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Tumor Microenvironment Abnormalities: Causes, Consequences, and Strategies to Normalize

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Abstract A solid tumor is an organ-like entity comprised of neoplastic cells and non-transformed host stromal cells embedded in an extracellular matrix. The expression of various genes is influenced by interactions among these cells, surrounding matrix, and their local physical and biochemical microenvironment. The products encoded by these genes, in turn, control the pathophysiological characteristics of the tumor, and give rise to the abnormal organization, structure, and function of tumor blood vessels. These abnormalities contribute to heterogeneous blood flow, vascular permeability, and microenvironment. Proliferating tumor cells produce solid stress which compresses blood and lymphatic vessels. As a result of vessel leakiness and lack of functional lymphatics, interstitial fluid pressure is significantly elevated in solid tumors. Each of these abnormalities forms a physiological barrier to the delivery of therapeutic agents to tumors. Furthermore, the metabolic microenvironment in tumors such as hypoxia and acidosis hinder the efficacy of anti-tumor treatments such as radiation therapy and chemotherapy. A judicious application of anti-angiogenic therapy has the potential to overcome these problems by normalizing the tumor vessels and making them more efficient for delivery of oxygen and drugs. Combined anti-angiogenic and conventional therapies have shown promise in the clinic. J. Cell. Biochem. 101: 937–949, 2007.

Key words: microenvironment; hypoxia; acidosis; interstitial fluid pressure; angiogenesis; microcircualtion; lymphangiogenesis; lymphatic metastasis; stromal cells; vascular endothelial growth factor; intravital microscopy

Tumors consist not only of cancer cells, but also of host stromal cells—non-malignant cells including endothelial cells, peri-vascular cells, fibroblasts, myofibroblasts, macrophages, lymphocytes, dendritic cells, and mast cells. These cells, embedded within a protein-rich extracellular matrix and interstitial fluid, face a hostile metabolic microenvironment characterized by hypoxia and acidosis. The tumor pathophysiology governs tumor growth, invasion, and metastasis as well as response to various therapies. In this review, we will discuss various pathophysiological parameters that characterize the vascular and extra-vascular compartments as well as metabolic environment in a tumor, the mechanisms governing the formation and function of these compartments, and normalization of these parameters using a judicious application of anti-angiogenic agents.

VASCULAR COMPARTMENT

Cells require oxygen and other nutrients for their survival and growth. Exchange of gas, nutrients, and metabolites over the capillary wall satisfies these requirements and maintains normal tissue homeostasis. Likewise, cells undergoing neoplastic transformation depend on nearby capillaries for growth [Goldman, 1907]. Once the size of the cellular aggregate reaches the diffusion limit for critical nutrients and oxygen, however, the aggregate as a whole can become dormant. Indeed, some human tumors can remain dormant for a number of years at a

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stage where tumor cell proliferation and death balance [Folkman, 2000]. But once new blood vessel formation is initiated, which can be as early as during the hyperplastic or dysplastic phase [Hagendoorn et al., 2006], tumorigenesis, tumor progression, and metastasis may follow [Gullino, 1978]. What triggers the growth of new vessels? How do these vessels compare with normal vessels with respect to structure and function? What are the consequences of their abnormal function on the tumor microenvironment? These questions will be addressed in this review.

Angiogenesis

New vessel formation (the process called angiogenesis) in both normal and disease tissues is governed by the net balance between pro- and anti-angiogenic factors [Carmeliet and Jain, 2000]. Under normal physiological conditions, this balance is strictly regulated so that angiogenesis is "on" when needed (e.g., embryonic development, wound healing, formation of the corpus luteum) and "off" otherwise. This balance becomes disturbed during neoplastic transformation and tumor progression.

Multiple pro- and anti-angiogenic molecules regulate the different steps in vessel formation as well as vascular function. Vascular endothelial growth factor (VEGF) is a strong mitogen and survival factor of vascular endothelial cells, one of the most potent angiogenic factors and the target of the first FDA approved anti-angiogenic agent, Avastin [Ferrara et al., 2003]. VEGF also contributes to the angiogenic phenotype by increasing the permeability of existing vessels, which permits extravasation of fibrin, plasmin, and clotting factors, resulting in a fibrin-rich stroma that supports the migration of endothelial and peri-endothelial cells and the formation of new vasculature [Dvorak, 2002]. Furthermore, VEGF induces the expression of adhesion molecules on vascular endothelial cells [Melder et al., 1996] and the mobilization and recruitment of bone marrow-derived cells [Rafii et al., 2002]. These circulating cells recruited to the tumor can directly and indirectly contribute to the angiogenic process. Furthermore, the number of proangiogenic factors produced by a tumor can increase as it progresses [Yoshiji et al., 1997; Fidler, 2001]. Thus, even if VEGF signaling is blocked, a tumor may rely on alternative angiogenic molecules (e.g., basic fibroblast growth factor [bFGF], interleukin-8 [IL-8]) to promote angiogenesis.

