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Vascular Targeting: Recent Advances and Therapeutic Perspectives

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The ability to deliver therapeutics site—specifically in vivo—remains a major challenge for the treatment of malignant, inflammatory, cardiovascular, and degenerative diseases. The need to target agents safely, efficiently, and selectively has become increasingly evident because of progress in vascular targeting. The vascular endothelium is a central target for intervention, given its multiple roles in the physiology (in health) and pathophysiology (in disease) and its direct accessibility to circulating ligands. In cancer, the expression of specific molecules on the surface of vascular endothelial and perivascular cells might enable direct therapeutic targeting. The use of in vivo phage display has significantly contributed to the identification of such targets, which have been successfully used for directed vascular targeting in preclinical animal models. Several animal studies have been performed by using fused molecules between tumor endothelium-directed molecules and immunomodulatory, procoagulant, or cytotoxic molecules. In addition to delivery of therapeutic agents, vascular targeted gene therapies based on both ligand-directed delivery of gene vectors to tumor endothelium and transcriptional targeting have also emerged. In this review, we discuss ligand-directed vascular targeting strategies with an emphasis on recent developments related to phage-display-based screenings. (Trends Cardiovasc Med 2006;16:80–88) © 2006, Elsevier Inc.

The search for anticancer therapeutic agents that target tumor cells specifically and selectively with limited toxicity has long been a quest in oncology. Systemic cytotoxics do not target cancer cells

selectively and lead to adverse effects because of a narrow therapeutic window. The development of targeted anticancer drugs, with improved discrimination between tumor cells and

nonmalignant cells, is arguably one of the most important goals of current anticancer research. Most chemotherapeutic agents do not preferentially accumulate at the disease sites. Indeed, the dose that reaches the tumor (normalized per gram of tissue) may be as little as 5%–10% of the dose that accumulates in normal organs (Bosslet et al. 1998). High interstitial pressure and the irregular tumor vasculature may account, at least in part, for the poor drug uptake by tumor cells (Jain 1987, Folli et al. 1993). Moreover, the activity of multidrug resistance mechanisms may further decrease drug uptake (Bradley et al. 1988). One avenue toward the development of more selective anticancer drugs consists of the targeted delivery of bioactive molecules (including, but not limited to, drugs, cytokines, procoagulant factors, photosensitizers, radionuclides, etc.) to the tumor environment by means of binding molecules specific for tumor-associated markers. The search

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for novel targets (cell surface receptors) and ligands is just as important as the development of strategies that convert a ligand (capable of selective homing to the tumor environment) into a therapeutic agent. Ligand-based tumor targeting approaches can enable ligand localization in the tumor microenvironment, with tumor-to-control organ ratios >10:1 reached only a few hours after intravenous administration (Buchegger et al. 1983, Wu et al. 1996, Adams 1998, King et al. 1994, Tarli et al. 1999, Demartis et al. 2001, Carnemolla et al. 2002, Borsi et al. 2002).

Several malignant, cardiovascular, inflammatory, and degenerative diseases are characterized by the onset of marked angiogenesis (Folkman 1995). In cancer, it has long been proposed that angiogenesis contributes to tumor progression by promoting both primary tumor growth and metastatic dissemination (Folkman 1971). Tumor growth requires the formation of new blood vessels. This process of neovascularization, or angiogenesis, appears to be essential for solid tumors. Tumor endothelial cells are central in this neovascularization process. Tumor cells promote new blood vessel formation by releasing endothelial cell growth factors that support endothelial cell proliferation, migration, and survival. In solid tumors, the vasculature is an attractive target because the vascular endothelium is composed of genetically stable nontransformed cells that are presumably less adaptive than tumor cells and less likely to acquire drug resistance (St Croix et al. 2000, Kolonin et al. 2001), although this notion has been recently challenged (Hida and Klagsburn 2005). In addition, endothelial cells lining tumor blood vessels are directly accessible to drugs via the systemic circulation and express several cell surface markers that are absent or barely detectable in established or quiescent blood vessels. Ligand-directed vascular targeting can be accomplished by antibodies, specific peptides or growth factors complexed with immunomodulatory, procoagulant, or cytotoxic molecules (Thorpe and Burrows 1995, Brekken and Thorpe 2001, Thorpe et al. 2003, Thorpe 2004). Targeted molecules are selectively up-regulated on angiogenic tumor endothelial cells and include α v integrins, vascular endothelial growth factor (VEGF) receptors, cell

