Review

Molecular Mechanisms Regulating the Angiogenic Phenotype in Tumors: Clinical Impact in the Future

KARINE AUBRY[1], GUILAINE BARRIERE[2], HELENE CHABLE-RABINOVITCH[2], AURELIE DUTOUR[2], FRANCOIS PARAF[3], JACQUES MONTEIL[4] and MICHEL RIGAUD[2]

Departments of [1]E.N.T., [2]Biochemistry and Molecular Biology, [3]Pathology and [4]Nuclear Medicine, University Hospital Center, Limoges, France

Abstract. *Tumor progression depends on the angiogenic switch. In this review, we recapitulate the molecular mechanisms involved in this angiogenic switch. The rat osteosarcoma model employed would permit further studies in the sequential events leading to initial recruitment of blood vessels and could lead to development of an angiogenesis-based panel of circulating blood cells (endothelial cells, endothelial progenitor cells and accessory cells) that can be quantified and used to detect microscopic tumors or to follow the efficacy of antiangiogenic therapy. Such a result would lead to the possibility of early therapy in cancer progression.*

The concept of the angiogenesis dependency of tumor growth and metastasis was born through the pioneering work of J. Folkman more than 30 years ago. However, as early the 19th century, the role of vascularization in tumors was underlined by Paget (1). Like normal tissue, tumors require an adequate supply of oxygen and nutrients and a way to remove waste. A tumor cannot grow beyond a size of 2 mm^3 without gaining access to the host's vasculature, thus establishing its own vessels.

This review is focused on: i) endothelial cells, whose phenotypic characteristics make them a target for antiangiogenic therapy, ii) their cellular markers and signalling pathways, and iii) the development of antiangiogenesis in cancer therapy and to the closely related problem of tumor dormancy.

Correspondence to: Dr. Karine Aubry, E.N.T. Department, University Hospital Center, 2 Avenue Martin Luther-King, 87000 Limoges, France. Tel: +33 555056239, Fax: +33555056287, e-mail: aubry.k@caramail.com

Keys Words: Angiogenesis, circulating cells, endostatin, cancer dormancy, review.

Endothelial Cells

"The endothelium is more than a sheet of nucleated cellophane" (2). It consists of a rather homogenous population of cells that have a propensity to assemble in tubes. However endothelial cells (EC) exhibit remarkable heterogeneity which leads to different phenotypes, and EC vessels have been classified as fenestrated, discontinuous and continuous (3). In this way capillaries show organ-specific differences, with a continuous endothelium for muscle, lung and brain, and a fenestrated type for endocrine and exocrine glands, choroid plexus and intestinal villi (4). The hallmark of the different phenotypes relies on the molecular differences between the EC populations. Technologies such as microarray screening and *in vivo* phage display enable the establishment of a map of endothelial cell 'addresses' (5) and provides a basis for targeting therapeutic molecules to tissues affected by cancer and other diseases.

Induced Phenotype Expression

The molecular phenotype of endothelial cells (ECs) is modulated by drugs and physiological inducers, leading to EC activation. Such modifications of gene expression have been reported in many experiments, *in vivo* and *in vitro*. When first passage human umbilical cells (HUVECs) are treated for 6 hours with lipopolysaccharide (LPS) or tumor necrosis factor-alpha (TNFα), expression of 191 common genes was increased and those of 102 genes decreased. Regulated transcripts encoded for a large number of chemokines, adhesion molecules, procoagulant factors and molecules that affect cell integrity (6). Using quantitative real-time PCR, the expression profile of a selected group of 74 glycosilation-related genes has been determined in HUVECs and human foreskin microvascular endothelial cells (FMVECs), under control and TNFα-induced

conditions. Thus, Garcia-Vallejo *et al.* demonstrated that induction of an inflamed phenotype of the cells by treatment with TNFα modulates a set of these genes in HUVECs and FMVECs, resulting in a change in the cell membrane-associated glycans (7).

Schulteiss *et al.* demonstrated, *in vivo*, the activation of brain endothelial cells in APP23 mice, a transgenic model of Alzheimer's disease (8). In these mice, brain amyloid deposition is followed by the expression of β3-integrins. The total number of vessels detected on brain histological sections, by CD31 staining (CD31 is a panendothelial marker) did not change with age, whereas the number of vessels staining positive for β3 integrins (marker of the endothelium during angiogenesis) significantly rose between 11 and 23 months of age in APP23 mice. Numerous other scientific papers which dealt with these molecular phenotype changes of ECs, are referred to below.

Cytogenetics of Endothelial Cells

For many years it was assumed that tumor ECs would be chromosomally normal and thus genetically stable. Therefore certain antiangiogenic drugs might constitute a treatment strategy that would avoid drug resistance (9). Possibly, ECs in solid tumor blood vessels have cytogenetic differences as well and display a degree of aneuploidy as evidenced by extra chromosomes and multiple centrosomes. These results were obtained in xenograft models (10). Such observations were also found in circulating endothelial cells in patients with B-cell non Hodgkin's lymphoma. These surprising findings might have different explanations: circulating endothelial cells and neoplasic cells in lymphoma and myeloma might have a common hemangioblast precursor, or might arise from dedifferentiation towards the endothelial phenotype, in the presence of angiogenic factors, or might be issued from fusion between neoplasic and ECs (11).