Vascular Architecture

The normal blood vessels form a well-organized architecture consisting of arterioles, capillaries, and venules (Fig. 1). Arterioles are circumferentially covered by contractile cells

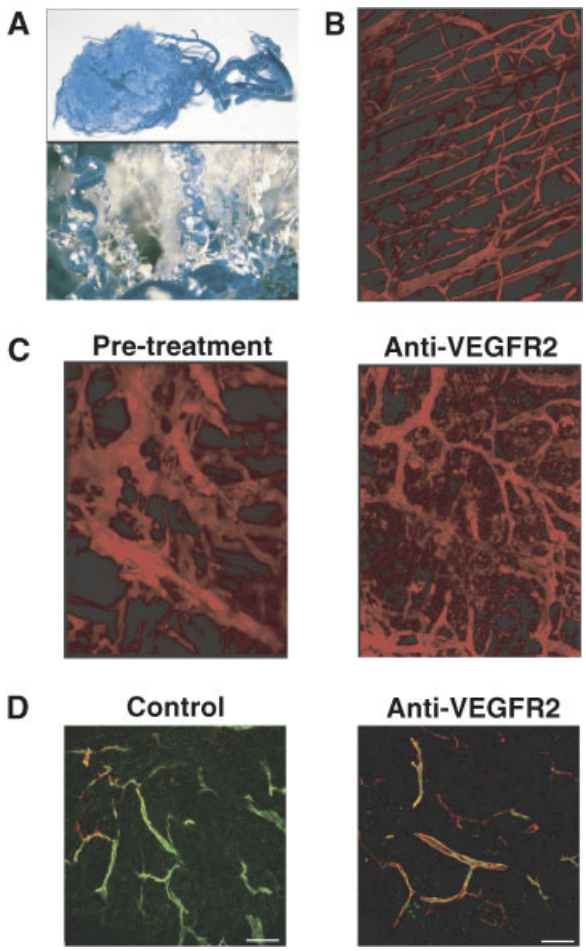

Fig. 1. Abnormal blood vessels in tumors. **A**: Vascular cast of a human colon cancer. **B**: Multiphoton laser-scanning microscopy image of normal blood vessels in a mouse dorsal skin chamber. Normal vessels are well organized with even diameters. **C**: Microangiography of tumor vessels (MCaIV tumor in the dorsal skin chamber). In contrast, tumor vessels are tortuous with increased vessel diameter and irregular shape. Anti-VEGFR2 treatment (DC101) reduces vessel diameter and prunes immature vessels. **D**: Immunohistochemistry of perfused blood vessels (green) and NG2-positive perivascular cells (red) in U87 tumor grown in the mouse cranium. Perivascular cell coverage is increased after 2–5 days of anti-VEGFR2 treatment. (A) reproduced from Jain [1994], B, C, courtesy of Dr. Ricky Tong, D, reproduced from Winkler et al. [2004].

and control blood flow by regulating vessel tone. From arterioles, blood flows into capillaries, which represent the major portion of the microvasculature and have the smallest diameter and thinnest vessel wall. Venules, the downstream portion of the microcirculation, have intermediate wall thickness and noncircumferential perivascular cell coverage. In contrast, tumor vessels are dilated, saccular, tortuous, and disorganized in their patterns of interconnection (Fig. 1) [Jain, 1988]. Normal vasculature is characterized by dichotomous branching, but tumor vasculature is unorganized and can present trifurcations and branches with uneven diameters. Perivascular cells in tumor vessels have abnormal morphology and heterogeneous association with vessels [McDonald and Choyke, 2003].

The molecular mechanisms causing this abnormal vascular architecture are not well understood, but the uncontrolled VEGF signaling may be a key contributor. "Normalization" of the tumor vasculature has been observed by interfering with VEGF signaling: that is, treatments directly targeting VEGF or VEGF receptor, therapies that reduce VEGF (e.g., hormone withdrawal from a hormone-dependent tumor), or agents mimicking an anti-angiogenic cocktail (e.g., Herceptin treatment of a HER2 overexpressing tumor) [Jain et al., 1998; Jain and Carmeliet, 2001; Kadambi et al., 2001; Izumi et al., 2002]. Solid (mechanical) stress generated by proliferating tumor cells also compresses vessels in tumors [Padera et al., 2004]. Thus, the combination of both molecular and mechanical factors renders the tumor vasculature abnormal, and both types of factors must be taken into account when designing novel strategies for cancer treatment.

Blood Flow and Microcirculation

Whether normal or abnormal, arterio-venous pressure difference and flow resistance govern blood flow in a vascular network. Flow resistance is a function of the vascular architecture (referred to as geometric resistance) and of the blood viscosity (rheology, referred to as viscous resistance) [Jain, 1988]. Abnormalities in both vasculature and viscosity increase the resistance to blood flow in tumors. As a result, overall perfusion rates (blood flow rate per unit volume) in tumors are lower than in many normal tissues and the average RBC velocity can be an order of magnitude lower than normal [Yuan et al., 1994]. Furthermore, tumor blood flow is unevenly distributed, fluctuates with time and can even reverse its direction in some vessels—therefore, regions with poor perfusion, or none at all, are commonly seen. The heterogeneity of tumor blood flow, caused by the factors above and some that will be discussed in the following, is an important contributor to both acute and chronic hypoxia in tumors—which in turn is a major cause of resistance to radiation and other therapies.

Considerable effort has gone into increasing tumor blood flow to improve radiation therapy, or decreasing tumor perfusion in the case of vascular-disruption therapies, including hyperthermia and PDT. This has been difficult to achieve reproducibly because tumor vessel network consists of heterogeneous vessels, and thus response to vasoactive agents is not uniform. As a result, efforts to increase the tumor blood flow by pharmacological or physical agents have not always been reproducible or successful [Jain, 1988]. On the other hand, it appears that judiciously applied anti-angiogenic therapy can "normalize" the abnormal tumor microcirculation by pruning the immature vessels, thus rendering the remaining vasculature more efficient for the delivery of drugs (Fig. 1) [Jain, 2001].