adhesion molecules (vascular cell adhesion molecule [VCAM] and E-selectin), and molecules associated with procoagulant changes on angiogenic endothelium such as phosphatidylserine (Brekken and Thorpe 2001, Brekken et al. 2002). To identify specific targets on angiogenic tumor endothelium and to gather more information on their cellular distribution and location, phage display has been extensively used (Zurita et al. 2004). Finally, phage display technologies are also used to map human vascular diversity (Arap et al. 2002, Zurita et al. 2004, Pentz et al. 2003, 2005).

• Targeted Tumor Infarction

The therapeutic potentials of approaches aiming to interfere with the blood supply can be easily appreciated when one considers the estimate that 100 tumor cells are fed by a single endothelial cell (Folkman 1997). Thus, the number of endothelial target cells is limited, in contrast to the number of tumor cells to be killed by a tumor cell-directed approach. Proofs of concept from many studies support the hypothesis put forward more than 3 decades ago that selectively interfering with the tumor blood supply would lead to strong anti-tumor effects.

Once specific vascular targets are identified, several different effector molecules could be targeted to the tumor vasculature, including radiolabeled, or cytotoxic agents (Oh et al. 2004, Veenendaal et al. 2002) as well as antiangiogenic and immunostimulatory cytokines (Halin et al. 2002). Another notable effector molecule is tissue factor (TF), which initiates the blood coagulation cascade. In the seminal work, the cell surface domain of TF was targeted to tumor blood vessels (Huang et al. 1997). This truncated extracellular domain of TF is soluble and five orders of magnitude less potent than the membrane form because the TF-factor VIIa complex requires negatively charged cell surface phospholipids to activate the downstream factors IX and X, which in turn catalyze the formation of thrombin (Ruf et al. 1991). Soluble TF targeted to tumor vasculature initiates an explosive coagulation cascade that leads to intravascular thrombosis and tumor infarction

(Ran et al. 1998). Targeting TF to tumor vessels has been accomplished with antibodies and peptides against tumor vasculature markers including major histocompatibility complex class II (Burrows and Thorpe 1993).

Dienst et al. (2005) have also evaluated a novel single-chain anti-VCAM-1 antibody/soluble TF fusion protein as a tumor vascular targeting agent. VCAM-1 is a type I transmembrane glycoprotein that is generally not expressed by quiescent endothelium but is induced by inflammation. The tumor vasculature and some tumor cells generally express VCAM-1. The authors observed tumor tissue necrosis and intratumor vascular fibrin thrombosis after a single systemic administration of the infusion protein. In addition, long-term treatment produced delays in tumor growth under certain conditions. Of note, after extensive analyses, the authors could find no evidence of treatment-related vascular thrombosis and necrosis in normal tissues and no evidence of systemic activation of the coagulation cascade. This work adds further support to the notion that strategies aimed at producing vascular occlusion in tumor tissues can be effective and selective.

In another elegant study, Hu et al. (2003) compared the therapeutic potency of three recombinant fusion proteins consisting of truncated TF fused to the Arg-Gly-Asp (RGD)-tripeptide-targeting integrins, to antibodies against DNA exposed in degenerative areas, or against the EDB domain of fibronectin (Nilsson et al. 2001). Interestingly, although fibronectin- and α v-integrin-targeted tissue factor induced thrombosis in small- and medium-sized blood vessels, DNA-targeted tissue factor did so in large blood vessels. Combining all three so-called "coagulgands" was the most effective treatment strategy to inhibit tumor outgrowth. Thus, it is possible that controlling vascular diversity of the endothelium during tumor progression will require such multi-target approach.

