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Ronit Satchi-Fainaro

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Editorial/Mini Review

Targeting Tumor Vasculature: Reality or a Dream?

RONIT SATCHI-FAINARO*

Department of Surgery, Children's Hospital and Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA

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In recent years, it has become clear that angiogenesis is important not only in physiological processes such as embryonic development, the female reproductive cycle, wound healing, and organ and tissue regeneration but also in pathological processes such as tumor progression and metastasis (Hanahan and Folkman, 1996). The process of angiogenesis, new capillary blood vessel growth from pre-existing vasculature, is now recognized as an important control point in cancer, mainly because the hypothesis that tumors are angiogenesis-dependent has been confirmed by a variety of experiments, but especially by genetic methods. Most tumors do not start out angiogenic, but remain as small, pinpoint dormant tumors for years or a life-time. They cannot grow until they can recruit new blood vessels, i.e. switch to the angiogenic phenotype. As a result, the microvascular endothelial cell, recruited by a tumor, has become an important second target in cancer therapy, a target that unlike the tumor cells themselves, is genetically stable (Folkman, 2001a,b). Angiogenesis is a complex multicomponent process involving many growth factors and their receptors, cytokines, proteases and adhesion molecules (Carmeliet and Jain, 2000); thus multiple targets for therapeutic intervention and targeting opportunities for anti-angiogenic therapy for cancer exist.

It has become feasible to propose that treating both the cancer cell and the endothelial cell in a tumor may be more effective than treating the cancer cell alone. Table I summarizes the advantages of targeting the vessels of the tumor instead of, or in addition to treating the tumor itself. As the target is the genetically normal endothelial cell, resistance to treatment due to somatic mutations in the target cell does not occur.

Angiogenesis inhibitors are emerging as a new class of drugs. In the U.S. there are currently 24 angiogenesis inhibitors in various clinical trials for late stage cancer, 8 are in clinical trial Phase III (Table II). Members of this family of drugs differ by their targets and vary from low MW molecules to polypeptides and antibodies. Some are cytostatic (Endostatin, Angiostatin and TNP-470, VEGF antagonists or VEGFR inhibitors) and some, like the vascular targeting agents (VTA), are cytotoxic. VTAs allow rapid destruction of existing blood vessels in tumors containing activated endothelial cells (EC). They consist of antitubulin agents such as combretastatin (Hill *et al.*, 2002) analogs (CA4P, CA1P, AVE 8062A, AVE 063), and colchicine analogs (i.e. ZD6126). Other drugs such as flavone acetic acid (FAA) analog and dimethyl-xanthenone-4-acetic acid (DMXAA) induce TNF-α and serotonin and inhibit blood flow.

In spite of the difference between various angiogenesis inhibitors the common ground is that all can benefit from specific targeting. A proper delivery system would enable optimization of their pharmacokinetic profile. The development of biocompatible, controlled release systems for macromolecules has provided the opportunity for researchers and clinicians to target and deliver biologically active entities. Systems releasing such biologically important polypeptides, as growth factors as well as a number of important inhibitory factors or low MW drugs, are beginning to be utilized.

The first *in vivo* screening of a peptide library binding to the human vasculature opens new possibilities for inhibiting angiogenesis and tumor growth. The hypothesis that tumor growth is angiogenesis-dependent (Folkman, 1971) and its subsequent confirmation by genetic methods (Folkman, 2001a,b; Lyden *et al.*, 2001) provided strong incentive for scientists to try to target peptides specifically to the vascular bed of tumors. Pasqualini and Ruoslahti achieved the first step towards this goal in 1997 when they reported a novel *in vivo* phage display that distinguished between active proliferating microvascular EC in a tumor and quiescent nonproliferating EC elsewhere in the vasculature (Pasqualini *et al.*, 1997). This methodology permitted

*E-mail: ronit.satchi-fainaro@tch.harvard.edu

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TABLE I Targeting tumor cells versus endothelial cells

	Conventional chemotherapy	Antiangiogenic therapy	VTA: Vascular targeting agents
Genome of target	Unstable Resistance	Stable (diploid and nontransformed) No resistance if direct targets used	Stable
Effect	Cytotoxic Therapy has to reach all tumor cells	Cytostatic Inhibition of new blood vessel formation 1 EC supplies O_2 and nutrients for 50–100 TC Applicable to all solid tumors Indirect killing of TC	Cytotoxic Rapid destruction of existing tumor blood vessels propagating from the center of the tumor out
Accessibility	"Hidden" in the tumor tissue	EC are in direct contact with the circulation	Direct contact
Targeting therapy	Mainly rapid dividing cells	Tumor associated-EC possess unique phenotypic characteristics	Activated endothelial cells
Side effects/Toxicity	Yes	Few or no side effects	Yes
Duration of treatment	Short	Long	Long
Expected regression	Fast	Slow	Long (viable rim remains around the tumor)
Goal	Eradication of tumor cells	Stable disease or growth delay Regression to avascular state or slow tumor regression	Acute tumor regression

