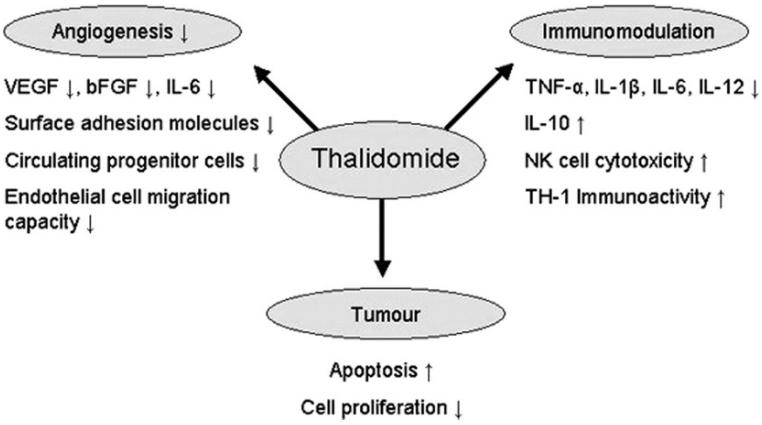

Fig. 12.4 Antitumoral and stroma-targeted effects of thalidomide

Antineoplastic Activities of Thalidomide

Beside direct anticancer effects such as induction of apoptosis and inhibition of cell proliferation, thalidomide possesses immunomodulatory and antiangiogenetic properties (Teo 2005). In human umbilical vein endothelial cells thalidomide decreased

the density of cell surface adhesion molecules involved in the adhesion cascade such as E-selectin, VCAM-1 and ICAM-1 (Geitz et al. 1996). Due to increased degradation of TNF-α mRNA, thalidomide and its analogues are potent inhibitors of TNFα production. Also levels of other cytokines like IL-1β, IL-6, IL-12 and granulocyte macrophage-colony stimulation factor (GM-CSF) are decreased while IL-10 synthesis is stimulated. Furthermore, an augmentation of NK cell cytotoxicity and enhancement of TH-1 type immune activity by thalidomide are described (Teo 2005).

Thalidomide and its analogues have been shown to inhibit tumour associated angiogenesis by affecting different pathways. Via downregulation of COX 2, thalidomide decreases the production of proangiogenetic eicosanoides. In addition, IMiD's reduce the levels of VEGF, IL-6 and IL-8 and thus shift the balance between pro- and antiangiogenetic factors. Suppression of bFGF has also been reported after treatment with thalidomide. Another mechanism underlying the antiangiogenetic effects might be the inhibition of endothelial cell migration capacity and the reduction of circulating endothelial progenitor cells.

Thalidomide in Clinical Trials

Thalidomide and its analogues have already entered clinical trials and showed efficiency in glioma, metastatic melanoma, pancreatic cancer and multiple myeloma. In women with advanced ovarian cancer the effects of a therapy with oral thalidomide appeared to be comparable with those of a single agent intravenous chemotherapy (Gordinier et al. 2007). In another clinical trial, two out of three patients with Kaposi sarcoma showed a complete response after 12 month of treatment with thalidomide (Rubegni et al. 2007). Furthermore, the combination of IMiD's with cytotoxic agents might augment the anticancer activity while reducing toxicity. For this reason, the application of IMiD's in cancer therapy warrants further investigation (Table 12.1).

Combinatorial Treatment with Biomodulators

The principle of stroma-targeted approaches is to reduce the risk of acquired drug resistance by affecting stromal cells instead of the genetically instable tumour cells. However, escape mechanisms might exist against this kind of therapy when single agents are used. As angiogenesis is a complex interplay between pro- and antiangiogenetic factors, the blockade of one single growth factor or its receptor could easily be compensated by increased production of redundant proangiogenetic growth factors. Furthermore, targeting tumour vasculature may foster the selection of tumour cell clones which tolerate nutrient starvation and hypoxia (Kerbel et al. 2001). Also epigenetic changes in stromal cells could modify the metabolism of administered drugs. Thus, in order of achieving long term disease control, combinatorial treatment with several biomodulating or cytotoxic agents is suggested.

