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Review

Tumor Vasculature Directed Drug Targeting: Applying New Technologies and Knowledge to the Development of Clinically Relevant Therapies[1]

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Recognition of the dependence of solid tumor growth on the formation of new blood vessels has ignited an enormous research effort aimed at the development of new therapeutic strategies for cancer. Besides direct application of drugs inhibiting endothelial cell function during angiogenesis, tumor vasculature directed drug-targeting strategies have been investigated for this purpose. In animal models of disease, proof of principle regarding the potential of selective interference with tumor blood flow as a powerful tumor therapy has been generated to its full extent. The challenge for the coming years will be to develop these strategies into clinically applicable ones. New insights into the molecular mechanisms prevailing in the endothelium during angiogenesis and into the mechanism(s) of action of drugs with anti-angiogenic activities, as well as new techniques to identify useful tumor endothelium specific target epitopes have in recent years been exploited to meet this challenge. This review summarizes vasculature directed therapeutic strategies proven to be successful in pre-clinical models and new (drug targeting) technologies enabling the development of more effective therapeutics for the treatment of cancer.

KEY WORDS: angiogenesis; anti-angiogenic drugs; drug targeting; vascular targeting; cancer.

INTRODUCTION

Neovascularization is a hallmark of tumor growth. The newly formed blood vessels facilitate nutrient and oxygen supply for tumor cell proliferation. Endothelial cells are central in this neovascularization process. In addition, they are an important source of cytokines and chemokines, potentiating cellular activation at the site of tumor growth. Therefore, the endothelium is considered an important target for therapeutic intervention.

The hypothesis in the early 1970s that the tumor blood supply represents a vulnerable element that could be therapeutically exploited was in recent years experimentally corroborated in different animal studies (1–3). The subsequent identification of molecules specifically expressed by tumor endothelial cells and of molecular processes taking place during neovascularization has opened up an array of possibilities to attack tumors at their most fragile fundament.

This mini-review addresses new advances in the development of anti-angiogenic drug targeting therapies explored in recent years. Powerful effector molecules and techniques to identify new target epitopes for vasculature targeting are discussed, with emphasis on tumor vasculature directed therapies. Since vascular remodeling is also a crucial event in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, some of the targeted anti-angiogenic approaches have been explored in these diseases as well. Due to space limitation, these are however, not discussed here. For in-depth reviews on targeted anti-angiogenic strategies, the reader is referred to e.g., (4–8).

General Considerations Concerning Vasculature Directed Therapies

The angiogenic process is a complex interplay between endothelial cells, smooth muscle cells, pericytes, fibroblasts and various leukocyte subsets. The vascular network in growing tumors is structurally and functionally abnormal, with tortuous, dilated blood vessels. Within the network the endothelium is highly heterogeneous with respect to activation status as exemplified by differential expression of vascular markers. The majority of anti-angiogenic therapeutics developed so far affect endothelial cell behavior by interference with intracellular signal transduction cascades (e.g., Vascular Endothelial Growth Factor (VEGF)-Receptor and basic Fibroblast Growth Factor (bFGF or FGF-2)-Receptor inhibitors), cellu-

[1] In remembrance of my colleague and friend, Dr. Pauline van Wachem, who died of cancer December 2 2001. Pauline's unselfish way of helping other people and her continuous effort to make her research of use for clinical application, is an example to all of us.

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ABBREVIATIONS: Ab, antibody; bFGF, basic fibroblastgrowth factor; B-FN, ED-B domain of Fibronectin; BsAb, bispecific antibody; MHC, major histocompatibility complex; MMP, metalloproteinase; scFv, single-chain Fv fragment; TEM, tumor endothelial marker; tTF, truncated Tissue Factor; VCAM-1, vascular cell adhesion molecule-1; VEGF(R), vascular endothelial growth factor (receptor).

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lar movement into the interstitium (inhibitors of matrix metalloproteinases), or interaction with the extracellular matrix for proper adhesion and cell survival (thrombospondin -1 and αvβ3 integrin inhibitors). Some anti-angiogenic therapies create a situation of "vessel normalization" by eliminating excess of endothelial cell mass within the growing neovasculature. As a result, the blood vessels become more conductive to the delivery of nutrients and therapeutics (9). This phenomenon can not only facilitate tumor growth, but can also make the tumor more responsive to subsequent radiation or chemotherapy (10).