Circulating Endothelial Cells in Malignant Diseases

Measurement of tumor angiogenesis is mainly based on the evaluation of microvascular density (MVD). In this procedure, blood vessels of tumor samples are stained with antibodies and counted under light microscopy (Figure 1). This approach is invasive and MVD might not correlate with the vasculature of the whole tumor. Moreover the MVD cannot always predict the efficacy of antiangiogenic drugs or the clinical outcome in most tumor types. Despite its importance, the MVD has led to serious misconceptions delineated by Folkman *et al.* (12): i) MVD is not a measure of the angiogenic dependance of the tumor. ii) The measurement of MVD is not predictive of tumor response under antiangiogenic treatment. iii) Tumor MVD may not vary in accordance with the tissue or blood levels of any single proangiogenic factor. iv) A minimum vessel density is determined by the metabolic demand of tumor cells. v) Rapid tumor growth does not imply high vascular density. If we subscribe to these assertions, and we do, it is necessary to develop molecular profiles of tumor blood vessels. This goal could be achieved by studying the microvascular endothelium in tumor and/or circulating ECs (CECs) and endothelial progenitor cells (EPCs). Moreover some markers from biological fluids could be indicators of the presence of tumors in a dormant state and circulating cancer cells could give major information on their proangiogenic activity.

Novel methods of assessing vascular endothelial function in cancer *via* the quantification and the characterization of CECs are under development in many laboratories. Recent research suggests that another cell type, the EPC, has an important role in tumor vasculogenesis. Vasculogenesis is the formation of new blood vessels in embryoid bodies, a process characterized by the recruitment of EPCs to sites of new vessel formation with differentiation towards mature ECs. EPCs also exist in the adult and contribute to new vessel formation, a process called post natal vasculogenesis (13). Thus the use of CECs, EPCs and endothelial microparticles (EMPs) – a marker investigated in vascular dysfunction and coagulopathy – might represent a new method for evaluation of the vascular status of cancer patients. CECs belong to the population of rare non hematopoietic cells present at a very low level in the peripheral blood. To detect them, cell enrichment from the whole blood and use of a specific marker are necessary. Use of magnetic beads coupled to a monoclonal antibody (S-endo-1) directed against the endothelial antigen CD 146 fulfils these conditions. CD 146 is an adhesion molecule belonging to the Ig superfamily and is largely distributed on all subpopulations of ECs. It is not detectable on hematopoietic cells (14). A consensus protocol has been proposed for isolation and enumeration of circulating endothelial cells. This protocol relies on CD146-driven immunomagnetic isolation and a subsequent confirmatory step with *Ulex europeaus* lectin-1 staining (15). Goon *et al.* (16) demonstrated that there were no statistically significant differences in CEC levels determined using the immunomagnetic bead (CD146 and FITC *Ulex europeaus* lectin-1) method and the flow cytometry method (CD45, CD34 and CD146).

To understand the contribution of CECs to tumor progression, it will be important to obtain a detailed phenotypical analysis of these circulating endothelial cells. There is no definitive answer yet to this concept.

CECs and EPCs, Valid Surrogate Markers for Neovascularisation

By using eight inbred mouse strains and assessed sprouting angiogenesis, Bertolini *et al.* (11) demonstrated a strong angiogenesis responsiveness to vascular endothelial growth

factor (VEGF) in the corneal neovascular micropocket assay. Pellets that contain angiogenic factors are inserted into the mouse corneal stroma next to the temporal limbus. Capillaries sprout from pre-existing vessels, grow towards the pellet, invade the corneal avascular tissue and can be counted by microscopy. When C57/Black6J and C3h/HeJ strains were tested, a limited response was observed, different from the one of the previous eight inbred mouse strains. A striking correlation was subsequently found between this heterogeneous response and the absolute number of CECs and EPCs in normal mice. These results suggest that both the angiogenic and vasculogenic phenotypes are probably regulated by the same genetic influences.

Another problem is that of the determination of the optimal dose of antiangiogenic therapeutic drug. Might the quantification of CECs and EPCs be used to determine it? Bertolini *et al.* (11) showed in a mouse preclinical tumor model that the optimal dose of a rat monoclonal antibody specific for vascular endothelial growth factor receptor-2 (VEGFR2) ranged from 800 to 1000 μg per mouse, every three days. This dose induced the lowest level of viable EPCs and the greatest decrease in tumor volume. With other tested antiangiogenic drugs, there was a striking correlation between suppressed levels of viable EPCs and the optimal dose of each particular drug. Hence the counting of CECs and EPCs could be used to determine the optimal drug dose thought to have an antiangiogenesis effect, in the process of low-dose metronomic chemotherapy.

CECs and EPCs as a Biomarker in Cancer

CEC levels are elevated for some cancer patients at diagnosis compared to normal levels and this CEC number is reduced at complete remission (17). In mice treated with endostatin, the majority of apoptotic or dead circulating cells are endothelial cells. This parameter could provide a rational biomarker in clinical trials involving antiangiogenic therapy (18). The last major point that must be underlined is the possibility of using *ex vivo*-modified EPCs to deliver drugs at tumor vasculature sites. The use of autologous EPCs might avoid immune rejection (19).