Vascular Permeability

Extravasation of molecules from the bloodstream occurs by diffusion, convection, and to some extent, by transcytosis in an exchange vessel. Diffusion is considered to be the major form of transvascular transport in tumors [Jain, 1987]. The diffusive permeability of a molecule depends on its size, shape, charge, and flexibility as well as the transvascular transport pathway. Widened inter-endothelial junctions, increased numbers of fenestrations, vesicles and vesico-vacuolar channels, and a lack of normal basement membrane and perivascular cells were found in tumor vessels [Dvorak et al., 2002]. In agreement with these ultrastructural alterations, vascular permeability in solid tumors is generally higher than that in various normal tissues [Yuan et al., 1994].

Despite increased overall permeability, not all blood vessels of a tumor are leaky. Not only does the vascular permeability vary from one tumor to the next, but it also varies spatially and temporally within the same tumor as well as during tumor growth, regression, and relapse

[Hobbs et al., 1998]. The local microenvironment plays an important role in controlling vascular permeability. For example, a human glioma (HGL21) has fairly leaky vessels when grown subcutaneously in immunodeficient mice, but it exhibits blood-brain barrier properties in the cranial window [Yuan et al., 1994]. The host–tumor interactions may control the production and secretion of cytokines associated with permeability increase (e.g., VEGF) and decrease (e.g., angiopoietin 1) [Fukumura et al., 1997]. Furthermore, the response of the blood vessels to a given stimuli may also vary depending on the host organ site and host–tumor interaction [Monsky et al., 1999]. A better understanding of the molecular mechanisms of permeability regulation in tumors is likely to yield strategies for improved delivery of molecular medicine to tumors as discussed in the following [Weis and Cheresh, 2005].

EXTRAVASCULAR COMPARTMENT

Lymphangiogenesis and Lymphatic Transport

By transporting both immune cells and interstitial fluid out of tissue, the normal lymphatic network plays an important role in immune function and in the maintenance of tissue interstitial fluid balance. Tumor cells grow in a confined space and thus create mechanical stress (solid stress), which compresses the intra-tumor blood and lymph vessels [Padera et al., 2004]. Consequently, there are no functional lymphatic vessels inside solid tumors [Leu et al., 2000; Padera et al., 2002]. Instead, functional lymphatic vessels are present in the tumor margin and the peri-tumoral tissue. Tumor cells can invade these peripheral lymphatic vessels and form metastases within the lymphatic system. Furthermore, the abnormal lymphatic valves allow retrograde flow in these lymphatic vessels and may facilitate lymphatic metastasis [Isaka et al., 2004].

The molecules involved in angiogenesis are also involved in lymphangiogenesis. For example, VEGF-C and -D can induce both angiogenesis and lymphangiogenesis and are associated with lymphatic metastasis in a variety of tumors [Alitalo et al., 2005]. Their receptor VEGFR3 is present in both lymphatic and vascular endothelium in tumors. As is the case with vascular angiogenesis, other positive and negative regulators (e.g., angiopoietins and PDGF) are also involved in lymphangiogenesis. The mechanical and/or molecular signals that could trigger the lymphangiogenic switch are unknown. Because lymphatic vessels help maintain the balance of fluid in tissues, hydrostatic pressure is a likely trigger [Boardman and Swartz, 2003]. Whether the hyperplasia and the increased density of lymphatic vessels seen in the tumor margins are a response to elevated hydrostatic pressure in tumors is an open question. Microlymphangiography, intravital microscopy techniques, and molecular targeting reagents revealed that these peri-tumor lymphatic vessels, which are induced by VEGF-C/D and yet-to-be-discovered lymphangiogenic factors, are able to carry cancer cells and mediate tumor metastasis. Furthermore, blockade of VEGFR3—by inhibiting peri-tumor lymphatic hyperplasia—can inhibit early steps of lymphatic metastasis as well as the delivery of cancer cells to the lymph node [Hoshida et al., 2006].

Interstitial Hypertension

Unlike normal tissues, in which the interstitial fluid pressure (IFP) is around 0 mmHg, both animal and human tumors exhibit interstitial hypertension (Fig. 2) [Jain, 2004]. Two major mechanisms contribute to interstitial hypertension in tumors. In normal tissues, the lymphatics maintain fluid homeostasis; thus, the lack of functional lymphatics in tumors is a key contributor. Indeed, DiResta et al. [2000] were able to lower the IFP by placing "artificial lymphatics" in tumors. The second contributor is the high permeability of tumor vessels. The tumor IFP begins to increase as soon as the host vessels become leaky in response to angiogenic molecules such as VEGF. Furthermore, tumor IFP goes up and down with the microvascular pressure within seconds [Netti et al., 1995]. As a result, the hydrostatic and oncotic (colloid osmotic) pressures become almost equal between the intravascular and extravascular spaces [Boucher and Jain, 1992]. Reduced or lack of transmural pressure gradients decrease convection across tumor vessel walls and thus compromise the delivery of therapeutic agents. Furthermore, IFP is nearly uniform throughout a tumor and drops precipitously in the tumor margin [Boucher et al., 1990]. Thus, the interstitial fluid oozes out of the tumor into the surrounding normal tissue, carrying away the drug with it. Finally, transmural coupling between IFP and microvascular pressure due