• In Vivo Phage Display and Discovery of Vascular Receptors for Ligand-Based Vascular Targeting Strategies

Phage display technology presents a rapid means by which proteins and

Table 1. Angiogenic cell surface receptors/homing motifs isolated by in vivo phage display

Receptor	Function/class	Carrier?	Localization	Homing motif
GRP 78	Stress response	Yes	Tumor cells	WIFPWIQL
α v Integrins	Cell adhesion	Yes	EC, tumor cells	RGD4C
CD13	Protease	Yes	EC, pericytes	CNGRC
APA	Protease	N/D	Pericytes, stromal cell	CPRECES
NG2/HMWMAA	Proteoglycan	N/D	Pericytes, tumor cells	GSL
MMP-2/MMP-9	Protease	Yes	EC, tumor cells	CTTHWGFTLC
IL-11-R	Cytokine receptor	Yes	EC, tumor cells	CGRRAGGSC
Prohibitin	Membrane chaperone	Yes	EC	CKGGRAKDC
HSP90	Heat shock	Yes	EC, tumor cells	CVPELGHEC

EC, endothelial cells; N/D, not determined.

peptides that bind specifically to predefined molecular targets can be selected and isolated from complex combinatorial peptide libraries. Phage display libraries consist of polypeptides expressed within the coat proteins of filamentous bacteriophage. In the minor pIII coat protein, as many as 10^9 unique peptide sequences can be displayed on the surface of the phage particles (Zacher et al. 1980, Scott and Smith 1990, Zurita et al. 2003).

To identify probes that home selectively to various vascular beds of normal and tumor angiogenic vasculatures, our group and others have developed an in vivo phage display technology to isolate peptides that bind selectively to target receptors that are expressed only on certain blood vessels. Both tissue-specific (Rajotte et al. 1998, Arap et al. 2002, Kolonin et al. 2002, 2004) and angiogenesis-related (Pasqualini et al. 1997, 2000a, Arap et al. 1998, 2002, 2004, Koivunen et al. 1999, Marchiò et al. 2004) vascular ligand receptor pairs have been identified through this technology (Pasqualini et al. 2000b). In this method, phage libraries are directly administered to mice into the tail vein, and tissues are collected and examined for phage bound to tissue-specific endothelial cell markers. Within a short circulation time (~5 min), the phage particles are unlikely to be able to leave the circulation; thus, after perfusion, only the phage population bound to the endothelium (vascular receptors) is recovered from the tissues. In vivo panning has the advantage that the isolated phage-displayed peptides

home selectively to “intact” targets of interest; moreover, these ligand peptides may be useful for the functional analysis of the corresponding receptors and potential identification of novel drug target candidates because some of the isolated peptides have been found to bind to endothelial receptors expressed in the vasculature of specific tissues. Although monoclonal antibodies have demonstrated clinical potentials as tumor-targeting agents, poor tumor penetration of the antibodies due to the size of molecules and liver/bone marrow toxicity by nonspecific uptake of the antibodies remain the two major limitations of antibody-based therapy (Shockley et al. 1991). Peptidic targeting agents may ease the problems associated with antibody cancer therapy. Peptides obtained from in vivo phage display technology can exert anticancer effect by inhibiting angiogenesis, decreasing tumor metastasis activity, or inhibiting enzymes important for neoplastic cell spread. They can also be used themselves as targeting molecules of receptor-targeted toxins and gene therapy, imaging and/or therapeutic agents, and nanomedical devices. Thus, our approach provides a straightforward way to identify drug-accessible tumor cell surface receptors and to discover peptide ligands that can serve as mimetic prototype drugs. Unlike genomic or proteomic-based approaches that rely on differential expression levels of transcripts or protein products, this discovery platform directly addresses functional protein–protein interactions at the

level of physical binding. In contrast to protein array systems, it is possible to select binding peptides even if the ligand–receptor interaction is mediated by conformational (rather than linear) epitopes. In summary, ligand-directed screening of combinatorial libraries on tumor cell surfaces may lead to improved selection of functionally relevant peptides that can be developed for targeting “druggable” molecular targets.

• Vascular Targeting Peptides

After the introduction of in vivo biopanning of peptide phage-display libraries (Pasqualini and Ruoslahti 1996), several vascular targeting peptides have been introduced as organ- or tumor-specific vascular targeting agents; in addition, many ligand/receptor pairs have been identified (Table 1).