Accessory Cells

Grunewald *et al.* demonstrated that VEGF plays a major role for organ homing of circulating mononuclear myeloid cells (20). These recruited bone marrow-derived circulating cells (RBCCs) are different from endothelial progenitor cells. Their homing in close proximity to angiogenic vessels is mediated by the chemokine CXCL12 known as stromal-derived factor (SDF1). RBCCs enhance *in situ* proliferation of endothelial cells by secreting proangiogenic factors. The spatial relationship between RBCCs and endothelial cells suggested a paracrine angiogenic role for RBCCs rather than a role as endothelial progenitors. The recruited RBCCs express VEGFR1, but not VEGFR2, and the typical myeloid markers CD45, CX3CR1 and CD11b. Moreover CXCR4 was found to be expressed by the majority of CD45+ cells that had been recruited. SDF1 is minimally expressed in quiescent tissues but *via* a conditional switch of VEGF expression, SDF1 expression increased markedly in perivascular fibroblasts and smooth muscle cells. Analogs of SDF1 and/or CXCR4 antibody may block CXCR4/VEGFR1 cell recruitment. Moreover RBCCs released matrix metalloproteinase-9 (MMP9) and thus facilitated endothelial cell sprouting. Blocking SDF1/CXCR4 signalling would be expected to inhibit angiogenesis in pathological conditions.

Antiangiogenesis in Cancer Therapy

The first angiogenesis inhibitors for cancer have now been approved by the U.S. Food and Drug Administration and in other countries. The majority of them target VEGF. However, it is well known that during therapy tumor cells are able to produce redundant angiogenic factors. Moreover, after treatment ends, the tumor vascularity can start up again. In the RIP-1Tag2 model, Mancuso *et al.* (21) demonstrated that 7 days' therapy targeting the VEGF signalling pathway left empty sleeves of basement membranes and pericytes. Empty sleeves act as a scaffold for tumor vessel regrowth. Bergers *et al.* (22) demonstrated that combination of an inhibitor targeting VEGFRs in endothelial cells (SU 5416) and a selective kinase inhibitor for platelet derived growth factor receptors (PDGFRs) (SU 6668) was more efficacious against all stages of pancreatic islet cancer than either single agent. Therefore for long term use in cancer, a combination of angiogenesis inhibitors or broad spectrum angiogenesis inhibitors will be needed.

Angiogenesis inhibitors can be divided into two classes: direct inhibitors and indirect inhibitors (23). Direct inhibitors target endothelial cells by arresting proliferation and migration of these cells or by inducing their apoptosis. Indirect angiogenesis inhibitors act on the signaling pathways induced by angiogenic stimuli, by sequestering the angiogenic factors secreted by tumor cells or by blocking the signal transduction pathways that are activated when binding factors meet their receptors on endothelial cells. Endostatin is one model of inhibition of endothelial cell migration and proliferation.

Endostatin

Endostatin (24, 25) is a 20 kDa internal fragment of the carboxy-terminus of collagen XVIII (26). Endostatin protein was initially purified from tumor-bearing mouse urine and then recombinant mouse endostatin was produced

in *Escherichia coli*. As methods of resolubilization gave very low yields of active protein (insoluble purified endostatin had the consistency of toothpaste), the insoluble protein was injected subcutaneously in mice, leading to a small deposit that disappears over 2-3 days. The antitumor activity was dramatic (24). Most remarkably, cyclic rounds of endostatin treatment not only inhibited tumor growth during the treatment stages, but after several rounds, the tumor entered remission. A considerable amount of data suggests that at least one of the major activities of endostatin appears to be its inhibition of endothelial cell migration and proliferation. Endostatin initiates a complex network of signaling at the gene level. The data reported by Abdollahi *et al.* (27) support the concept of a large scale and highly interactive angiogenic signalling network, where eight pathway groups are down-regulated. These latter include inhibitor of differentiation/DNA binding (Id) proteins (28), hypoxia-inducing transcription factor-1-alpha (HIF1-α) (29), Ephrins (30), tumor necrosis factor (TNF) (31), nuclear factor kappaB (NF-κB) (32), activator protein-1 (AP-1) (27), thrombin receptors and ETS transcription factor-1 (Ets-1) (27). Thus endostatin is a paradigm of a broad-spectrum endogenous antiangiogenic molecule.

Several reports suggest that certain small molecules that can be taken orally will raise the endogenous expression of specific angiogenesis inhibitors. For exemple, celecoxib can increase the serum endostatin level (33). Thus a possible new pharmaceutical field could be developed around the future discovery of low molecular weight, orally available drugs that could protect against cancer by increasing endogenous angiogenesis inhibitors. The endostatin antiangiogenic therapy can be delivered either as endostatin protein or by means of a gene delivery approach. The ease of accessing ECs of the blood vessels supports this latter possibility (34) but gene therapy must overcome obstacles: limited access of ECs and selection of specific targets against microvascular ECs that have been recruited into the tumor bed.