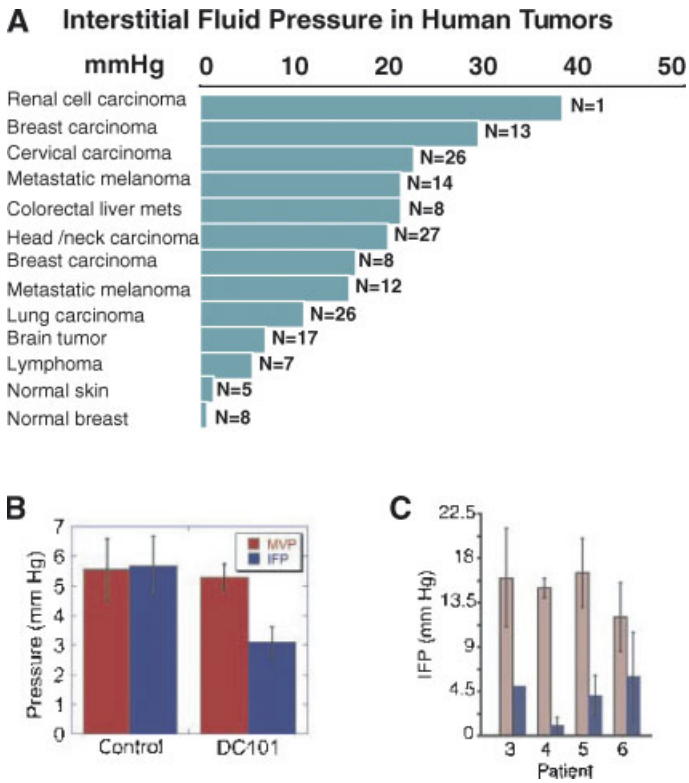

Fig. 2. Elevated interstitial fluid pressure in tumors. **A**: Interstitial fluid pressure (IFP) in various human tumors. IFP is elevated in all solid tumors examined. **B**: Microvascular pressure (MVP) and IFP in MCaIV tumors grown in the dorsal skin chambers. MVP and IFP are almost equal. Anti-VEGFR2 treatment (DC101) significantly lowers tumor IFP while not changing MVP. As a result, a pressure gradient between blood vessels and intesititum is re-established. **C**: IFP in human colorectal cancers during anti-VEGF (bevacizumab) treatment. Bevacizumab significantly lowers tumor IFP in patients. (A) adapted from Jain [2004], (B) reproduced from Tong et al. [2004], (C) reproduced from Willett et al. [2004].

to the high permeability of tumor vessels can abolish pressure difference between up- and down-stream tumor blood vessels and lead to blood flow stasis in tumors without physically occluding the vessels.

Since IFP is a reflection of the global pathophysiology of tumors, it may be used for diagnosis and/or prognosis (Fig. 2). The steep rise of IFP at the tumor periphery can be used to locate tumors during needle biopsy and improve diagnosis of patients [Jain et al., 1995]. Furthermore, a study of cervical cancer has shown that elevated tumor IFP can predict a poor outcome of radiation therapy [Jain, 2004]. Further studies are needed to evaluate the prognostic significance of IFP in human tumors. Decreasing vascular permeability might restore the transmural pressure gradients and potentially resume/re-establish blood flow in the non-perfused regions of tumors. Some direct and indirect anti-angiogenic therapies might "normalize" the tumor vasculature through this mechanism [Jain, 2005]. In fact, IFP can be lowered by antibodies against VEGF or VEGFR2 (Fig. 2) [Tong et al., 2004; Willett et al., 2004].

METABOLIC ENVIRONMENT

Hypoxia and acidosis are hallmarks of the metabolic environment in solid tumors (Fig. 3) [Vaupel et al., 1989; Harris, 2002]. Both oxygen tension (pO_2) and pH are important determinants of tumor growth, metabolism, and response to a variety of therapies such as radiation therapy, chemotherapy, hyperthermia, and photodynamic therapy.

Hypoxia

A key function of the vasculature is to provide adequate levels of nutrients and oxygen to the parenchymal cells and to remove waste products. Based on the anatomy of the capillary bed and a mathematical model of oxygen diffusion and consumption, the Nobel laureate August Krogh introduced the concept of a diffusion limit