angiogenesis-related targeting of tumor blood vessels. Two years later they demonstrated that a small peptide could be specifically targeted to tumor vasculature. The peptide inhibited two metalloproteinases, resulting in inhibition of angiogenesis, tumor growth, and invasion (Koivunen *et al.*, 1999; Folkman, 1999). Earlier this year, Arap and Pasqualini and their colleagues reported *in vivo* screening of a peptide library in a patient for the first time (Arap *et al.*, 2002). Circulating peptides containing 47,160 motifs localized to the vasculature of different organs in a nonrandom distribution. Furthermore, certain circulating peptides bound specifically to known receptors on the vascular endothelium of the organ from which the peptide was recovered, but not to endothelium from other organs. In this issue, "Use of a phage display library to identify oligopeptides binding to the luminal surface of polarized endothelium by *ex vivo* perfusion of human umbilical veins," by Maruta *et al.* extends the approach of using phage display to find targeting peptides to human EC. The authors utilize umbilical veins *ex vivo* to bypass the difficulties of using living human volunteers.

TABLE II Angiogenesis inhibitors in clinical trials for cancer

Drug	Sponsor	Mechanism
Phase I		
SU6668	Sugen	Blocks VEGF, FGF and EGF receptor signaling
Angiostatin	EntreMed	Inhibits endothelial proliferation
Vitaxin	MedImmune	Binds to $\alpha v\beta 3$
Combretastatin	Oxigene	Apoptosis in proliferating endothelium
MC-1C11	ImClone	Monoclonal antibody to KDR receptor
ZD6474	AstraZeneca	Inhibits VEGF receptor-associated tyrosine kinase
Phase II		
PTK787	Novartis	Inhibits VEGF receptor
CAI	NCI	Inhibits calcium influx
COL-3	Collagenex, NCI	Synthetic MMp inhibitor, tetracycline derivative
Endostatin	EntreMed	Inhibits endothelial proliferation
TNP-470	TAP Pharm.	Fumagilin analog: inhibits endothelial proliferation
2-methoxy-estradiol (Panzem)	EntreMed	Inhibits microtubule function
Interleukin-12	Genetics Inst.	Induces IFN-γ and IP-10
EMD 121974	Merck KcgaA	Blocks an endothelial integrin
Prinomastat	Agouron	Synthetic MMp inhibitor
Phase III		
Erbitux	Imclone	Blocks EGF receptor (VEGF, bFGF, IL-8)
Marimastat	British Biotech	Synthetic MMP inhibitor
Neovastat	Aeterna	Natural MMP inhibitor
Interferon-α	Commercially available	Inhibition of bFGF production
IM862	Cytran	Endothelial inhibitor
Thalidomide	Ceigene	Unknown
Anti-VEGF Ab	Genentech	Monoclonal Ab to VEGF
Squalamine	Magainin	Inhibits Na/H exchanger

In part from the National Institute Database (Updates February, 2002).

Some of the many potential clinical applications of this elegant technology were reviewed previously (Folkman, 1999). In their latest report (Arap *et al.*, 2002), the authors point out that it may ultimately become possible to determine molecular profiles of blood vessels in different organs and in specific conditions. If such a molecular map of the human vasculature is eventually achieved and the results are taken together with the recently identified genes which encode endothelial markers overexpressed during tumor angiogenesis (St. Croix *et al.*, 2000), a novel pharmacologic approach to angiogenesis-dependent diseases can be envisioned. Currently, antiangiogenic proteins are delivered into the circulation and achieve their high therapeutic index by selective inhibition of proliferating and migrating EC in an angiogenic focus, without having a similar effect on quiescent endothelium in the remaining vasculature. If these direct angiogenesis inhibitors, which include thrombospondin, angiostatin, endostatin and tumstatin (Maeshima *et al.*, 2002) could be targeted to the angiogenic focus in a tumor, potency could be potentially enhanced.