Of importance in the development and evaluation of anti-angiogenic therapies are the spatiotemporal differences in neovascularization stages within a growing tumor. In animal tumors the induction of tumor growth is often rapid, leading to a situation of synchronization of neovessel formation. The clinical situation is, however, notably different. Parts of the tumor can be dormant with respect to cellular proliferation and associated angiogenesis, while other parts are actively proliferating with concomitant intense neovascularization. Furthermore, the stages of neovascularization seen in these tumors are thought to be more heterogeneous than those observed in the animal models. To ultimately affect all vessels in a human tumor, anti-angiogenic therapy should be clinically administered for a prolonged period of time. As a result, normal vasculature will also be extensively exposed to the drugs, possibly leading to loss of function and/or toxicity not observed in pre-clinical animal studies. Bridging this gap between pre-clinical testing and clinical trials requires an approach in which desired and undesired effects should be meticulously analyzed.

Effector Mechanisms to Interfere with Endothelial Function

In general, tumor vasculature directed therapies either aim at the specific interference with endothelial cell behavior during the angiogenic process, or at the direct induction of tumor endothelial cell death or tumor blood flow inhibition.

A significant part of research on angiogenesis inhibition has been dedicated to the VEGF signal transduction cascade, as VEGF has been most extensively studied for its central role in tumor growth associated angiogenesis. Furthermore, inhibitors of bFGF, metalloproteinases, antagonists of αvβ3 integrins, and small molecular weight inhibitors of enzymes associated with endothelial cell function (e.g., Cycloxygenase (COX)-2 and mitogen activated protein (MAP) kinase inhibitors), are under development or have entered clinical testing (for a concise review on pharmacological strategies for anti-angiogenic therapy, see (11)). Of the peptides and proteins with vasculostatic properties, especially endostatin and angiostatin have attracted attention by their potential to induce tumor dormancy in animal models upon repeated dosing (12,13). Likely, their mechanism of action is a combination of effects, including endothelial cell apoptosis induction and inhibition of tumor and endothelial cell migratory capacity. Some of the angiogenesis inhibitors can be administered to patients without concomitant (dose limiting) toxicities. Others, however, have been withdrawn from clinical studies. Especially the latter category of drugs represents candidate drugs for further application in a drug targeting formulation.

Of interest is the recent observation that the immunosuppressive drug rapamycin strongly inhibits VEGF driven angiogenesis in animal tumor models at relatively low doses. Selective delivery of this drug into the tumor neovascular endothelium may dissociate the desired anti-angiogenic effects from the immunosuppressive effects that unfavorably affect tumor growth (14).

In the case of gene therapy based strategies, genes encoding dominant negative forms of (endogenous) inhibitors of enzymes involved in pro-angiogenic signaling cascades can affect endothelial cell behavior. Furthermore, genes encoding anti-angiogenic proteins such as platelet factor 4, thrombospondin, and Tie-2 receptor have been applied for anti-angiogenic effects (11). Especially for these latter genes, specific delivery of the genes into the tumor endothelium *per se* is not a prerequisite for obtaining anti-tumor effects. Moreover, other cells in the body may be much better producers of the anti-angiogenic proteins encoded by the plasmid.

Direct endothelial cell killing can be induced using a variety of toxic drugs. Bacterial toxins have been studied in great detail in the last thirty years as part of tumor cell directed immunotoxin therapy. Their applicability, limited to hematological tumors due to poor solid tumor penetration characteristics, has been hampered by the occurrence of so called "vascular leak syndrome". This toxicity is likely based on endothelial cell damage exhibited by a three amino acid motif present in the toxins (15). Deletion or mutation of this motif while maintaining toxin activity may open up new opportunities for clinical application of toxin based therapeutics aimed at tumor vasculature. Besides using toxins as effector drugs, conventional chemotherapeutics like doxorubicin can cause endothelial cell death, although sensitivity of endothelial cells to these drugs can significantly differ from that of tumor cells.

Recently, molecular sized generators of alpha-emitting isotope cascades were developed as tumor cell selective killing devices by attachment of Actinium-225 to tumor antigen specific antibodies (16). Actinium-225 has a 10-day half-life and decays via alpha emission through three atoms, each of which emits high linear energy transfer helium nuclei (alpha particles) that locally exert strong cytotoxicity. Considering the better accessibility of tumor endothelial cells, this therapeutic concept may prove even more effective for solid tumor therapy when delivered (in)to tumor endothelium.