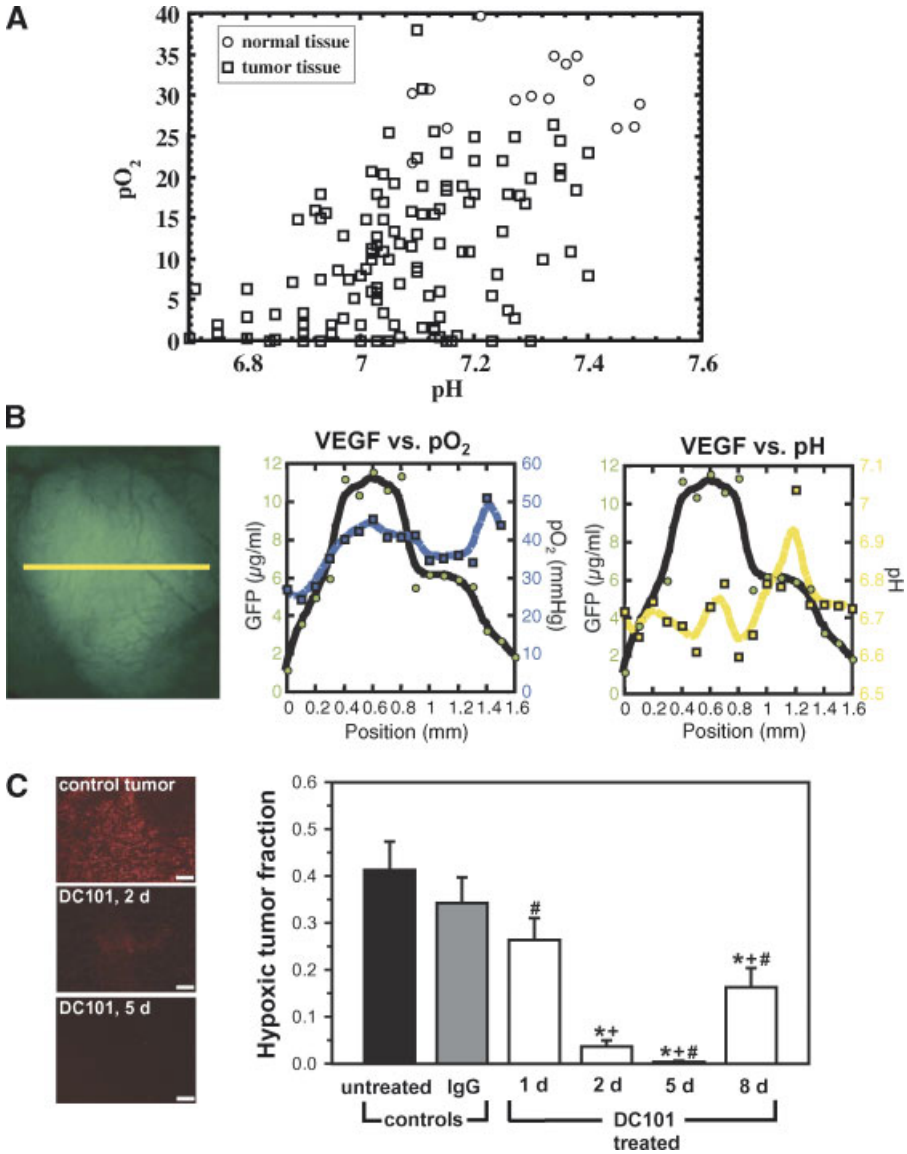

Fig. 3. Hypoxia and acidosis in tumors. **A**: Tissue pO_2 (phosphorescence quenching microscopy) and pH (fluorescence ratio imaging microscopy) in LS174T tumors and normal tissues in the dorsal skin chamber. Tumors are hypoxic and acidic. There is no clear relationship between tissue pO_2 and pH in tumors. **B**: VEGF promoter activity (green), tissue pO_2 (blue), and pH (yellow) in U87 tumors. Left, intravital microscopy image of GFP driven by VEGF promoter. The three parameters are determined along the yellow line. This tumor is well oxygenated and there is no correlation between tissue pO_2 and VEGF promoter activity. On the other hand, the peak of VEGF promoter activity is observed in acidic pH region. **C**: Alteration of tissue oxygenation during anti-VEGFR2 treatment. Tissue hypoxia is determined by immunostaining of a redox marker pimonidazole (left). Hypoxic area is decreased after 2–5 days of anti-VEGFR2 treatment (DC101). (A) adapted from Helmlinger et al. [1997]; (B) adapted from Fukumura et al. [2001]; (C) reproduced from Willett et al. [2004].

for oxygen of 100–200 μm nearly a century ago [Krogh, 1922]. This unit of tissue—a single capillary surrounded by a 100–200 μm radius cylinder—is referred to as a "Krogh cylinder" in physiology. Fifty years ago, Thomlinson and Gray [1955] identified similar "cords" in human lung cancer and found necrotic cells beyond 180 μm away from blood vessels, presumably due to lack of oxygen. This is referred to as "chronic hypoxia" or "diffusion-limited" hypoxia. Although various hypoxia markers and microelectrodes have suggested these gradients, the first direct measurements of these perivascular gradients—along with pO_2 and blood flow rate of the same vessels—became possible only with the development of

phosphorescence quenching microscopy (Fig. 3) [Torres-Filho et al., 1994; Helmlinger et al., 1997].

As discussed previously, blood flow in tumor vessels is intermittent, and thus, some regions of a tumor are starved for oxygen periodically. The resulting hypoxia is referred to as "acute hypoxia" or "perfusion-limited hypoxia" [Brown and Giaccia, 1998; Dewhirst, 1998]. A necessary consequence of intermittent blood flow is the resumption of blood flow after shutdown, and the resulting production of free radicals can lead to "reperfusion injury" or "reoxygenation injury," applying additional selection pressure on cancer cells.