In antiangiogenic therapy of cancer, such increased potency may be useful in the case of tumor cells deficient in p53. It has been suggested that because these tumor cells have a diminished rate of apoptosis under hypoxic conditions, that they might be less responsive to antiangiogenic therapy (Yu *et al.*, 2002). For those angiogenesis inhibitors which have shown virtually no toxicity or side effects in animals or humans (e.g. angiostatin, endostatin), increasing the dose or combining two or more inhibitors should obviate the problem of p53 $-/-$ tumor cells. Viable tumor cells form microcylinders around each capillary blood vessel that has been recruited to the tumor (Folkman, 2001a,b). With increasing distance from the nearest blood vessel, tumor cells live under increasing hypoxia. However, beyond a given oxygen diffusion limit (which may be in the range of 110 μm for tumor cells which are p53 $+/+$ but greater, i.e. in the range of 150 μm, for p53 null tumor cells), anoxic conditions cause tumor cells to die. Because one endothelial cell controls the survival of approximately 50–100 tumor cells, a direct angiogenesis inhibitor of sufficient potency and dose to cause endothelial apoptosis would result in tumor cell death in the vessel neighborhood (Browder *et al.*, 2000). However, for those angiogenesis inhibitors where dose cannot be increased because of side-effects [e.g. TNP-470, a Fumagillin analog (Ingber *et al.*, 1990)] or for those whose efficacy is not improved by dose escalation, targeting to the microvascular endothelium in a tumor bed may greatly increase the usefulness of the inhibitor. We have been developing a drug delivery system for angiogenesis inhibitors using water-soluble polymers as carriers for anticancer therapy (Satchi-Fainaro *et al.*, 2002), to specifically target drugs to tumor EC and to allow their accumulation in the tumor bed by the enhanced permeability and retention (EPR) effect (Maeda *et al.*, 2000).

For polypeptides, the difficulties of large-scale protein production, long-term storage of bioactive protein, and cumbersome daily administration may be overcome through transfer of the genes encoding the antiangiogenic proteins. Experience with antiangiogenic proteins delivered by gene transfer is still in its infancy but rigorous research is exploring this option. Several groups (Sauter *et al.*, 2000; Gyorffy *et al.*, 2001; Kuo *et al.*, 2001) have generated recombinant adenoviruses encoding angiostatin, endostatin, and the ligand-binding ectodomain of the vascular endothelial growth factor receptors Flk1, Flt1, and neuropilin, and used them to systemically deliver the gene products in several different murine tumor models. Viruses encoding soluble forms of Flk1 or Flt1 resulted in $\sim$80% tumor inhibition. In contrast, adenoviruses encoding angiostatin, endostatin or neuropilin were significantly less effective compared to their protein counterparts and to the VEGFR adenoviruses. In these studies the antiangiogenic adenoviruses were given by i.v. tail-vein injections and in other studies intratumoral administration allowed the viruses to get to all body tissues. In order to avoid the lack of specificity, elegant methods to activate promoter of genes in specific cellular targets were suggested. Conditionally replicating adenoviruses (CRADs, Savontaus *et al.*, 2002) were constructed to target proliferating EC by replacing the promoters of immediate early genes in the adenoviral genome with promoters that are induced only in activated EC. Tissue specific expression of a protein (GFP) was achieved using an adeno-based vector containing the murine preproendothelin-1 (PPE-1) promoter. Genes activated by the PPE-1 promoter were highly expressed in the neovasculature of primary tumors and metastasis (Bloom *et al.*, 2001). This system has the potential to target the expression of the protein specifically to the endothelium, however the vectors will still distribute systemically to all tissues.

The specificity of vascular-directed gene therapy can also be improved through the use of macromolecular polymeric carriers. Delivery systems redirecting biodistribution and controlling gene/drug rate of release are an attractive option with potentially great applications in cancer in general and angiogenesis in particular. Water-soluble polymers and polymerized-liposomes have raised considerable interest as drug carriers in cancer chemotherapy (Gregoriadis, 1995; Brocchini and Duncan, 1999; Duncan *et al.*, 2001). Their versatility, biocompatibility, and lack of immunogenicity confer them intrinsic advantages as pharmaceutical devices for drug delivery. Significant technological advances in the last decade enabling the production of large-scale batches with rigorous specification and high standards of reproducibility and shelf stability have made polymers and liposomes an acceptable pharmaceutical entity. The rational for the use of these carriers in cancer drug delivery is based on the following pharmacological principles (Gabizon, 2001): (1) Slow drug release: Drug bioavailability depends on drug release from the carrier. Entrapment of drug in liposomes or by a covalent bond

between the polymeric carrier and the drug will slow drug release and reduce renal clearance to a variable extent. Slow release may range from a mere blunting of the peak plasma levels of free drug, to a sustained release of drug mimicking continuous infusion. These pharmacokinetic changes may have important pharmacodynamic consequences with regard to toxicity and efficacy of the carrier-delivered agents. (2) Site avoidance of specific tissues: The biodistribution pattern of polymers and liposomes may lead to a relative reduction of drug concentration in tissues specifically sensitive to the drug. This may have implications with regard to the therapeutic window of various cytotoxic drugs. (3) Accumulation in tumors: Prolongation of the circulation time results in significant accumulation in tissues with increased vascular permeability, such as tumors (Jain, 1998), especially in those areas with active angiogenesis.