Whereas many inhibitors of intracellular signal transduction pathways indirectly induce apoptosis of the endothelial cells, increased knowledge on the function and activation mechanisms of caspase enzymes involved in apoptosis signaling may be applied for the development of new therapeutic entities with anti-angiogenic properties (17). In theory, specific inhibitors of e.g., VEGF and bFGF signaling have the advantage of interacting with pro-angiogenic endothelium only. For direct inducers of caspase activity, however, it is pivotal to selectively deliver them into the target endothelium, to circumvent toxicity elsewhere in the body.

Massive tumor load that cannot be surgically removed may benefit most from tumor mass debulking strategies based on instant blockade of tumor blood flow. The most effective approach reported to date to completely block tumor blood flow was the induction of blood coagulation by selective delivery of a coagulation factor at the tumor endothelial cell membrane. Cross linking of tissue factor to the greater part of tumor blood vessels led to massive tumor mass reduction (Fig. 1) and even cure of tumor bearing mice (2).

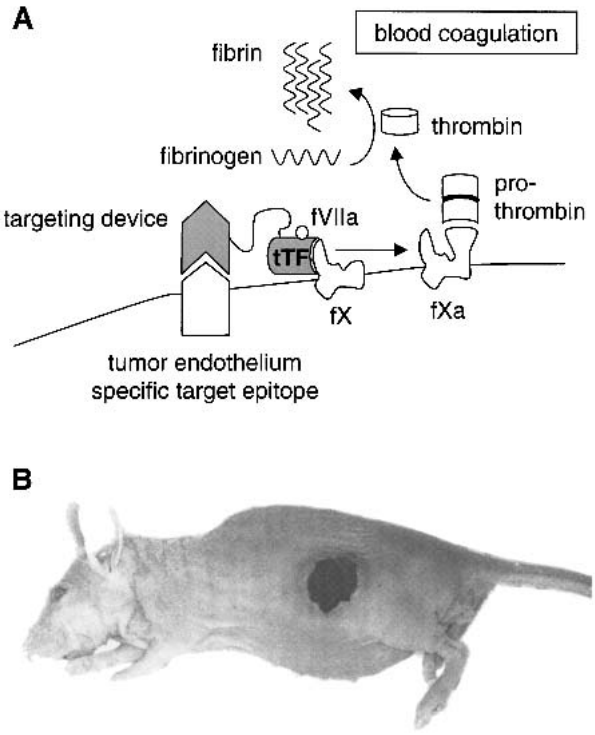

Fig. 1. A. Schematic representation of blood coagulation induction by selective delivery of truncated Tissue Factor (tTF) at the membrane of tumor vascular endothelial cells, leading to massive tumor cell death and tumor mass reduction in established tumors. As targets on the tumor endothelial cells, MHC class II, VCAM-1 and ED-B domain of Fibronectin have been explored in different studies. Following bridging of tTF to membrane associated factor X (fX) in the presence of factor VIIa (fVIIa), the coagulation cascade is initiated via activated fXa. **B.** Within hours after intravenous administration of a mixture of MHC class II directed bispecific antibody and truncated Tissue Factor into a mouse bearing an established C1300$\mu\gamma$ tumor, the tumor became haemorrhagic. Six days after treatment (photograph), more than 95%of the tumor mass was eradicated (unpublished).

In general, *in vitro* anti-angiogenic effects of (targeted) therapeutics are being studied in primary endothelial cells from different species and endothelial cell lines (18,19). Depending on their origin and the culturing protocol, the endothelium behaves differently in response to pharmacological stimuli and inhibitors thereof. Furthermore, the behavior of endothelium under conditions of tumor growth is a trait difficult to mimic *in vitro*. *In vivo* studies are therefore crucial to assess the added value of the therapeutic approach under development. The observation that shear stress significantly affects gene expression profiles of endothelial cells (20,21) is an additional reason to evaluate the effectiveness of (targeted) anti-angiogenic strategies *in vivo* in an early phase of development.

New Technologies towards Identification of Target Molecules for Vascular Targeting

Knowledge on the cellular processes taking place during angiogenesis has been the basis for the identification of molecules expressed by endothelial cells that can be used for drug targeting purposes. With the advent of new molecular biologic and biochemical techniques, the rate of target identification has significantly increased. Technologies with proven power to identify new targets include antibody fragment or peptide phage display libraries, the isolation of endothelial plasma membranes using colloidal silica followed by protein mapping by monoclonal antibody generation, analysis of differential gene expression using e.g., serial analysis of gene expression (SAGE) or cDNA microarray technology, and systemic evolution of ligands by exponential enrichment (SELEX).