We have identified peptides that selectively target normal organs in mice; so far, prostate, kidney, skin, pancreas, retina, intestine, uterus, and adrenal gland have been targeted in this manner (Kolonin et al. 2001, Trepel et al. 2002). These results indicate that the vascular endothelium of normal organs is modified in ways that enable differential targeting with peptide probes. Recently, our group used in vivo phage display to isolate a peptide motif CKGGRAKDC that homes to white fat vasculature by targeting prohibitin, a multifunctional membrane protein (Table 1). The CKGGRAKDC sequence conjugated to apoptosis-inducing peptide (KLAKLAK)² caused ablation of white fat (Kolonin et al. 2004).

We have also devised a means of identifying peptides that home to the angiogenic vasculature of tumors (Table 1). Moreover, we have assembled a panel of peptide motifs that target the blood vessels of tumor xenografts (reviewed in Kolonin et al. 2001). These motifs include the sequences CDCRGDCFC (termed *RGD-4C*), NGR, CPRECES, and GSL. The RGD-4C peptide has previously been identified as selectively binding α v integrins and has been shown to home to the vasculature of tumor xenografts in nude mice. The tumor homing is possible because α v integrins play an important role in angiogenesis: the α v β 3 and α v β 5 integrins are absent or expressed at low levels in normal endothelial cells but are induced

in the angiogenic vasculature of tumors (Pasqualini et al. 1997, Brooks et al. 1994a, b, Hammes et al. 1996, Wickham et al. 1997, Sipkins et al. 1998, Hood et al. 2002, Dickerson et al. 2004). Phage displaying a double cyclic RGD-4C peptide exhibited 10–20 times higher tumor-homing ability than the negative control phages (Arap et al. 1998, Pasqualini et al. 1997). To date, $\alpha v\beta 3$ integrin, VEGF receptors, and the EDB domain of fibronectin have been extensively studied as targets for the delivery of toxic drugs into tumor endothelial cells. We reported that RGD-4C or the cyclic CNGRC conjugated to the chemotherapeutic drug doxorubicin or to the proapoptotic peptide (KLA-KLAK)₂ increases the therapeutic index and reduces toxicity in vivo (Arap et al. 1998, Ellerby et al. 1999). Moreover, the NGR peptide has been shown to improve the antitumor effects of tumor necrosis factor and interferon- γ when fused to these cytokines (Curnis et al. 2000, 2005). We recently identified aminopeptidase N/CD13 as the angiogenic receptor for the NGR motif (Pasqualini et al. 2000a) and aminopeptidase A (APA) as the angiogenic receptor for the CPRECE-SIC motif (Marchiò et al. 2004). Our group showed that APA is strongly up-regulated in angiogenic tumor blood vessels but barely detectable in normal blood vessels; the enzymatic activity of APA colocalizes to its expression pattern in human tumors. We showed that the CPRECE-SIC motif inhibits its enzymatic activity, homes to tumor vasculature in vivo, and inhibits tumor growth (Marchiò et al. 2004). Some of the targets in tumor blood vessels turned out to be matrix metalloproteinases (Koivunen et al. 1999, Marchiò et al. 2004). Similarly, selective peptide inhibitors of gelatinases MMP-2 and MMP-9 localized to murine tumors and prevented tumor growth upon intravenous injection (Koivunen et al. 1999).

Our studies clearly show that αv integrins, aminopeptidase N, aminopeptidase A, NG2, and MMP-2/MMP-9 are specifically expressed in angiogenic endothelial cells and pericytes of both human and murine tissue origin. Markers in the angiogenic neovasculature are either expressed at very low levels or not at all in nonproliferating endothelial cells. Interestingly, many of these tumor vascular markers are proteases (somewhat intuitive, given that

malignant tumors are invasive); some of the markers also serve as viral receptors, such as αv integrins, receptors for adenoviruses (Wickham et al. 1993); CD13, a receptor for coronaviruses (Yeager et al. 1992); and MMP-2 and MMP-9, recently shown to be receptors for echoviruses (Pulli et al. 1997). It is tempting to speculate that bacteriophage (i.e., prokaryotic viruses) may use the same cellular receptors as do eukaryotic viruses. In fact, the structure of the phage capsid protein provides good evidence that bacteriophage share ancestry with animal viruses (Hendrix 1999). More than an evolutionary biology footnote, these findings do suggest that the receptors isolated by in vivo phage display will have cell internalization capability, a key feature if one wishes to use peptide motifs as therapy carriers targeted to selective cell subpopulations.