Research in the field of tumor angiogenesis inhibition not only focuses on the identification of new antiangiogenic agents but also implies the design of carriers that selectively target the site of the tumor (35). Cationic liposomes were shown to specifically target angiogenic vessels in tumor and sites of inflammation (36, 37). Krasnici *et al.* demonstrated that cationic liposomes exhibit a significantly enhanced accumulation in tumor tissue and tumor vasculature up to 3-fold compared to surrounding tissues (38). The binding mechanism is supposed to be charge related (39, 40). The positive charge on the liposomal surface interacts with a negative charge on the cell surface of angiogenic microvessels leading to internalization of the liposomes. Normally the endothelium of microvessels is covered by the negative glycocalyx and moreover the basal laminal is also negatively charged (41, 42). The negative charges are enhanced on the surface of tumor endothelium and one of the major findings which emerges from the study of Ran *et al.* (43) is that anionic lipids are exposed on the surface of tumor endothelium. This was demonstrated using a monoclonal antibody, 9D2, and annexin which bind selectively to anionic phospholipids. Normally anionic phospholipids are largely absent from the external leaflet of the plasma membrane of cells. Phosphatidyl serine (PS), the most abundant anionic phospholipids, is normally located at the internal leaflet. Exposure of PS on the cell surface occurs during apoptosis, cell activation and cell injury. This could be due to activation of scramblase or inhibition of aminophospholipid translocase. Thus finally, cDNA complexed to cationic liposomes (cDNA/CLP) would be one of the best systems to target endothelial tumor cells (Figures 2, 3). Such a system has been used *in vitro* and *in vivo* (Figure 4). The intravenous administration of cDNA/CLP targets ECs but expression from the transgene remains transient. For durable gene transfer the group of Flechter (44) utilized the integrating capability of the 'Sleeping Beauty' (SB) transposon. A plasmid-based transposon with activity in vertebrate cells leads to a mechanism by which one can facilitate genomic integration. Transposons are more mobile genetic elements found in a variety of species, including bacteria, plants and insects. Transposition of SB occurs by a 'cut and paste' mechanism inserting a transposon flanked by two inverted repeat/direct repeat elements into a TA dinucleotide sequence found within plasmid or genomic DNA. This activity requires only the expression of the transposase. Ohlfest *et al.* (45) assessed the antitumor activity of SB transposon vectors that encode two potent antiangiogenic proteins, a soluble vascular endothelial growth factor receptor (Sftl-1) and an angiostatin-endostatin fusion protein (statin AE). Their experiment demonstrates that non viral gene transfer reduces tumor vessel density and inhibits growth tumor.

One of the latest papers dealing with gene therapy reports the use of an artificial gene leading to the expression of recombinant anginex (46). Most antiangiogenic agents have been discovered by identifying endogenous molecules, primarily proteins, which inhibit EC growth. This traditional approach has produced several major antiangiogenic agents: platelet factor-4 (47), thrombospondin-1 (48), interferon-γ-inducible protein-10 (49), angiostatin (50), endostatin (25), vasostatin (51), tumstatin (52) and bactericidal/permeability-increasing protein (BPI) (53).

Another approach to discover antiangiogenic agents is that of Thijssen *et al.* (54). By using yeast-two-hybrid technology, they identified galectin-1 as an endothelial cell receptor of the potent antiangiogenic anginex. The interaction was confirmed by Biacore analysis and NMR. From the 33-mer peptide anginex, they constructed an artificial gene encoding this peptide. Their results suggest

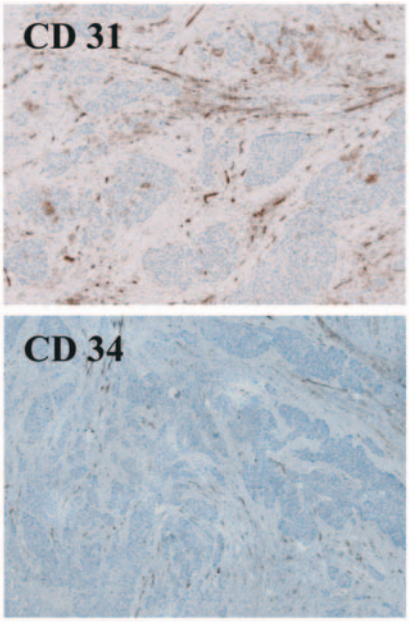

Figure 1. *Microvascular density (MVD) assessment. Representative micrographs of human epidermoïd carcinoma of larynx stained with endothelial cell markers CD31 and CD34.*

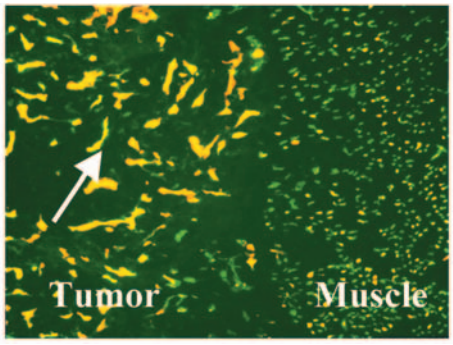

Figure 2. *Biodistribution of cationic liposomes. The distribution of liposomes in tumor and organs was recorded 20 minutes after intravenous administration of liposomes (circulation time of liposomes) and shows the tumor/muscle interface (the tumor model is an orthotopic grafted osteosarcoma in immunocompetent rat). A massive accumulation of rhodamine/FITC double fluorescence was seen in the tumor (arrow), whereas double staining of the surrounding muscles was weak.*

that the artificial anginex gene can be used in gene therapy approaches for antitumor treatment. The involvement of human lectins in disease progression accounts for the interest in designing potent inhibitors. The frequent occurrence of glycoconjugate epitopes plays a salient role as molecular signals of biochemical communication. The knowledge of these cell surface epitopes highlights the importance of galectins as targets for drug design in developing angiogenesis inhibitors (55, 56) (Figure 5).