Tumor localization of long-circulating liposomes, such as pegylated liposomes [sometimes referred to as Stealth or sterically stabilized (Papahadjopoulos *et al.*, 1991)], is a passive targeting effect which may enable substantial accumulation of liposome-encapsulated drug in the interstitial fluid at the tumor site (Symon *et al.*, 1999)). This accumulation is followed by gradual release of drug *in situ* and its subsequent diffusion to the intracellular tumor compartment. A reasonable expectation is that an increased exposure of EC in the tumor vicinity to angiogenesis inhibitors with regard to concentration and time parameters will enhance the therapeutic effect.

A further step in liposomal drug delivery to tumor angiogenesis is to devise an active targeting strategy by coupling ligands to the liposome surface that will recognize specific receptors of the target endothelial cell. Although active targeting may further diminish unwanted interactions with normal tissues and cells, its main advantage over above mentioned passive targeting is the ability to deliver a large drug payload directly into the EC.

This rationale is the basis for the development of polymerized liposomal cationic nanoparticle (NP) linked-integrin αvβ3-targeting ligand conjugated to a mutant Raf gene, ATP^{μ}-Raf, which blocks endothelial signaling and angiogenesis in response to multiple growth factors (Hood *et al.*, 2002). In this formulation, polymer coating protects the liposomes from opsonization and recognition by the reticulo-endothelial system resulting in prolonged circulation time and enhanced accumulation in tumors (Gabizon and Martin, 1997). During vascular remodeling and angiogenesis, EC show increased expression of several cell surface molecules that potentiate cell invasion and proliferation (Yancopoulos *et al.*, 1998; Eliceiri and Cheresh, 2001). One such molecule is αvβ3, which is preferentially expressed in angiogenic endothelium *in vivo*. In addition to its role in cell matrix recognition, αvβ3 may be of particular use in gene delivery strategies because this receptor facilitates gene transfer. This system delivers the pro-apoptotic form Raf, ATP^{μ}-Raf. Systemic injection of the NP into mice resulted in apoptosis of the tumor-associated endothelium, ultimately leading to tumor cell apoptosis and sustained regression of established primary and metastatic tumors.

In general, an ideal delivery system is one that can enable the conjugation of any targeting moiety and the active entity in a simple chemical platform fashion.

In the near future the various fields of drug delivery will be combined to target pathological angiogenesis.

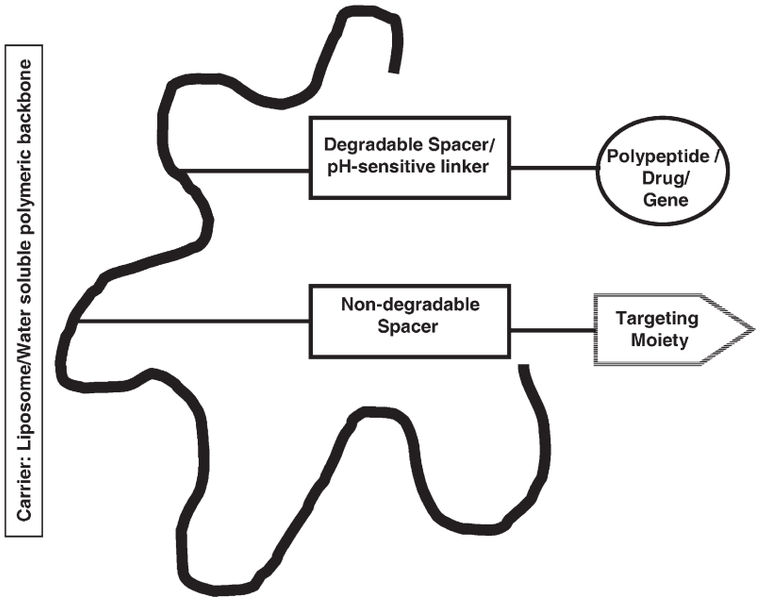

FIGURE 1 Schematic diagram of a soluble polymeric drug carrier (modified from Ringsdorf, 1975).

Polymers, polymerized liposomes, encapsulated nano/microparticles, dendrimers and microspheres will have two arms (Fig. 1). One will be a specific targeting moiety to proliferating EC; such as an antibody (to VEGF, endosialin, EC caveolae proteins, endoglin, VCAM-1, PMSA; ED-B Domain in FN), a peptide ($\alpha v\beta 3$-ligand like RGD) or any ligand to the upregulated molecules present on the surface of proliferating EC. The other arm will be the active entity which may be an angiogenesis inhibitor protein (endostatin, angiostatin, tumstatin), a low MW drug (TNP-470 or any of the VTAs), a viral vector/gene expressing an angiogenesis inhibiting-protein or toxins (ricin, gelonin), coagulation factors (tTF, phosphatidyl serine), IL-12, vasoactive molecules: TNF-α, cytotoxic drugs (CPA). Paul Ehrlich's magic bullet dream (1906) may not have materialized yet, but we are definitely heading to that direction in the next 5–10 years!

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