Not only proteins can serve as vascular targets: Burg et al. (1999) isolated peptides that bind in vitro to NG2, a proteoglycan selectively expressed in angiogenic vasculature, and demonstrated homing of these peptides to mouse tumors.

To avoid challenges with species specificity, we started a program in cancer patients. By using the in vivo phage display approach in humans, our group reported the first in vivo screening of a peptide library in a patient and led to the identification (Arap et al. 2002, Pentz et al. 2003) of a mimic motif of interleukin 11 (IL-11) from prostate biopsies. We also demonstrated that the IL-11 phage mimic (displaying the cyclic peptide CGRRAGGSC) bound specifically to a corresponding IL-11 receptor (IL-11R α) and that IL-11R α is a potential target for intervention in human prostate cancer (Arap et al. 2002, Zurita et al. 2004).

Cancers express tumor antigens; the immune response against these antigens can be explored to identify markers of disease aggressiveness or targets for therapy. We have reported a methodology to select peptide motifs recognized by tumor-associated antibodies; this phage-display-based approach (termed *fingerprinting*) enables isolation of mimic peptides of antigens eliciting humoral response (Mintz et al. 2003). In this method, antibody fingerprinting is a combinatorial screening in which phage display random peptide libraries

are selected on in vitro pools of immobilized patient-derived immunoglobulins. Thus, by fingerprinting the circulating repertoire of antibodies from patients with prostate cancer, we have identified glucose-regulated protein-78 (GRP78) as a relevant molecular target expressed in metastatic tumors (Mintz et al. 2003). Synthetic chimeric peptides composed of GRP78 binding motifs fused to a proapoptotic peptide suppressed tumor growth in mice (Arap et al. 2004).

We also fingerprinted the pool of antibodies purified from a patient with ovarian-cancer-derived ascites (Vidal et al. 2004) and identified a peptide motif mimicking the heat-shock protein 90 kDa (HSP90), which has been reported for its implication in cancer (Kamal et al. 2004). Then we found that HSP90 is widely expressed in ovarian cancer. The humoral response against HSP90 is tumor-associated and stage-specific.

The data obtained with these various peptides from phage display screenings support their potential as targeting ligands in drug development for clinical applications.

Finally, our group has developed a novel technology, termed *biopanning and rapid analysis of selective interacting ligands*, enabling fast selection of peptides binding to cell surface receptors (Giordano et al. 2001).

Biopanning and rapid analysis of selective interacting ligands is based on differential centrifugation in which cells incubated with phage in an aqueous upper phase are centrifuged through a nonmiscible organic lower phase to separate specific phage–cell complexes from unbound phage. This method has several advantages over conventional methods relying on washing steps or limiting dilution. It is faster, more sensitive and more specific, and directly applicable to cells, which are more likely to preserve the native conformation of proteins. Potential isolation of ligand–receptor pairs from cells derived from clinical samples and automation for high-throughput screening are just two examples of the vast potential this system offers. As a proof of principle, we constructed a ligand–receptor map of the VEGF-receptor family based on peptides, and a new motif targeting VEGF receptor-1 was validated (Giordano et al. 2001).