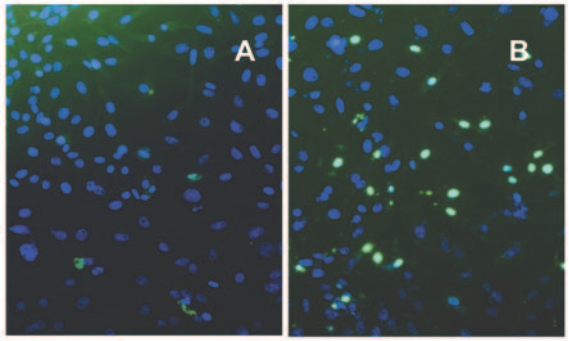

Figure 3. *Detection of apotosis under fluorescence microscopy, after DAPI and T.U.N.E.L. staining of EJG cells (Bovine capillary endothelial cells). EJG cells were transfected using cDNA endostatin lipoplexes at 2 μg/ml. Apoptosis was detected 18 hours after transfection (magnification x200). A: untransfected EJG cells, B: lipoplexe/pSecEndo transfected cells.*

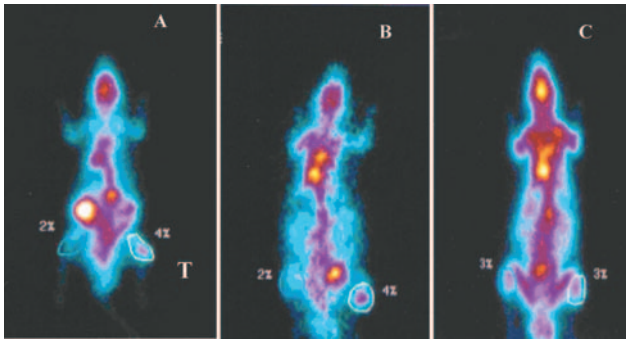

Figure 4. *Inhibition of proliferation in vivo (^{18}FDG tomoscintigraphy). In an orthopic osteosarcoma model grafted in an immunocompetent rat, a tumor was visualized using ^{18}FDG petscan. The animal received intravenous injections of cDNA endostatin lipoplexes (37) three times a week. After one week of such a therapy, the tumor returned to dormancy. This absence of tumor progression is the result of the turning off of the angiogenesis switch, and then to an equilibrium between the multiplication of cancer cells and apoptosis. The percentage indicates ^{18}FDG tumor fixation compared to whole body fixation. (T: Tumor). A: first tumor detection (beginning of the treatment), B: tumor detection after 2 weeks of treatment, C: tumor detection after 4 weeks of treatment.*

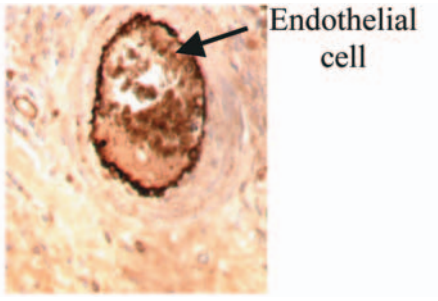

Figure 5. *Ulex europeaus UEA-1. Lectins are glycoproteins which interact with N acetyl-b-D-glucosamine and a-L fucosyl residues of cell membranes. The best system will be to target distinct glycan epitopes and especially those which imply galactose as a key site (Galectin family). Micrographs of vascularization of tumors: staining of endothelial cells by lectin Ulex europeaus UEA-1.*

Metalloproteinases as Drug Targets

Metalloproteinases (MMPs) are a family of enzymes that proteolytically degrade various components of the extracellular matrix (ECM). Some of them have been shown to enhance angiogenesis: detachment of pericytes from vessels undergoing angiogenesis, release of ECM-bound angiogenic factors, exposure of cryptic proangiogenic integrin-binding sites in the ECM, generation of promigratory component fragments, cleavage of endothelial cell–cell adhesion. Others can also contribute negatively to angiogenesis: liberation of angiogenic inhibitors by cleavage of certain collagen chains and plasminogen, modulation of cell receptor signaling. These MMPs, also called matrixins, are a family of more than 20 zinc-containing endopeptidases. All their characteristics and implications have been reviewed by Rundhaug (57). Endogenous or synthetic MMP inhibitors have been used to block pathological angiogenesis (58, 59). The most important problem in the metalloproteinase therapy field is to distinguish MMPs as drug targets or antitargets. It is important not only to consider the factors produced by host stroma but also those produced by the tumor itself. Thus MMP inhibition might be useful in treating patients with early stage cancer, but MMP9 is an antitarget in patients with advanced disease.

Tyrsine Kinase Inhibitors

Analogous problems could arise with tyrosine kinase inhibitors of VEGFRs. These compounds are generally low molecular weight molecules that bind to the ATP-binding catalytic site of the tyrosine kinase domain of VEGFR, leading to the blockade of intracellular signaling. An update of recent developments concerning tyrosine kinase inhibitors in the treatment of solid tumors is delineated in the review of Steeghs *et al.* (60). Inhibition of oncogenic and angiogenic tyrosine kinases is one of the most exciting developments in cancer research. But some of these agents have been troubled with issues of side-effects which can induce heart damage.

RGD-based Strategies for Selective Delivery of Therapeutics to the Tumor Vasculature

The ability of fibronectin to bind cells can be accounted for by the tetrapeptide L-arginyl-glycyl-L-aspartyl-L-serine, a sequence which is part of the cell attachment domain of fibronectin and present in at least five other proteins. This tetrapeptide may constitute a cellular recognition determinant common to several proteins. The arginine-glycine-aspartic acid sequence (RGD) was discovered as a cell attachment site in fibronectin twenty years ago (61). Thereafter, RGD-recognition sites were reported in other ECM proteins (62, 63). The receptors for these ECM proteins were identified and classified in the integrin family. The RGD sequence is the basic model for a variety of RGD-containing peptides that preferentially target αVβ3 integrins and related αV integrins. One of the best studied RGD-peptide ligands for αVβ3 integrin is c(RGD(-NMe-V) known as Cilengitide (64, 65) which has reached phase II clinical trials for the treatment of melanoma, glioblastoma and prostate cancer. αVβ3 Integrin is expressed on angiogenic but not on resting endothelial cells. This restricted expression profile makes it an excellent target. For drug delivery and imaging, αVβ3 integrin-specific RGD-peptides have been introduced into proteins, liposomes, viruses and other delivery vehicles. With 18-F-galacto-RGD, the first RGD-based imaging agent was tested recently in humans (66). Combining this diagnostic tool with designed RGD-targeted therapy could be a strong weapon against a variety of cancers.