Phage display technology is based on the construction of a large collection of bacteriophages, so called libraries, containing up to hundred of millions of related proteins or peptides displayed on the surface of the filamentous phages. By this means, the genetic code for the proteins or peptides is captured in the DNA vectors in the phages. Both *in vitro* and *in vivo* selection strategies have been explored for target identification (Fig. 2). *In vitro* selection on endothelial cells is often based on culturing the cells under disease specific conditions, followed by selection of phages displaying e.g., single chain Fv (ScFv) fragments or peptides that selectively bind to the cells (22,23). Improvement of the selection procedure by Brasil (biopanning and rapid analysis of selective interactive ligands) may overcome major drawbacks (i.e., recovery of non-specific clones, labor intensity and inefficiency of selection rounds) of *in vitro* phage selection (24). Since mimicking of angiogenic endothelium *in vitro* is rather cumbersome if not impossible, *in vivo* selection is considered a more appropriate approach to identify endothelium binding ligands (25). For drug targeting strategies aiming to deliver drugs intracellularly in the vascular cells, *in vivo* phage display may potentially be combined with the identification of subsets of phages that display internalizing capacity (26).

In vivo phage selection has mainly been performed in animal models of disease. Considering the divergence in gene expression patterns between mouse and man, knowledge on the human vasculature is pivotal to rationally extrapolate data from animal model studies towards the human situation. The first step towards mapping the human vasculature by phage display was reported by Arap *et al.* (27). For this purpose, a 2×10^8 diversity containing phage random peptide library displaying a constrained cyclic loop within the pIII capsid protein, was i.v. injected into a brain-dead patient. Fifteen minutes after injection, tissue biopsies were obtained and phages retrieved. Initial high throughput analysis of the peptide motifs revealed several similarities to ligands for differentially expressed cell-surface proteins, among others.

Using a *biochemical approach,* Schnitzer *et al.* (28) isolated luminal plasma membranes from endothelial cells of both normal and diseased tissues by *in situ* lamination of the vasculature with cationic silica and polyanion perfusion. The isolated membrane fraction was enriched in caveolae, which can mediate (receptor mediated) endocytosis as well as transcytosis of blood borne molecules (29). One of the monoclonal antibodies raised against the isolated endothelial plasma membrane of rat lungs specifically targeted endothelial caveolae in the lungs upon i.v. injection. Within minutes, the antibody was transported into the perivascular space. This technology formed the basis for the elucidation of caveolae function in cellular processes, yet no studies have been reported on the use of such antibody molecules in disease related drug targeting strategies.

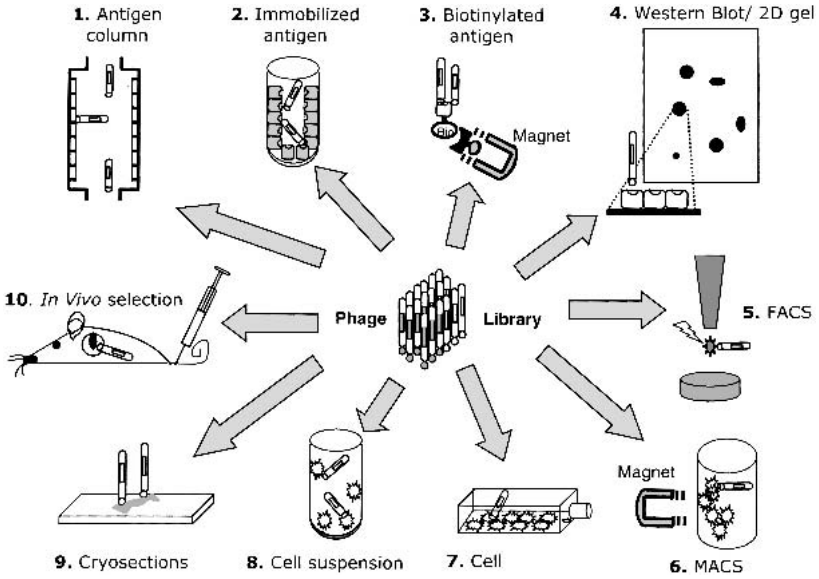

Fig. 2. Selection strategies for obtaining specific phage ligands for e.g., drug targeting applications, can be performed *in vitro* (**1**: panning on an antigen column; **2**: panning on an antigen absorbed onto a solid support; **3**: to avoid conformational changes of antigen during coating, selection on biotinylated antigen can be preferred; **4**: selection on proteins isolated by electrophoresis; **5**: selection and subtraction by flow cytometry or **6**: by magnetic beads; **7**: selection on cell monolayers, or **8**: on cells in suspension; **9**: selection on tissues) and in vivo (**10**: in vivo selection in tumor bearing animals) (reprinted with permission from (22)).