- **Vascular Targeting in Atherosclerosis**

Atherosclerosis is regarded as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation. This condition is the leading cause of death in developed countries. The development of targeted imaging agents for timely identification of unstable atherosclerotic lesions is an unmet need because the rupture of these plaques often results in myocardial infarctions and strokes (Fuster 1994). Several targeted nuclear imaging agents have been developed to report on high-risk features of atherosclerotic lesions (Mari and Strauss 2002). Molecular changes on the endothelial surface are considered important contributors to the initiation, progression, and thrombotic complications of atherosclerosis (Ross 1993a, 1993b, Fuster 1994). However, the profile of the endothelial surface protein display during atherogenesis is still poorly characterized. Although adhesive cell-surface glycoproteins, such as selectin (Dong et al. 1998) and VCAM-1 (Cybulsky and Gimbrone 1991), appear to play a role in atherogenesis, systematic exploration of the affected endothelial surfaces has been hampered by technical limitations. Liu et al. (2003), have used in vivo phage display technology for identification of peptide probes that selectively bind to atherosclerotic lesions induced in apoE knockout mice. The cellular events in this model are thought to mimic, at least in part, molecular changes that occur in the hypercholesterolemic animal models and perhaps in the human disease (Boisvert et al. 1999, Curtiss and Boisvert 2000). Strikingly, GRP78 has again been identified as the endothelial surface target of one selected peptide. This peptide preferentially bound ex vivo to resected human arterial atherosclerotic lesions. The three other selected peptides homologous to TIMP-2 recapitulated the binding to lesions and were, in turn, inhibited by TIMP-2 protein.

Peptides have been used to target pathologic features, which are sometimes associated with angiogenesis. For example, Tepe et al. (2001), described radiolabeled endothelin derivatives, which preferentially accumulate on atherosclerotic plaques after intravenous administration. MMPs have also been broadly implicated in several cardiovas-

cular diseases, including atherosclerosis (Libby 2002, Galis and Khatri 2002), aortic aneurysms (Pyo et al. 2000), and heart failure (Lee and Libby 2000) and, therefore, may represent a relevant target for cardiovascular molecular imaging. In atherosclerosis, MMPs are expressed in macrophages and vascular cells and are involved in atherosclerotic plaque disruption via enzymatic degradation of the fibrous cap or endothelial basement membrane (Libby 2002, Galis and Khatri 2002). Among these MMPs, MMP-9 (gelatinase B) appears to participate in several stages of atherosclerosis, and it is a candidate target for in vivo molecular imaging of atherosclerosis.

- **Targeting Gene Viral Vectors to Vascular Receptors**

Progress in gene therapy depends on the development of ligand-directed vectors that will enable the systemic targeted delivery of genes. The rational design, construction, and testing of vascular-targeted gene delivery vectors is a promising route to improve the safety and efficacy of gene therapy. It would be highly advantageous if gene delivery to vascular cells could be improved in efficiency (thus enabling reduced doses to be used and potentially reduced immunogenicity) and improved selectivity (thus reducing potential deleterious side-effects of transgene expression at nontarget sites). Vector targeting strategies have advanced substantially in the recent past, enabling construction of vectors that can home to defined sites in vivo after systemic application. Adenovirus (Ad) and adeno-associated virus (AAV) are commonly used for cardiovascular gene therapy. The most commonly used Ad vectors for gene therapy are based on serotypes 2 and 5. In cardiovascular disease, adenoviruses can transduce the endothelium and smooth muscle cells within the blood vessel wall, albeit only upon administration of high titers (Lemarchand et al. 1993, French et al. 1994). This has not hindered preclinical progression of Ad serotype 5 vectors to clinical trials for ischemia (Grines et al. 2002, Hedman et al. 2003). It would therefore be of benefit for cardiovascular gene therapy to develop adenoviruses with a more favorable profile. This can be achieved in several ways, including the use of anti-

bodies to retarget the virus to alternative receptors such as angiotensin-converting enzyme or E-selectin (Harari et al. 1999, Reynolds et al. 2000), targeting peptides inserted into the HI loop of the fiber structure to modulate receptor binding (Nicklin et al. 2000, 2004), molecular adaptors (Trepel et al. 2000a, 2000b), or by pseudotyping (involving the exchange of the Ad fiber for a fiber from an alternative serotype that possesses a more favorable cell binding profile (Chillon et al. 1999, Havenga et al. 2001). All these strategies have provided proof of concept for improvement in the transduction of vascular cells (Nicklin and Baker 2002).

