Research article – Histology and cell biology

Tumor angiogenesis. From bench to bedside

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Abstract

Starting with the hypothesis of Judah Folkman that tumor growth is angiogenesis dependent, this area of research has a solid scientific foundation. More than 30 years ago, Folkman found a revolutionary new way to think about cancer. He postulated that in order to survive and grow, tumors require blood vessels and that by cutting off the blood supply a cancer could be starved into remission. Several clinical studies have shown a positive correlation between the number of vessels in the tumor, metastasis formation and prognosis. The genetic instability of tumor cells permits the occurrence of multiple genetic alterations that facilitate tumor progression and metastasis, and cell clones with diverse biological aggressiveness may coexist within the same tumor. These two properties allow tumors to acquire resistance to cytotoxic agents. Inhibiting angiogenesis is a major area of therapeutic development for the treatment of cancer. Whereas conventional chemotherapy, radiotherapy and immunotherapy are directed against tumor cells, anti-angiogenic therapy is aimed at the vasculature of a tumor and will either cause total tumor regression or keep tumors in a state of dormancy. Even though numerous compounds inhibit angiogenesis, few of them have proved effective *in vivo*, and only a couple of agents have been able to induce tumor regression. Bevacizumab is considered to be the first specific angiogenesis inhibitor for clinical oncology.

Introduction

In 1945, Algire and Chalkley were the first to appreciate that growing malignant tumors could continuously elicit new capillary growth from the host (Algire and Chalkley, 1945). In 1971, Judah Folkman isolated the first angiogenic factor, and called it "Tumor Angiogenesis Factor" (TAF). He fractioned by gel-filtration on Sephadex G100 the homogenate of a Walker 256 carcinoma and obtained a fraction with a strong angiogenic activity with a molecular weight of about 10,000 Dalton, consisting of 25% RNA, 10% proteins, 58% carbohydrates, and a lipid residue. Several other low molecular weight angiogenic factors were isolated from the Walker 256 carcinoma, capable to induce an angiogenic response in vivo when tested on rabbit cornea or chick embryo chorioallantoic membrane (CAM), and in vitro on cultured endothelial cells (Folkman et al., 1971). Subsequently, TAF was extracted from several tumor cell lines. Starting from the discovery of TAF, other pro-angiogenic molecules have

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been isolated, namely basic fibroblast growth factor (bFGF)/fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF), and placental growth factor (PlGF). In the meantime, it has been demonstrated the angiogenic activity of non-classic angiogenic molecules, including hematopoietic cytokines, namely granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), and erythropoietin (EPO).

In this context, Folkman (1971) hypothesized that tumor growth is angiogenic-dependent and that inhibition of angiogenesis could be therapeutic, introducing the term anti-angiogenesis. Investigations on neoplastic transformation have focused on transformed cells and in the meantime have addressed the tumor microenvironment and documented its importance in tumor progression. The pathogenesis of most cancers, in fact, includes complex and mutual interactions affecting tumor cells, inflammatory cells and various components of the extracellular matrix. These concepts are now widely accepted and supported by experimental and clinical studies.

Tumor growth occurs through an avascular phase followed by a vascular phase. Assuming that such growth is dependent on angiogenesis and that this depends on the release of angiogenic factors, the acquisition of an angiogenic ability can be seen as an expression of progression from neoplastic transformation to tumor growth and metastasis (Ribatti et al., 1999).

Practically all solid tumors, including those of the colon, lung, breast, cervix, bladder, prostate and pancreas, progress through these two phases. The role of angiogenesis in the growth and survival of leukemias and other hematological malignancies has only become evident since 1994, thanks to a series of studies demonstrating that progression in several forms is clearly related to their degree of angiogenesis (Vacca and Ribatti, 2006).

In the 1970s Gullino's group (Brem et al., 1977, 1978; Maiorana and Gullino, 1978) observed that experimental breast cancer in rat and mouse gave rise to marked breast angiogenic activity that was lacking in adult gland. Moreover, just like the hyperplastic and dysplastic breast lesions more frequently subject to malignant change, pre-malignant lesions also induce a strong vasoproliferative response long before any morphological sign of malignant transformation can be observed.