Low pH

Another consequence of the abnormal microcirculation of the tumor is low extracellular pH. There are at least two sources of H^+ ions in tumors—lactic acid and carbonic acid [Helmlinger et al., 2002; Pouyssegur et al., 2006]. The former results from anaerobic glycolysis and the latter from conversion of CO_2 and H_2O via carbonic anhydrase. The intracellular pH of cancer cells remains neutral or alkaline (pH 7.4), however, in spite of the acidic extracellular pH. One would expect low extracellular pH and hypoxia to track each other and to co-localize with regions of low blood flow. Surprisingly, there is a lack of spatial correlation among these parameters (Fig. 3), a discovery made possible by recent developments in optical techniques that permit the simultaneous high-resolution mapping of multiple physiological parameters [Helmlinger et al., 1997]. A potential explanation for this lack of concordance is that some perfused tumor vessels carry hypoxic blood [Helmlinger et al., 1997]. Thus, although they might not be able to deliver adequate oxygen to the surrounding cells, they may be able to carry away the waste products (e.g., lactic acid).

Regulation of Angiogenic Gene Expression by Metabolic Microenvironment

Generation of pro- and anti-angiogenic molecules can be triggered by metabolic stress [Fukumura, 2005]. Hypoxia upregulates various angiogenic growth factors, including VEGF, Ang2, PDGF, placenta growth factor (PlGF), transforming growth factor (TGF), IL-8, and hepatocyte growth factor (HGF) [Harris, 2002]. Of the various molecules involved in sensing and responding to hypoxia, Hypoxia inducible factor-1α (HIF-1α) is considered to be the master regulator of oxygen homeostasis [Semenza, 2003]. This transcription factor is upregulated in a number of human tumors [Harris, 2002]. Hypoxia may play an important role in the angiogenic switch [Hanahan and Weinberg, 2000] which is required for tumor growth and expansion.

Low extracellular pH causes stress-induced alteration of gene expression, including the upregulation of VEGF and IL-8 in tumor cells in vitro [Xu et al., 2002]. Despite its importance, the effect of the low and heterogeneous interstitial pH on VEGF expression in vivo, especially in relationship with hypoxia remained unknown for many years due to the lack of appropriate techniques and animal models. The combination of fluorescence ratio imaging microscopy for pH measurements [Martin and Jain, 1993], phosphorescence quenching microscopy for pO_2 measurements [Torres-Filho et al., 1994], and the transgenic technology for visualization of VEGF promoter activity [Fukumura et al., 1998] has allowed the coordinated study of pH, pO_2, and VEGF expression in vivo [Fukumura et al., 2001]. Overall, tissue pO_2 but not pH was inversely correlated with VEGF promoter activity. However, detailed analysis revealed an important insight into the regulation of VEGF by the metabolic environment. Under low pH or oxygenated conditions, tissue pH, but not pO_2, is related to VEGF promoter activity [Fukumura et al., 2001]. Conversely, under hypoxic or neutral pH conditions, tissue pO_2 and not pH is correlated with VEGF expression. These results indicated that VEGF transcription in tumors is independently regulated by the tissue pO_2 and pH. In fact, subsequent analysis of the VEGF promoter region revealed that acidic pH induces VEGF expression distinct from the HIF-HRE mediated pathway [Xu et al., 2002]. Taken together these data suggest that two major metabolic microenvironments in solid tumors regulate angiogenic factors in a complimentary manner.

Therapeutic Consequences

Oxygen is an important component of radiation therapy [Brown, 1999]. Ionized radiation directly and indirectly damages DNA, and the effect of both is dependent on oxygen. Therefore, hypoxia in solid tumors significantly reduces

their radiation sensitivity. Tumor hypoxia is also associated with resistance to some chemotherapeutics such as bleomycin and neocarzinostatin [Brown, 1999]. Tumor hypoxia correlated with poor outcome even when surgery was the only treatment and is now considered as a prognostic factor for overall tumor aggressiveness and resistance to therapy [Hockel and Vaupel, 2001].

Hypoxia induces apoptosis via p53 and HIF-1-dependent mechanisms [Carmeliet et al., 1998]. On the other hand, tumor cells develop many mechanisms to survive under hypoxic conditions including HIF-HRE mediated inductions of the genes for angiogenesis, vasodilation, glycolysis, and hematopoiesis [Harris, 2002]. Mutations in p53 make tumor cells resistant to apoptosis and more prone to further mutations. The balance between hypoxia-induced apoptosis/necrosis and the increased resistance to cell death mediated by various hypoxia-induced pathways determines whether a tumor can survive and even grow under hypoxic conditions. Immune cells targeting tumor cells cannot be fully functional under hypoxic conditions and thus, allow tumors to evade the host immune response and cell based therapies. Furthermore, exposure to hypoxia alters tumor cells to be highly invasive and metastatic [Pennacchietti et al., 2003; Erler et al., 2006]. Ultimately, hypoxia might select for tumor cells that are more malignant, more aggressive, and genetically unstable, and less susceptible to apoptosis, thus rendering them resistant to various therapies. Therefore, several molecules in the hypoxia-induced pathways are now being targeted in the development of diagnostic and therapeutic agents [Semenza, 2003; Pouyssegur et al., 2006].