Serial analysis of gene expression (SAGE) associates individual mRNA transcripts with 14-base pair tags derived from a specific position near their 3′- termini. The abundance of each tag provides a quantitative measure of the transcript level present in the mRNA population studied and as such, allows to study differences in gene expression levels by different cell types. Applying SAGE to endothelium isolated from normal human colon and tumor colon tissue, St. Croix and colleagues identified a number of genes specifically expressed by the tumor vasculature (30). Subsequent investigation of a selection of these tumor endothelial markers (TEM) with regard to protein structure identified TEM1, TEM5, and TEM8 as potential targets for the development of anti-angiogenic therapies. Not only do these proteins contain transmembrane domains of importance for accessibility by systemically applied (targeted) therapeutics, they furthermore are conserved between mouse and men with regard to expression in tumor vessels. This latter characteristic is of importance for pre-clinical development and testing of therapeutics aimed at these, currently unidentified, proteins (31).

Besides the use of SAGE and GeneCalling™ to identify either entirely new genes or known genes not previously known to be involved in the cellular processes studied, techniques such as differential display and large-scale arrays of cDNA sequences or oligonucleotides are now widely employed for this purpose. The reader is referred to a concise review on gene profiling techniques applied in angiogenesis research for more indepth details on the methodologies, useful websites, and reported experimental data (32). Furthermore, the rapid development of tools for in silico analysis of gene expression, among others supported by the online availability of data via the Cancer Genome Anatomy Project (CGAP) (33), offers additional opportunities to identify new genes of interest for vascular targeting.

Systematic evolution of ligands by exponential enrichment (SELEX) is based on *in vitro* selection and amplification to identify novel nucleic acid sequences that bind to target molecules of interest. The resulting aptamers are theoretically suitable for application in diagnostics and therapeutics. A SELEX library consists of random sequences obtained from combinatorial chemical synthesis of DNA. The ~10^{14}–10^{15} sequences are in consecutive screening rounds selected for their binding to specific target molecules. Upon purification, the bound sequences are amplified by PCR and subjected to additional selection rounds (34). Aptamers typically have high affinity for their target proteins, ranging from 0.05–10 nM. Therapeutically, a liposome-aptamer approach to neutralize VEGF in chorioallantoic membrane angiogenesis has been reported (35).

Using these techniques, over 20 molecular targets and targeting ligands have been identified so far. Some of these targets or ligands have now been explored for anti-angiogenic drug targeting strategies, a selection of which will be discussed later.

In Vivo Anti-Tumor Effects of (Vasculature) Targeted Therapeutics

Every animal model of disease has particular characteristics that may, in theory, be of influence on the effectiveness of the therapeutic strategy under study. For example, permeability of tumor blood vessels leading to target independent enhanced retention of the drug targeting construct at the tumor site, differs significantly between models used (36). Furthermore, different tumor models can exhibit different de-

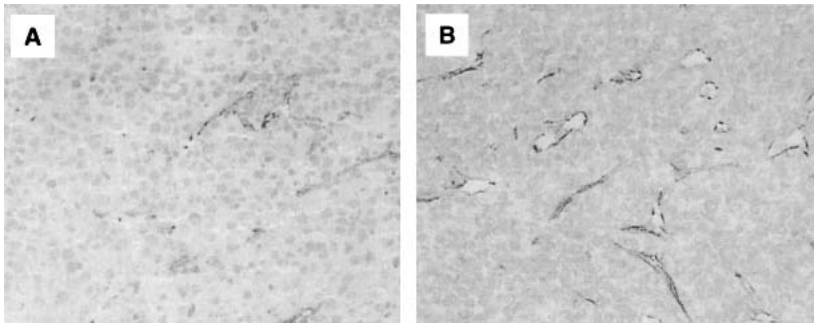

Fig. 3. Different tumor models can exhibit different degrees of neovascularization. Whereas the B16.f10 melanoma tumor s.c. grown in C57bl/6 mice displays a moderate microvascular density (A), C26 colon carcinoma tumor s.c. grown in Balb/c mice exhibits intense microvascular density (B). Snap frozen tumor sections were cryostat cut and immunohistochemically stained for the vascular marker CD31.