The avascular phase appears to correspond to the histopathological picture presented by a small colony of neoplastic cells (500,000–1 million cells/1–2mm in diameter) that reaches a steady state before it proliferates and becomes rapidly invasive. Here metabolites and catabolites are transferred by simple diffusion through the surrounding tissue. The cells at the periphery of the tumor continue to reproduce, whereas those in the deeper portion die away. Some of these prevascular lesions can be seen in the skin or in the bladder, but the majority of prevascular tumors in the breast, colon, and other organs are microscopic, clinically undetectable, and asymptomatic.

Tumor angiogenesis may occur through several mechanisms: i) sprouting angiogenesis; ii) recruitment of bone marrow-derived endothelial progenitor cells to form new vessels (postnatal vasculogenesis) ; iii) vasculogenic mimicry (the transdifferentiation of cancer cells allowing them to form tubular structures themselves); iv) mosaic vessel formation (the incorporation of cancer cells into the vessel wall or vascular cooption);v) intussusception.

Morphofunctional properties of tumor blood vessels

In tumors, the organ- and tissue-specific vascular architecture is not retained. Tumor blood vessels are irregular in size, shape, and branching pattern, lack the normal hierarchy, and do not display recognizable features of arterioles, capillaries, or venules (Ribatti et al., 2007a). Tumor-associated endothelium is structurally defective: intercellular gaps, transendothelial holes, vesiculo-vacuolar organelles, and endothelial fenestrae are present in the endothelium of tumor vessels (Dvorak et al., 1988). Defects in endothelial cell barrier function, due to abnormal cell–cell junctions and other changes, are responsible of vascular leakiness, which has been attributed also to highly active angiogenesis and microvascular remodeling. Leakiness correlates with histological grade and malignant potential (Doldrup et al., 1998) and can be exploited in locating tumors by imaging contrast media and in the delivery of macromolecular therapeutics (Mc Donald and Choyke, 2003). Furthermore, it results in extravasation of plasma proteins and even erythrocytes and may facilitate the traffic of tumor cells into the bloodstream and the formation of metastases (Doldrup et al., 1998). Whereas endothelial cells of mature, quiescent vessels are characteristically low proliferative and their estimated turnover times are measured in years, those of tumor vessels are markedly dependent on growth factors for survival. VEGF plays a central role in the induction of host vessels into a growing tumor. When endothelial cells invade a newly formed tumor, they come into contact with tumor cells that produce VEGF, which may be responsible not only for vascular proliferation but also for the altered permeability of the newly formed vessels (Senger et al., 1995; Dvorak et al., 1987). Although tumor cells represent the major source of VEGF, tumor-associated stroma is also an important site of VEGF production (Fukumura et al., 1998).

In tumors, pericytes are located near blood vessels at the growing front of tumors, where angiogenesis is most active, and show morphological abnormalities (Morikawa et al., 2002). Moreover, pericyte deficiency may be partly responsible for vessel abnormalities in tumor blood vessels (Gerhardt and Semb, 2008) and partial dissociation of pericytes (Hobbs et al., 1998; Hashizume et al., 2000) contributes to increased tumor vascular permeability.

Inflammation and tumor angiogenesis

Rudolf Virchow in 1863 critically recognized for the first time the presence of inflammatory cells infiltrating neoplastic tissues and first established a causative connection between the "lymphoreticular infiltrate" at sites of chronic inflammation and the development of cancer.

In 1986, Dvorak noted that wound healing and tumor stroma formation shared many important properties (Dvorak, 1986). They both were rich of newly formed vessels and fibrin matrix. He likened tumors to "wounds that do not heal", because tumor cells secreted a vascular permeability factor, now referred to as VEGF, that could lead to persistent extravasation of fibrin and fibronectin and continuous generation of extracellular matrix. It is well established that tumor cells are able to secrete pro-angiogenic and pro-inflammatory factors as well as mediators for inflammatory cells. They produce indeed cytokines such as VEGF, FGF-2, interleukin-8 (IL-8), PlGF,

transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), angiopoietins (Angs) and others. These are exported from tumor cells or mobilized from the extracellular matrix. As a consequence, tumor cells are surrounded by an infiltrate of inflammatory cells, namely lymphocytes, neutrophils, macrophages and mast cells. These cells communicate via a complex network of intercellular signaling pathways, mediated by surface adhesion molecules, cytokines and their receptors. Results point to the importance of a cross-talk between several host cells for promoting angiogenic effects in tumor areas.