Recombinant AAV serotype 2 is yet another promising vector for gene therapy because it can achieve long-term stable transgene expression in animal and human subjects after direct administration of the vectors into various target tissues (Kay et al. 2000). Unfortunately, endothelial cells are apparently poorly transduced by AAV-2 (Richter et al. 2000, Nicklin et al. 2001). Indeed, the deficiency of AAV-2 for endothelial transduction was recognized by Richter et al., (2000), who showed that local delivery of AAV-2 to blood vessels led to transduction of underlying vascular smooth muscle cells, even in the presence of intact endothelium. Subsequent studies have reported that sequestration of AAV-2 within the extracellular matrix around endothelial cells (thus preventing cell binding and entry) and degradation of internalized AAV-2 particles in the proteasome are the two factors responsible for the inefficient transduction of endothelial cells by AAV-2 (Nicklin et al. 2001, Pajusola et al. 2002).

As for Ad, strategies include nongenetic modification of AAV-2, genetic insertion of targeting peptides, and pseudotyping with alternative serotype capsids (Nicklin and Baker 2002). In the case of targeting peptides, two main methods have been assessed thus far for vascular cells. First, phage-display-derived peptides have been incorporated into AAV-2 capsids at position 587 (for optimal peptide insertion within the capsid to display the inserted peptide on the surface of the virion (Girod et al. 1999). Subsequent studies showed increased efficiency and selectivity of endothelial cell transduction in vitro and in vivo after intravenous administra-

tions using either targeting peptides with seven or 12 residues (Nicklin et al. 2001, White et al. 2004). In an elegant alternative, Muller et al. (2003) introduced random peptide libraries displayed on the capsid of AAV-2. Screening of this AAV library on human primary coronary artery endothelial cells yielded a motif that enhanced infectivity of these endothelial cells, as compared with nonendothelial cells. It remains to be determined whether this approach will be successful in vivo (Muller et al. 2003, Lieber 2003).

Retroviruses have also been engineered so that they can be coated with an antibody (e.g., anti-VEGF Flk1/KDR receptor) for selective gene delivery to tumor endothelium (Masood et al. 2001). The combined use of regulatory elements controlling two independent markers of tumor endothelium, Flk-1, and endoglin gave synergistic effects on targeting specificity in vitro (Savontaus et al. 2002). A recent strategy for attaining specific effects on tumor blood vessels is to use tumor cell-specific cytotoxic T lymphocytes to deliver a retrovirus containing a gene encoding a VEGF toxin fusion protein to tumor cells. The VEGF toxin synthesized by the tumor cells is expected to destroy the adjacent tumor endothelium but not angiogenic blood vessels in normal tissues distant from the tumor (Jin et al. 2002).

• **Vascular Targeting of Gene Delivery with Nonviral Vectors**

In addition to viruses, liposomes conjugated to the cyclic RGD-4C were used as delivery device selective to endothelial cells and melanoma cells expressing $\alpha\beta 3$ integrins (Fahr et al. 2002). Peptides containing RGD and a short polylysine segment for electrostatic binding of DNA efficiently enhanced the transfer of DNA to different cell types (Suh et al. 2002, Kunath et al. 2003).

Similar vascular targeting strategies were employed to selectively deliver genes to tumor blood vessels. Hood et al. (2002) have described the use of cationic nanoparticles complexed to $\alpha\beta 3$ integrin-ligand to selectively deliver a mutant raf protein that inhibits endothelial cell survival signaling to block angiogenesis in response to different growth factors. Systemic administration of the nanoparticles to tumor-bearing mice resulted in apoptosis of the tumor

endothelium leading to tumor cell apoptosis and even regression of primary and metastatic tumors. A similar strategy using small interfering RNA, specifically knocking down VEGF receptor 2 expression, led to inhibition of tumor outgrowth (Schiffelers et al. 2004).