Cancer Dormancy

The future of cancer would be to induce dormancy (Figure 4) and/or to eradicate dormant disease. Most human tumors arise in the absence of angiogenic activity and exist in a microscopic dormant state for months to years without neovascularization. Hanahan *et al.* (67) have developed an animal model, the RIP1-tag2 model, in which 4% of tumors are angiogenic whereas the remaining 96% remain microscopic and non angiogenic (68). The passage from non angiogenic lesions to the angiogenic phenotype was termed the angiogenic switch. What are the determinants and mechanism for the 'clock' that governs the angiogenic switch? During the dormancy period, non angiogenic tumors had high proliferation rates and were not quiescent. The angiogenic switch appears to be independent of the tumor cell proliferation/apoptosis ratio. The future would be to develop a panel of assays to detect specific circulating cells as new blood markers of the angiogenic switch. If this approach is feasible, microscopic tumors could possibly be treated before they become symptomatic or their anatomical site is detectable.

References

1 Paget S: The distribution of secondary growths in cancer of the breast. Lancet *1*: 571-573, 1889.

2 Cleaver O and Melton DA: Endothelial signaling during development. Nat Med *9(6)*: 661-668, 2003.

3 Majno G and Joris I: Endothelium 1977: a review. Adv Exp Med Biol *104*: 169-225, 481-526, 1978.

4 Pasqualini R, Arap W and McDonald DM: Probing the structural and molecular diversity of tumor vasculature. Trends Mol Med *8(12)*: 563-571, 2002.

5 Arap W, Kolonin MG, Trepel M, Lahdenranta J, Cardo-Vila M, Giordano RJ, Mintz PJ, Ardelt PU, Yao VJ, Vidal CI, Chen L, Flamm A, Valtanen H, Weaving LM, Hicks ME, Pollock RE, Botz GH, Bucana CD, Koivunen E, Cahill D, Troncoso P, Baggerly KA, Pentz RD, Do KA, Logothetis CJ and Pasqualini R: Steps toward mapping the human vasculature by phage display. Nat Med *8(2)*: 121-127, 2002.

6 Magder S, Neculcea J, Neculcea V and Sladek R: Lipopolysaccharide and TNF-alpha produce very similar changes in gene expression in human endothelial cells. J Vasc Res *43(5)*: 447-461, 2006.

7 Garcia-Vallejo JJ, Van Dijk W, Van Het Hof B, Van Die I, Engelse MA, Van Hinsbergh VW and Gringhuis SI: Activation of human endothelial cells by tumor necrosis factor-alpha results in profound changes in the expression of glycosylation-related genes. J Cell Physiol *206(1)*: 203-210, 2006.

8 Schultheiss C, Blechert B, Gaertner FC, Drecoll E, Mueller J, Weber GF, Drzezga A and Essler M: *In vivo* characterization of endothelial cell activation in a transgenic mouse model of Alzheimer's disease. Angiogenesis *9(2)*: 59-65, 2006.

9 Kerbel RS: Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents. Bioessays *13(1)*: 31-36, 1991.

10 Hida K and Klagsbrun M: A new perspective on tumor endothelial cells: unexpected chromosome and centrosome abnormalities. Cancer Res *65(7)*: 2507-2510, 2005.

11 Bertolini F, Shaked Y, Mancuso P and Kerbel RS: The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer *6(11)*: 835-845, 2006.

12 Folkman J: Tumor angiogenesis. Adv Cancer Res *43*: 175-203, 1985.

13 Rumpold H, Wolf D, Koeck R and Gunsilius E: Endothelial progenitor cells: a source for therapeutic vasculogenesis? J Cell Mol Med *8(4)*: 509-518, 2004.

14 Dignat-George F and Sampol J: Circulating endothelial cells in vascular disorders: new insights into an old concept. Eur J Haematol *65(4)*: 215-220, 2000.

15 Woywodt A, Blann AD, Kirsch T, Erdbruegger U, Banzet N, Haubitz M and Dignat-George F: Isolation and enumeration of circulating endothelial cells by immunomagnetic isolation: proposal of a definition and a consensus protocol. J Thromb Haemost *4(3)*: 671-677, 2006.

16 Goon PK, Boos CJ, Stonelake PS, Blann AD and Lip GY: Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. Thromb Haemost *96(1)*: 45-52, 2006.

17 Beaudry P, Force J, Naumov GN, Wang A, Baker CH, Ryan A, Soker S, Johnson BE, Folkman J and Heymach JV: Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. Clin Cancer Res *11(9)*: 3514-3522, 2005.

18 Monestiroli S, Mancuso P, Burlini A, Pruneri G, Dell'Agnola C, Gobbi A, Martinelli G and Bertolini F: Kinetics and viability of circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. Cancer Res *61(11)*: 4341-4344, 2001.

19 Arbab AS, Pandit SD, Anderson SA, Yocum GT, Bur M, Frenkel V, Khuu HM, Read EJ and Frank JA: Magnetic resonance imaging and confocal microscopy studies of magnetically labeled endothelial progenitor cells trafficking to sites of tumor angiogenesis. Stem Cells *24(3)*: 671-678, 2006.

20 Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R and Keshet E: VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell *124(1)*: 175-189, 2006.