grees of neovascularization, as exemplified by the moderate microvascular density in s.c. growing B16.F10 melanoma in C57bl/6 mice vs. the intense microvascular density observed in s.c. growing C26 colon carcinoma in Balb/c mice (Fig. 3). Although the molecular background for these differences are unknown, the differences clearly reflect heterogeneity in local growth factor balances and activation status of the cells present in the diseased site. Lastly, data obtained in tumor models exploiting immune compromised animals should be interpreted with great care, since the immune system cannot only strongly affect anti-tumor effects of (vasculature) targeted drugs, but can also play an important role in the neovascularization process itself.

In Table I, a selection of tumor vascular targeting strategies is summarized. Some of these studies will be addressed in more detail, with emphasis on approaches that showed strong anti-tumor effects in animal models and on new developments and concepts for vascular targeting strategies.

Inhibition of Angiogenesis by (Targeted) Gene Therapy

Anti-angiogenic gene therapy strategies can be divided into strategies aiming at the selective delivery of genes into the angiogenic endothelium to inhibit endothelial cell function, and strategies aiming at the production of anti-angiogenic proteins at sites in the body distant from the tumor. Adenoviruses have been extensively studied for their

Table I. Selection of Tumor Vasculature Directed Drug Targeting Studies as Reported in the Literature (Adapted from [8])

Target epitope	Homing device	Effector molecule
Aminopeptidase N (CD13)	NGR peptide	doxorubicin
		$(KLAKLAK)_2$ (pro-apoptotic peptide)
		TNFα
αvβ3	RGD-4C peptide	doxorubicin
	Anti-αvβ3 Ab (LM609)	magnetic contrast agent (for MRI)
	RGD-4C peptide	$(KLAKLAK)_2$ (pro-apoptotic peptide)
	cRGDfV/cRGDyK peptides	radionuclide
B-FN	Anti-B-FN scFv	tTF
		radionuclide
Endoglin (CD105)	Anti-endoglin Ab	toxin
		radionuclide
	Bispecific diabody	adenovirus
MHC II	BsAb	tTF
MMP-2/-9	HWGF peptide	
VCAM-1	Anti-VCAM-1 Ab	tTF
VEGFR	$rVEGF_{121/165}$	toxin
VEGF:VEGFR complex	Anti-VEGF: VEGFR Ab	radionuclide

Abbreviations; Ab = antibody, B-FN = ED-B domain of Fibronectin, BsAb = bispecific antibody, MHC = major histocompatibility complex, MMP = metalloproteinase, scFv = single-chain Fv fragment, tTf = truncated Tissue Factor, VCAM-1 = vascular cell adhesion molecule-1, VEGF(R) = vascular endothelial growth factor (receptor).

application in anti-angiogenic gene therapy (see reviews on this subject (4,7)). Advances in the molecular construction of endothelium specific, coxsackievirus adenovirus receptor (CAR) defective vectors include the combination of insertion of endothelial cell binding peptides with fiber mutations to block CAR binding (37). Furthermore, specificity of transgene expression may be obtained by combining transductional and transcriptional targeting in one approach. Reynolds *et al.* (38) showed that a bifunctional anti-adenovirus knob Fab fragment conjugated to an anti-angiotensin converting enzyme Fab fragment, combined with a VEGF-Receptor 1 specific promotor sequence, resulted in a 300,000 fold increase in selectivity in transgene expression in the lung.

It should be noted that the choice of the transgene to be expressed by gene therapy may be quite difficult to make based on studies with the original protein, as demonstrated by Kuo *et al.* (39). Furthermore, the prolonged production of anti-angiogenic proteins obtained by gene therapy may be of therapeutic value as it leads to prolonged exposure of tumor vasculature to anti-angiogenic proteins and therefore may improve therapeutic efficacy (40).

The observation that tumor angiogenesis is associated with recruitment of endothelial precursor cells via the circulation has led to the use of genetically modified endothelial cells to deliver genes at sites of active neovascularization. Administration of three sequential i.v. injections of 10^5 endothelial cells expressing a human interleukin-2 transgene abrogated tumor metastases and prolonged survival of B16 melanoma bearing mice (41).