Prokaryotic viral vectors have also been developed for delivery of genes to eukaryotic cells (Larocca et al. 1998, 1999, Monaci et al. 2001). Prokaryotic viruses such as bacteriophage (phage) might offer an attractive alternative to other viral and nonviral vectors because they can, in theory, overcome the drawbacks of other animal vectors. For example, a major advantage of phage over eukaryotic viral vectors is their lack of tropism for mammalian cells (Barrow and Soothill 1997, Barbas et al. 2000). Phage can also be safely administered to patients, as evidenced by the fact that humans received bacteriophage during the preantibiotic era without adverse effects (Barrow and Soothill 1997). Therefore, they can be engineered to deliver genes to such cells without the need to eliminate native tropism, a concern for current animal viral-vector targeting (Ivanenkov et al. 1999, Poul and Marks 1999, Larocca et al. 1999). Delivery of reporter genes has been achieved by genetically modified phage particles displaying peptides for surface receptors such as FGF-2, transferrin, or epidermal growth factor (Larocca et al. 1998) and ErbB-2 (Monaci et al. 2001). However, current phage-based vectors are otherwise generally considered poor gene therapy vectors. Recently, Piersanti et al. (2004), reported the construction of a lambda-phage-based vector displaying the full-length of the penton base of adenovirus. The penton base, which mediates attachment entry, endosomal escape, and proteasome resistance of adenovirus in mammalian cells, was able to confer these characteristics to the phage particles.

Finally, phage particles selected by in vivo phage display for their ability to home to cell surface receptors on blood vessels could provide targeted systemic gene delivery to the vascular endothelium. Moreover, they have advantages over existing gene delivery vectors for vascular targeted gene therapy because phage identified to home to vascular receptors in an in vivo screening do not require further targeting modifications.

• **Combination Therapy**

The success of a combined treatment modality, including targeted antivascular agents and conventional tumor therapy (radiochemotherapy) may have a complex dose and time dependence: dependence on its dose, schedule, and mode of action. Antiangiogenesis or antivascular therapies may reduce tumor blood flow, thus decreasing the tumor dose of a concurrently administered chemotherapeutic drug. Furthermore, inhibiting the blood supply leads to overexpression of hypoxia-inducible factor 1α , which is correlated with increased tumor invasiveness and resistance to chemotherapy (Blagosklonny 2004, Semenza 2000, Tran et al. 2002, Yu et al. 2002). To address this challenge, Sengupta et al. (2005) have recently reported the design of a delivery system, termed a *nanocell*, comprising a nuclear nanoparticle within an extracellular pegylated lipid envelope. The nanocell enabled a first temporal release of a vascular targeting agent (combretastatin-A4) trapped within the lipid envelope causing a vascular “shutdown”; then the inner nanoparticle released a chemotherapeutic drug (doxorubicin), which killed the tumor cells.

In tumor-bearing mice, the nanocell treatment showed superior antitumor efficacy when compared directly with the equivalent doses of the controls. Moreover, a significant increase was achieved in the lifespan of tumor-bearing animals treated with the nanocell.

• **Conclusions**

The identification of vascular markers opens the way to diagnostic and therapeutic strategies, based on the targeted delivery of imaging and therapeutic agents to the vasculature. Ligand-directed vascular targeting agents have proven their mode of action and antitumoral efficacy in several preclinical animal models. Currently there are more than a dozen vascular targeting agents under preclinical or clinical investigation being tested for systemic treatment of disseminated tumor disease. Endothelial cell surface receptors of the neovasculature are candidate therapeutic targets in cancer. In the last decade, the phage display library technique has been successfully used to discover cell surface

binding peptides. Peptides are excellent alternative targeting agents for human cancers, and they may alleviate some of the problems associated to antibody targeting.

Because of its central role in the pathogenesis of cardiovascular disease, the vascular endothelium is also quite an attractive therapeutic target for cardiovascular disease. Genetic modulation of endothelial function may offer new opportunities to modify the course of cardiovascular diseases such as hypertension, atherosclerosis, thrombosis, and ischemic artery disease. Gene therapy is a promising approach for the treatment of this endothelial dysfunction; however, successful therapy will require further development of vectors and delivery tools to improve specificity, safety, and efficiency of gene transfer. Ligand-directed gene delivery to the endothelium for cardiovascular disease offers a unique opportunity to treat cardiovascular pathologies.

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