21 Mancuso MR, Davis R, Norberg SM, O'Brien S, Sennino B, Nakahara T, Yao VJ, Inai T, Brooks P, Freimark B, Shalinsky DR, Hu-Lowe DD and McDonald DM: Rapid vascular regrowth in tumors after reversal of VEGF inhibition. J Clin Invest *116(10)*: 2610-2621, 2006.

22 Bergers G, Song S, Meyer-Morse N, Bergsland E and Hanahan D: Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest *111(9)*: 1287-1295, 2003.

23 Harper J and Moses MA: Molecular regulation of tumor angiogenesis: mechanisms and therapeutic implications. Exs *96*: 223-268, 2006.

24 Boehm T, Folkman J, Browder T and O'Reilly MS: Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature *390(6658)*: 404-407, 1997.

25 O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR and Folkman J: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell *88(2)*: 277-285, 1997.

26 Oh SP, Kamagata Y, Muragaki Y, Timmons S, Ooshima A and Olsen BR: Isolation and sequencing of cDNAs for proteins with multiple domains of Gly-Xaa-Yaa repeats identify a distinct family of collagenous proteins. Proc Natl Acad Sci USA *91(10)*: 4229-4233, 1994.

27 Abdollahi A, Hahnfeldt P, Maercker C, Grone HJ, Debus J, Ansorge W, Folkman J, Hlatky L and Huber PE: Endostatin's antiangiogenic signaling network. Mol Cell *13(5)*: 649-663, 2004.

28 Benezra R, Davis RL, Lockshon D, Turner DL and Weintraub H: The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell *61(1)*: 49-59, 1990.

29 Jiang BH, Agani F, Passaniti A and Semenza GL: V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Cancer Res *57(23)*: 5328-5335, 1997.

30 McBride JL and Ruiz JC: Ephrin-A1 is expressed at sites of vascular development in the mouse. Mech Dev *77(2)*: 201-204, 1998.

31 Cheng N and Chen J: Tumor necrosis factor-alpha induction of endothelial ephrin A1 expression is mediated by a p38 MAPK- and SAPK/JNK-dependent but nuclear factor-kappa B-independent mechanism. J Biol Chem *276(17)*: 13771-13777, 2001.

32 Ghosh S and Karin M: Missing pieces in the NF-kappaB puzzle. Cell *109 Suppl*: S81-96, 2002.

33 Ma L, del Soldato P and Wallace JL: Divergent effects of new cyclooxygenase inhibitors on gastric ulcer healing: Shifting the angiogenic balance. Proc Natl Acad Sci USA *99(20)*: 13243-13247, 2002.

34 El-Aneed A: Current strategies in cancer gene therapy. Eur J Pharmacol *498(1-3)*: 1-8, 2004.

35 Schmitt-Sody M, Strieth S, Krasnici S, Sauer B, Schulze B, Teifel M, Michaelis U, Naujoks K and Dellian M: Neovascular targeting therapy: paclitaxel encapsulated in cationic liposomes improves antitumoral efficacy. Clin Cancer Res *9(6)*: 2335-2341, 2003.

36 Thurston G, McLean JW, Rizen M, Baluk P, Haskell A, Murphy TJ, Hanahan D and McDonald DM: Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. J Clin Invest *101(7)*: 1401-1413, 1998.

37 Dutour A, Monteil J, Paraf F, Charissoux JL, Kaletta C, Sauer B, Naujoks K and Rigaud M: Endostatin cDNA/cationic liposome complexes as a promising therapy to prevent lung metastases in osteosarcoma: study in a human-like rat orthotopic tumor. Mol Ther *11(2)*: 311-319, 2005.

38 Krasnici S, Werner A, Eichhorn ME, Schmitt-Sody M, Pahernik SA, Sauer B, Schulze B, Teifel M, Michaelis U, Naujoks K and Dellian M: Effect of the surface charge of liposomes on their uptake by angiogenic tumor vessels. Int J Cancer *105(4)*: 561-567, 2003.

39 Lappalainen K, Miettinen R, Kellokoski J, Jaaskelainen I and Syrjanen S: Intracellular distribution of oligonucleotides delivered by cationic liposomes: light and electron microscopic study. J Histochem Cytochem *45(2)*: 265-274, 1997.

40 McLean JW, Fox EA, Baluk P, Bolton PB, Haskell A, Pearlman R, Thurston G, Umemoto EY and McDonald DM: Organ-specific endothelial cell uptake of cationic liposome-DNA complexes in mice. Am J Physiol *273(1 Pt 2)*: H387-404, 1997.

41 Simionescu M, Simionescu N and Palade GE: Partial chemical characterization of the anionic sites in the basal lamina of fenestrated capillaries. Microvasc Res *28(3)*: 352-267, 1984.

42 Vink H and Duling BR: Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries. Circ Res *79(3)*: 581-589, 1996.

43 Ran S, Downes A and Thorpe PE: Increased exposure of anionic phospholipids on the surface of tumor blood vessels. Cancer Res *62(21)*: 6132-6140, 2002.

44 Liu L, Sanz S, Heggestad AD, Antharam V, Notterpek L and Fletcher BS: Endothelial targeting of the Sleeping Beauty transposon within lung. Mol Ther *10(1)*: 97-105, 2004.

45 Ohlfest JR, Demorest ZL, Motooka Y, Vengco I, Oh S, Chen E, Scappaticci FA, Saplis RJ, Ekker SC, Low WC, Freese AB and Largaespada DA: Combinatorial antiangiogenic gene therapy by nonviral gene transfer using the sleeping beauty transposon causes tumor regression and improves survival in mice bearing intracranial human glioblastoma. Mol Ther *12(5)*: 778-788, 2005.