Besides ligand modified viral vectors for selective delivery of anti-angiogenic genes, also non-viral vehicles such as liposomes and artificial virus-like envelopes can be endowed with targeting ligands specific for tumor vascular endothelium (42,43). In theory, not only plasmids encoding anti-angiogenic proteins can be incorporated, but also toxins may be delivered by this means.

Tumor Endothelial Cell Killing by Vascular Targeting

The αvβ3/αvβ5 integrin specific RGD-peptides identified by phage display strongly induced anti-tumor effects *in vivo* when chemically conjugated to the chemotherapeutic drug doxorubicin or the apoptosis inducing peptide $(KLAKLAK)_2$ (44,45). Of note is the observation that the targeted delivery of doxorubicin circumvented cardio- and liver toxicity observed with the freely administered doxorubicin. In addition, the RGD peptide by itself could induce endothelial or tumor cell apoptosis, by interfering with cell—extracellular matrix interactions or by the direct induction of caspase activity (46). In parallel, a peptide specific for aminopeptidase N (CD13) on tumor vasculature was identified and explored as a homing ligand for drugs. Although selectivity towards tumor vasculature seemed to be superior in comparison with the RGD-peptide, no NGR-doxorubicin constructs have been reported to exert prominent anti-tumor effects *in vivo*. In contrast, NGR-TNFα fusion proteins exhibited increased tumor vascular toxicity in combination with a significant reduction in general toxicity in mice (47). Likely, NGR-peptide based drug targeting constructs specifically bind to tumor vasculature expressed CD13 without subsequent internalization. Internalization is, however, a requirement for proper doxorubicin delivery, but not for TNFα induced effects. Knowledge on the cellular routing of the target molecule/drug targeting preparation complex is therefore of importance in relation to the choice of effector molecule to be delivered.

To obtain an αvβ3/αvβ5 specific macromolecular carrier protein with improved drug loading capacity and pharmacokinetics, in our laboratory we chemically conjugated the αvβ3/αvβ5 binding peptide cRGDfK to a 150 kD protein backbone. The multivalent derivatives displayed an increased affinity for binding to αvβ3/αvβ5 integrins on endothelial cells, with an over 1200 fold increase at a peptide: protein loading ratio of ~22:1 (48) (Fig. 4). Furthermore, the macromolecular RGD-proteins exhibited improved pharmacokinetic behavior, as reflected by plasma $t_{1/2}$ values in mice of approximately 90 min. In s.c. growing tumors in mice, the RGD-protein conjugates localized at the tumor vasculature only, as demonstrated immunohistochemically (49). Theoretically, the exposure time of the pro-angiogenic endothelium to conjugates consisting of this macromolecular carrier protein and toxic drugs will be prolonged. Whether this will lead to improved anti-tumor effects and more favorable dosing schedules needs to be established.

Angiotoxins represent therapeutics consisting of angiogenesis related carrier molecules conjugated to toxin molecules. One example of angiotoxins developed for the selective killing of tumor endothelial cell are those consisting of VEGF as a carrier molecule. Upon binding to VEGF-Receptor over-expressed on tumor neovasculature, VEGF/VEGF-Receptor complex is internalized. This makes VEGF protein of use as a carrier for the intracellular delivery of pharmacologically active drugs, or toxins. Both chemical conjugates of Diphteria toxin (DT) and VEGF protein (50), and