For nearly half a century, considerable preclinical and clinical efforts have been focused on alleviating hypoxia. These efforts include improving tumor perfusion with mild hyperthermia or drugs; increasing oxygen content of the blood (via hyperbaric oxygenation, for example); or increasing hemoglobin/hematocrit (via erythropoeitin, for example). Unfortunately, these strategies have not shown much success in the clinic. One reason for the failure is abnormal structure and function of tumor vasculature causing uneven perfusion. This makes it difficult to increase pO_2 in all regions of tumors to optimal levels and/or to deliver radiation sensitizers or chemotherapeutic drugs to all regions of a tumor at therapeutically effective levels. Alternatively, one can exploit tumor hypoxia by using cytotoxic agents which are specifically activated under hypoxia [Brown, 1999]. Although this approach would allow high therapeutic index (ratio between effect on tumors and normal tissues), the physiological barrier in solid tumors may not permit the delivery of these drugs to all hypoxic cells.

Low extracellular pH can also affect the outcome of therapeutics adversely or in some case favorably [Gerweck et al., 2006]. Despite the low extracellular pH, the intracellular pH in tumor cells in vivo remains neutral. As a consequence, significant intracellular–extracellular pH difference exists in tumors. This trans-membrane pH gradient hinders the cellular uptake of weak base drugs such as adriamycin, doxorubicin, and mitoxantrone and thus, their efficacy [Vukovic and Tannock, 1997]. Acidic pH also causes dysfunction of immune cells. On the other hand, it may be exploitable for the treatment of cancer by weak acid drugs such as chlorambucil that are membrane permeable in their uncharged state. In an acidic extracellular environment, the non-ionized fraction of a weak acid increases, allowing more drugs to diffuse through the cell membrane into the relatively basic intracellular compartment where the ionized fraction increases, resulting in an increased intracellular drug concentration. Systemic injection of glucose could further acidify extracellular pH without changing intracellular pH and enhance tumor growth delay by a weak acid drug (chlorambucil), whereas worsen the effect of a basic drug (doxorubicin) [Gerweck et al., 2006].

There is, however, a caveat in acidifying tumor to enhance the efficacy of certain drugs. Exposure of tumor cells to acidic extracellular pH induces expression of proteinases which facilitates invasion and metastasis [Rofstad et al., 2006]. As discussed earlier, acidic pH induces expression of angiogenic factors [Xu et al., 2002] and thus contributes to growth of metastatic tumors [Rofstad et al., 2006]. Normalization of tumor vasculature by anti-angiogenic agents may neutralize the acidic extracellular pH in tumors. Increasing interstitial pH toward normal values may enhance efficacy of base drugs and reduce the metastatic potential of tumor cells.

NORMALIZATION OF TUMOR MICROENVIRONMENT BY ANTI-ANGIOGENIC AGENTS

The balance of endogenous pro- and anti-angiogenic factors is well maintained in normal tissues. Excess production of pro-angiogenic molecules and/or diminished production of anti-angiogenic molecules may cause abnormalities in vessels and microenvironment in tumors resulting in insufficient drug delivery and therapeutic efficacy [Jain, 2005]. Thus, if one were to judiciously downregulate angiogenic signaling such as VEGF, which is