Results of several preclinical as well as clinical studies outline the benefit of combining the agents discussed in this chapter with conventional anticancer strategies such as chemotherapy and radiation. In this context, metronomic chemotherapy represents another attractive combination partner. This daily low dose administration of cytotoxic drugs turned out to exhibit antiangiogenetic effects without causing severe toxicities and may thereby complement novel strategies using biomodulators for tumour stroma targeting. COX 2 inhibitors, PPARγ agonists and mTOR antagonists potentiated the antitumour effects of conventional chemotherapeutic drugs by enhancing apoptosis (Mabuchi et al. 2007, Zhang et al. 2007), inhibiting cell proliferation (Ponthan et al. 2007) or downregulation of VEGF production as well as VEGF-receptor 2 expression (Marimpietri et al. 2007). In addition, celecoxib significantly enhanced glioblastoma radiosensitivity and prolonged survival in glioblastoma-implanted mice. However, in other preclinical studies inhibition of COX 2 function did not lead to radiosensitation of cancer cells (Ohneseit et al. 2007) and even attenuated 5-fluorouracil-induced apoptosis (Lim et al. 2007). In multiple human melanoma cell lines, the B-Raf inhibitor BAY43-9006 and the mTOR inhibitor rapamycin synergistically inhibited serum-stimulated cell proliferation.

Combination of PPARγ agonists and COX 2 inhibitors particularly seems to be a promising strategy. There is evidence for a reciprocal crosstalk between PPARγ and COX 2 expression in several tumour entities (Konstantinopoulos et al. 2006, Stadlmann et al. 2006) as well as a downregulation of PPARγ by COX 2 activation (Han et al. 2003). Accordingly, combined targeting of PPARγ and COX 2 was shown to induce apoptosis and inhibit growth of human breast cancer cells synergistically (Michael et al. 2003). In addition, in a malignant melanoma cell line combination of celecoxib and rapamycin exerted additive growth inhibitory effects (Bundscherer et al.).

The evidence of promising results of combinatorial treatment is not limited to preclinical studies. In the meantime a variety of clinical trials combining biomodulators with conventional anticancer strategies are ongoing or already finished (Table 1).

The combination of thalidomide with carboplatin and etoposide as maintenance therapy for patients with small cell lung cancer appeared to be well tolerated. With an overall response rate of 68% (20% CR, 48% PR) this regimen showed encouraging results and led to the initiation of a randomized phase III trial (Lee et al. 2008). In a phase I/II trial combining temsirolimus and interferon α for the treatment of advanced renal cell carcinoma, 8% of the patients achieved partial response and 36% had stable disease for at least 24 month (Motzer et al. 2007). The application of metronomic chemotherapy combined with COX 2 inhibitors and PPARγ agonists turned out to be a promising approach in tumour palliation of angiosarcoma (Vogt et al. 2003), malignant melanoma and soft tissue sarcoma (Bader et al. 2006) as well as Kaposi sarcoma (Coras et al. 2004) and Langerhans' cell histiocytosis (Reichle et al. 2005).

Taken together, combined stroma-targeted therapy with biomodulators and conventional anticancer strategies appears to be a promising strategy in tumour palliation. Due to the possibility of an outpatient setting and low toxicities, this

kind of therapy is well accepted by most of the patients and can improve their quality of life compared with conventional high-dose chemotherapies. For this reason, stroma-targeted therapy warrants further clinical evaluation to achieve optimal therapy schedules for different tumour entities.

Abbreviations

bFGF	Basic Fibroblast Growth Factor
CDK	Cyclin dependent kinase
CNS	Central Nervous System
COX	Cyclooxygenase
HIF	Hypoxia inducibe factors
HUVEC	human umbilical vein endothelial cells
MMP	matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NK-cells	Natural killer cells
NSCLC	Non small cell lung cancer
PPAR	Peroxisome proliferator-activated receptor
TDZ	thiazolidinediones
VEGF	vascular endothelial growth factor

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