46 Brandwijk RJ, Dings RP, van der Linden E, Mayo KH, Thijssen VL and Griffioen AW: Anti-angiogenesis and anti-tumor activity of recombinant anginex. Biochem Biophys Res Commun *349(3)*: 1073-1078, 2006.

47 Gupta SK, Hassel T and Singh JP: A potent inhibitor of endothelial cell proliferation is generated by proteolytic cleavage of the chemokine platelet factor 4. Proc Natl Acad Sci USA *92(17)*: 7799-7803, 1995.

48 Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA and Bouck NP: A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA *87(17)*: 6624-6628, 1990.

49 Luster AD, Greenberg SM and Leder P: The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. J Exp Med *182(1)*: 219-231, 1995.

50 O'Reilly MS, Holmgren L, Shinq Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH and Folkman J: Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell *79(2)*: 315-328, 1994.

51 Pike SE, Yao L, Jones KD, Cherney B, Apella E, Sakaguchi K, Nakhasi H, Teruya-Feldstein J, Wirth P, Gupta G and Tosato G: Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. J Exp Med *188(12)*: 2349-2356, 1998.

52 Maeshima Y, Colorado PC, Torre A, Holthaus KA, Grunkemeyer JA, Ericksen MB, Hopfer H, Xiao Y, Stillman IE and Kalluri R: Distinct antitumor properties of a type IV collagen domain derived from basement membrane. J Biol Chem *275(28)*: 21340-21348, 2000.

53 van der Schaft DW, Toebes EA, Haseman JR, Mayo KH and Griffioen AW: Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis *via* induction of apoptosis in vascular endothelial cells. Blood *96(1)*: 176-181, 2000.

54 Thijssen VL, Postel R, Brandwijk RJ, Dings RP, Nesmelova I, Satijn S, Verhofstad N, Nakabeppu Y, Baum LG, Bakkers J, Mayo KH, Poirier F and Griffioen AW: Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. Proc Natl Acad Sci USA *103(43)*: 15975-15980, 2006.

55 Scott K and Weinberg C: Galectin-1: a bifunctional regulator of cellular proliferation. Glycoconj J *19(7-9)*: 467-477, 2004.

56 Andre S, Maljaars CE, Halkes KM, Gabius HJ and Kamerling JP: Discovery of galectin ligands in fully randomized combinatorial one-bead-one-compound (glyco)peptide libraries. Bioorg Med Chem Lett *17(3)*: 793-798, 2007.

57 Rundhaug JE: Matrix metalloproteinases and angiogenesis. J Cell Mol Med *9(2)*: 267-285, 2005.

58 Maekawa R, Maki H, Yoshida H, Hojo K, Tanaka H, Wada T, Uchida N, Takeda Y, Kasai H, Okamoto H, Tsuzuki H, Kambayashi Y, Watanabe F, Kawada K, Toda K, Ohtani M, Sugita K and Yoshioka T: Correlation of antiangiogenic and antitumor efficacy of *N*-biphenyl sulfonyl-phenylalanine hydroxiamic acid (BPHA), an orally-active, selective matrix metalloproteinase inhibitor. Cancer Res *59(6)*: 1231-1235, 1999.

59 Jiang Y, Goldberg ID and Shi YE: Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene *21(14)*: 2245-2252, 2002.

60 Steeghs N, Nortier JW and Gelderblom H: Small molecule tyrosine kinase inhibitors in the treatment of solid tumors: an update of recent developments. Ann Surg Oncol *14(2)*: 942-953, 2007.

61 Pierschbacher MD and Ruoslahti E: Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature *309(5963)*: 30-33, 1984.

62 Lawler J, Weinstein R and Hynes RO: Cell attachment to thrombospondin: the role of ARG-GLY-ASP, calcium, and integrin receptors. J Cell Biol *107(6 Pt 1)*: 2351-2361, 1988.

63 Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin GR and Kleinman HK: Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures *in vitro*. Cell *58(5)*: 933-943, 1989.

64 Dechantsreiter MA, Planker E, Matha B, Lohof E, Holzemann G, Jonczyk A, Goodman SL and Kessler H: *N*-Methylated cyclic RGD peptides as highly active and selective alpha(V)beta(3) integrin antagonists. J Med Chem *42(16)*: 3033-3040, 1999.

65 Duggan ME, Duong LT, Fisher JE, Hamill TG, Hoffman WF, Huff JR, Ihle NC, Leu CT, Nagy RM, Perkins JJ, Rodan SB, Wesolowski G, Whitman DB, Zartman AE, Rodan GA and Hartman GD: Nonpeptide alpha(v)beta(3) antagonists. 1. Transformation of a potent, integrin-selective alpha(IIb)beta(3) antagonist into a potent alpha(v)beta(3) antagonist. J Med Chem *43(20)*: 3736-3745, 2000.

66 Beer AJ, Haubner R, Goebel M, Luderschmidt S, Spilker ME, Wester HJ, Weber WA and Schwaiger M: Biodistribution and pharmacokinetics of the alphavbeta3-selective tracer 18F-galacto-RGD in cancer patients. J Nucl Med *46(8)*: 1333-1341, 2005.

67 Hanahan D and Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell *86(3)*: 353-364, 1996.

68 Folkman J, Watson K, Ingber D and Hanahan D: Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature *339(6219)*: 58-61, 1989.

Received May 4, 2007
Revised July 2, 2007
Accepted July 30, 2007