Inflammatory cells cooperate and synergise with stromal cells as well as malignant cells in stimulating endothelial cell proliferation and blood vessel formation. These synergies may represent important mechanisms for tumor development and metastasis by providing efficient vascular supply and easy pathway to escape host defense. Indeed, the most aggressive human cancers, such as malignant melanoma, breast carcinoma and colorectal adenocarcinoma, are associated with a dramatic host response composed of various inflammatory cells, especially macrophages and mast cells. It is well known that tumor vessels present a defective organization of the endothelium.

The angiogenic switch and the concept of normalization

Angiogenesis is controlled by the balance between molecules that have positive and negative regulatory activities and this concept has led to the notion of the angiogenic switch, which depends on an increased production of one or more positive regulators of angiogenesis (Ribatti et al., 2007b). Most human tumors arise and remain in situ without angiogenesis for a long time before switching to an angiogenic phenotype, through a pre-neoplastic stage as occurs in breast and cervical carcinomas, which becomes neovascularized before the malignant tumor appears. Activation of the angiogenic switch has been attributed to the synthesis or release of angiogenic factors, and accordingly to the balance hypothesis the level of angiogenesis inducers and inhibitors regulates angiogenesis in physiological conditions. This balance is altered in pathological conditions, including tumors, as a consequence of an increased bioavailability or activity of the inducer proteins, or reduced concentrations of endogenous angiogenesis inhibitors. Thrombospondin-1(TSP-1) was the first endogenous inhibitor for which there was compelling evidence of participation in the angiogenic switch, because it was down-regulated in tumors before angiogenesis could be triggered. By 1989, the switch itself was understood as the result of a shift in the 'net balance' of angiogenesis stimulators and inhibitors. This led to the discovery of angiostatin and endostatin. Angiostatin is a 38-kDa internal fragment of plasminogen, while endostatin is a 20-kDa internal fragment of collagen XVIII.

In the RIP-TAG transgenic mice expressing an oncogene in beta cells of the pancreas, Folkman et al. (1989) found that the angiogenic switch occurs in the early stage of cancerogenesis in hyperplastic islets before the onset of the tumor formation.

Restore of this balance may induce a normalization of structure of blood vessels. The concept of "normalization" of tumor blood vessels by anti-angiogenic drugs was introduced by Rakesh Jain in 2001 (Jain, 2001).The state of normalization is probably transient and dependent on the dose and duration of the treatment.

Anti-angiogenesis

Anti-angiogenic agents may be divided in two major groups: indirect agents that block the expression or the activity of angiogenic molecules or the expression of their receptors on endothelial cells, and agents able to directly affect endothelial cell function or survival. Beginning in the 1980s, the industry began exploiting the field of anti-angiogenesis for creating new therapeutic molecules in angiogenesis-dependent diseases. At present anti-angiogenic therapy is essentially anti-VEGF/VEGF receptor (VEGFR) therapy and has already fulfilled its promise in the clinic.

Bevacizumab (Avastin) was the first angiogenesis inhibitor approved by the Food and Drug Adminstration (FDA) for the treatment of colorectal cancer in February 2004, administered in combination with irinotecan, 5-fluorouracil and leucovirin (Hurwitz et al., 2004). It was subsequently approved for use, in combination with cytotoxic chemotherapy, in other cancers including non-small-cell lung cancer and breast cancer, demonstrating an improvement in overall survival or delayed tumor progression compared to chemotherapy alone (Sandler et al., 2006; Miller et al., 2007).

Encouraging results have also been obtained when chemotherapy has been used in combination with an anti-VEGF2 monoclonal antibody (cediranib; Goss et al., 2010) and an oral tyrosine kinase inhibitor of VEGFRs and epidermal growth factor receptor (EGFR) (vandetanib). The observation that combining chemotherapy with angiogenesis inhibitors causes increased apoptosis in tumors in vivo (Song et al., 2001; Inoue et al., 2003) suggests that angiogenesis inhibitors may have an additive effect when administered in combination with chemotherapy.