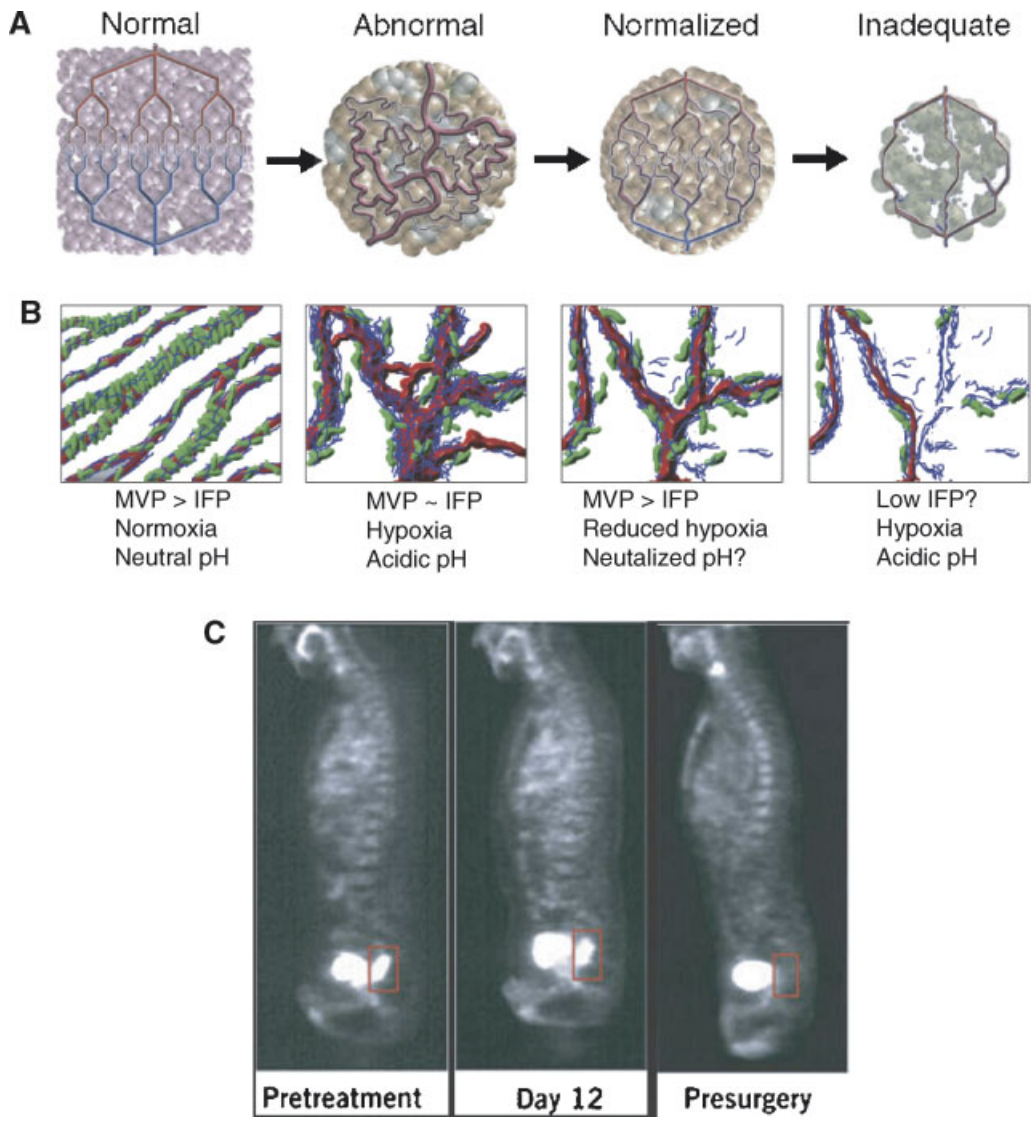

Fig. 4. Normalization of tumor vasculature and microenvironment. **A**: Tumor vasculature is structurally and functionally abnormal. It is proposed that anti-angiogenic therapies initially improve both the structure and the function of tumor vessels. However, sustained or aggressive anti-angiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for delivery of drugs or oxygen. **B**: Diagram depicting the concomitant changes in vessel morphology, perivascular cell (green) and basement membrane (blue) coverage, and tumor microenvironment during anti-angiogenic treatment. Anti-angiogenic treatment can transiently normalize the abnormal structure and function. **C**: Tumor FDG uptake before treatment (pretreatment), 12 days after bevacizumab treatment, and 6–7 weeks after completion of neoadjuvant therapy (presurgery). Sagittal projections of FDG-Pet scans of a colorectal cancer patient. Tumor is outlined in the box, posterior to bladder. Note that the FDG-uptake by the tumor is not decreased after bevacizumab treatment, even though the microvessel density is decreased by about 50%—suggesting that the normalized vessels are twice as efficient as the vessels before treatment with bevacizumab. (A, B) adapted from Jain [2005], (C) reproduced from Willett et al. [2004].

overexpressed in the majority of solid tumors, then the vasculature might revert back to a more "normal " state [Jain, 2005]. Indeed, neutralizing antibody against VEGF receptor 2 pruned the immature and leaky vessels of transplanted tumors in mice and actively remodeled the remaining vasculature so that it more closely resembled the normal vasculature (Fig. 1) [Tong et al., 2004; Winkler et al., 2004]. This "normalized" vasculature had less leaky, less dilated, and less tortuous vessels with a more normal basement membrane and greater coverage by perivascular cells (Fig. 4). These changes in tumor vasculature were accompanied by normalization of the tumor microenvironment—decreased IFP (Fig. 2), increased tumor oxygenation (Fig. 3), and presumably neutralized pH. As a result, penetration of drugs in these tumors and efficacy of radiation treatments were improved [Tong et al., 2004; Winkler et al., 2004]. Furthermore, we obtained clinical data mirroring these preclinical findings in rectal carcinoma patients receiving bevacizumab with chemo- and radiation therapies (Figs. 2 and 4) [Willett et al., 2004]. More recently, seven independent laboratories have published data that support our findings on vascular normalization using a variety of anti-angiogenic drugs and animal models [Wildiers et al., 2003; Inai et al., 2004; Ansiaux et al., 2005; Bang et al., 2005; Huber et al., 2005; Salnikov et al., 2005; Vosseler et al., 2005].

Anti-VEGFR2 antibody-induced vascular normalization may be transient (Fig. 3) [Tong et al., 2004; Winkler et al., 2004]. The combination of anti-angiogenic treatment and radiation therapy delayed tumor growth synergistically only when ionizing radiation was given during this "normalization window" [Winkler et al., 2004]. Therefore, prolongation of the normalization window would make the normalization strategy more clinically beneficial. Understanding of cellular and molecular mechanisms of vascular normalization would help such a development. Along that line, we found that perivascular cell recruitment via Tie2 signaling and normalization of basement membrane by balancing its synthesis and degradation appear to be involved in the vascular normalization induced by anti-VEGFR2 treatment [Winkler et al., 2004]. Finding and validating reliable surrogate markers is also urgently needed not only for clinical translation of the vascular normalization strategy, but also for anti-angiogenic treatment in general. Circulating endothelial cells appear to be a useful candidate [Willett et al., 2005; Duda et al., 2006]. Furthermore, it is critical to determine the presence and extent of vascular normalization with different clinically available anti-angiogenic agents in different type of orthotopic tumors. Our current goal is to exploit this knowledge to improve the therapeutic outcome and to prolong the overall survival of patients beyond the 2–5 months which is currently achievable with bevacizumab [Hurwitz et al., 2004]. To this end, we and others have initiated a number of preclinical studies and corresponding clinical trials in glioblastoma multiforme, head and neck, breast and ovarian cancers, and sarcoma patients using antibodies or tyrosine kinase inhibitors that target VEGF and/or PDGF pathways.

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