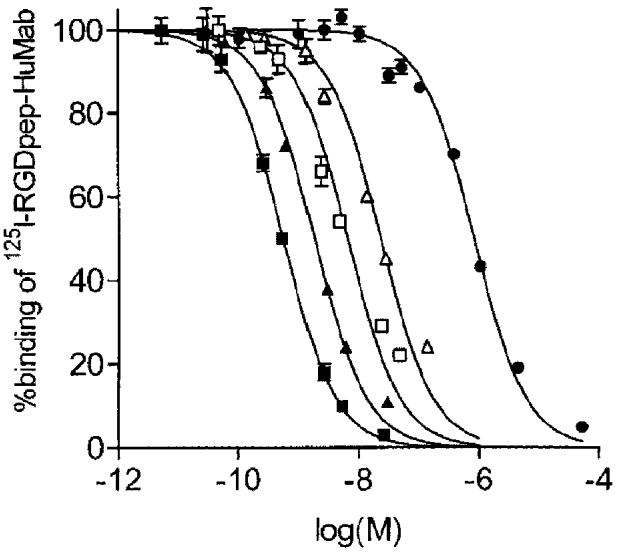

Fig. 4. Multivalent RGDpeptide-protein constructs exert increased binding affinity for αvβ3 / αvβ5 expressing endothelial cells as compared to parental RGDpeptide. RGDpeptides were covalently attached to the inert protein backbone HuMAb at increasing ratios RGD: protein. The resulting RGDpep-HuMAb conjugates [I-IV, with peptide: protein ratios ranging from 2 (construct I) to 22 (construct IV)] displaced ^{125}I-RGDpep-HuMAb (IV) from the endothelium in a dose dependent matter. Displacement was furthermore dependent on the density of RGDpeptide per protein backbone. ● RGDpep; △ RGDpep-HuMAb (I); □ RGDpep-HuMAb (II); ▲ RGDpep-HuMAb (III); ■ RGDpep-HuMAb(IV) (48).

fusion proteins of $VEGF_{165}$ or $VEGF_{121}$ and DT translocation and enzymatic domain (51) have been reported. Both approaches showed the potency of selective tumor endothelial cell killing as a therapeutic strategy for cancer. If the toxin moieties used in these constructs have vascular leak syndrome inducing, disintegrin like characteristics similar to ricin A chain toxin (15), mutation of the motif responsible for this toxicity may facilitate development of angiotoxins into clinically applicable therapeutics.

Tumor Mass Reduction by Targeted Blood Flow Blockade

Induction of local blood coagulation leading to instant tumor blood flow blockade showed enormous potential in reducing tumor mass of established tumors (2,52,53). Although in the first study reporting on the targeted coaguligand strategy for cancer therapy approximately 40% of mice were cured (i.e., they were tumor free for a prolonged period of time after therapy), the later studies did not report cure of animals. This difference in effectiveness may be related to the number of blood vessels blocked by the local blood coagulation induction as well as to differences in immunogenicity of the tumor cells and the immune status of the animals.

CONCLUSIONS AND PERSPECTIVES

Anti-angiogenic therapies have proven their potential in inhibiting tumor growth and reducing tumor mass in a variety of animal tumor models, and now a large number of clinical trials for cancer therapy exploiting anti-angiogenic drugs are being carried out. The challenge in anti-angiogenic therapy will be to completely abrogate neovascularization to occur (9). With the observation that at anti-angiogenic treatment, a second wave of angiogenesis can emerge from surviving tumor cells (54), the most promising therapeutic approach will be to subject the neovascular endothelium to drugs affecting multiple angiogenic factors for a prolonged period of time. Important issues in anti-angiogenesis clinical trials with this new class of drugs will be optimization of dosing schedules and the development of imaging techniques and surrogate marker assays to analyze the phenotypic changes of the tumor vasculature during the anti-angiogenic therapy (9).

The time period between the initial observations that specific compounds inhibited the proliferative behavior of endothelial cells *in vitro* and the start of clinical research with anti-angiogenic drugs has been relatively short. This emphasizes not only the need for new drugs for cancer therapy and the involvement of the pharmaceutical industry in this area of drug development, but also the rapidity of research leading to the unraveling of endothelial cell (dys)function in disease. This, in combination with increasing knowledge on the pharmacologic mechanisms of action of the molecules explored, will lead to the design of better drugs specifically interacting with the tumor endothelial cells (55).

Angiogenesis inhibitors strongly affect tumor outgrowth in different disease models, both when administered as protein therapeutics and in gene therapy studies. In animal models with established tumors, strongest anti-tumor effects are observed with strategies that directly inhibit tumor blood flow or kill tumor endothelial cells. A prerequisite for these latter strategies is the specific delivery of the effector molecules at or in the endothelial cells of the tumor vasculature. The direct relationship between the number of blood vessels attacked and the robust anti-tumor effects observed justifies exploitation of a combination of target epitopes instead of aiming at one single target. In addition, different tumors may induce the expression of different target molecules, complicating the development of one therapeutic strategy to be used for the treatment of multiple types of tumors.

In summary, various untargeted and targeted tumor vasculature directed therapeutic strategies can be considered candidate therapeutics for cancer treatment based on their strong anti-tumor effects in pre-clinical models. Concerted, multidisciplinary research efforts and the lessons learned from 40 years of drug targeting research, should form a solid basis for their further development for clinical use.

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