Removal of VEGF inhibition causes tumor re-growth due to the fact that pericytes provide a scaffold for the rapidly re-growing of tumor vessels (Mancuso et al., 2006). Pericytes have been indicated as putative targets in the pharmacological therapy of tumors by using the synergistic effect of anti-endothelial and anti-pericytic molecules. Removal of pericyte coverage leads to exposed tumor vessels, which may explain the enhanced effect of combining inhibitors that target both tumor vessels and pericytes. Bergers et al. (2003) showed that combined treatment or pre-treatment with anti-PDGF-B/PDGF receptor beta (PDGFR-β), reducing pericyte coverage, increases the success of anti-VEGF treatment in the mouse RIP1-TAG2 model.

A clinical challenge in anti-angiogenesis is the finding of biological markers that help to identify subsets of patients more likely to respond to a given anti-angiogenic therapy, to detect early clinical benefit or emerging resistances and to decide whether to change therapy in second-line treatments (Table 1).

The results from clinical trials have not shown the dramatic antitumor effects that were expected following preclinical studies, which revealed a much higher efficacy of these type of agent in animal models. Patients with different types of tumors respond differently to anti-angiogenic therapy. While colorectal, lung and breast cancer patients have responded, pancreatic cancer patients have not shown survival advantage when treated with anti-angiogenic monotherapy or combinations of anti-angiogenic agents with chemotherapy. Additionally, preclinical and clinical data have shown the possibility that tumors may acquire resistance to anti-angiogenic drugs or may escape anti-angiogenic therapy via compensatory mechanisms. Most of the FDA-approved drugs, as well as those in phase III clinical trials, target a single proangiogenic protein. However, multiple angiogenic molecules may be produced by tumors,

Table 1 – Predictive markers useful to identify subsets of patients more responsive to anti-angiogenic therapy.

Plasma and/or urinary levels of angiogenic cytokines

Circulating platelet levels

Serum levels of soluble VEGFRs

Serum levels of soluble v-CAM, E-selectin, and Tie-2

Circulating levels of endothelial precursor cells (EPCs) and endothelial cells (CECs)*

Immunohistochemical expression of thymidine phosphorylase (TP) in endothelial cells**

Cancer stem cells (CSCs) detection***

Functional imaging of tumor angiogenesis [single positron emission tomography (SPECT) and positron emission tomography)]****

* In general, but not always, the plasma level of the markers decrease parallel to tumor regression in response to the therapy, and the circulating levels of one or more of these factors has been reported as an indicator to predict patient survival.

** TP-positive endothelial cells correlate with CD34-positive endothelial cells and microvascular density in tumor bioptic specimens. Moreover, TP expression correlates with a better survival rate of patients.

*** CSCs secrete high levels of angiogenic cytokines and are more resistant to conventional chemotherapy and radiotherapy due to an increased expression of antiapoptotic proteins.

**** Imaging techniques allow an estimation on tumor angiogenesis and of the effect of anti-angiogenic therapy on tumor microcirculation.

and tumors at different stages of development may depend on different angiogenic factors for their blood supply. Therefore, blocking a single angiogenic molecule might have little or no impact on tumor growth. Cancer genomics and proteomics are likely to identify novel, tumor-specific endothelial targets and accelerate drug discovery.

Ligand-targeted therapeutic strategies

Since tumor endothelial cells express several markers that are not expressed in normal counterpart, it is possible to develop ligand-targeted chemotherapeutic strategies, based on peptides that are selective for tumor vasculature and involving vascular targeting agents (VTAs) or vascular disrupting agents (VDAs) (Spear et al., 2011). VTAs/VDAs directly attack the tumor endothelial cells and inhibit their proliferation and the destruction of existing tumor blood vessels results in tumor hypoxia and tumor cell necrosis. VDAs comprise two main classes: (1) small-molecules, that localize to the tumor endothelium by exploiting the known differences between tumor and normal endothelium to induce selective vascular dysfunction, and (2) ligand-based therapies, chemotherapeutics, toxins, procoagulant and proapoptotic effectors to the tumor endothelium (Siemann et al., 2004; Thorpe, 2004; Pilat and Lorusso, 2006).

Acknowledgment

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