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13. ABSTRACT (Maximum 200 Words) The identification of markers expressed on specific tumors would give valuable insights into the specialization of tumor vasculature, and would also provide a means of targeting distinct tumor sites. We have recently developed a novel approach for in vivo selection of peptides capable of homing into specific organs or tumors. We have demonstrated the feasibility of the approach by isolating a panel of targeting peptides that bind selectively to receptors either within the angiogenic vasculature of tumors or directly on tumor cells. In this proposal we intend to extend this approach by screening experimental breast carcinoma models in vivo. We will use phage display peptide libraries to identify tumor homing peptide motifs capable of recognizing breast tumors. Using targeting peptides, we have shown in animal experiments that an anthracyclin drug can be converted into a less toxic and more potent anti-cancer agent than the free drug. Here we propose to generate molecular conjugates that can target gene therapy vectors to breast tumors in vivo following intravenous administration. Since only a small subset of tumors are accessible to direct injection, targeted vectors would overcome the current limitations associated with the delivery of therapeutic genes.				
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INTRODUCTION

It is well known that angiogenesis, the recruitment of new blood vessels, is an important rate-limiting step in solid tumor growth. New anti-tumor therapies based on the premise that inhibiting angiogenesis suppresses tumor growth are currently being tested in multiple clinical trials (1, 2, 26). Angiogenesis is a multi-stage process that involves the release and activation of angiogenic factors, endothelial cell migration and proliferation, and differentiation into newly formed capillaries (3, 4).

Angiogenic neovasculature expresses markers that are either expressed at much lower levels or not at all in non-proliferating endothelial cells (5-7). The markers of angiogenic endothelium include receptors for vascular growth factors, such as specific subtypes of VEGF and basic FGF receptors (8-11), and the α_v integrins (12, 13). Identification of novel molecules characteristic of angiogenic vasculature will improve our understanding of the plasticity of the endothelial phenotype and suggest new therapeutic strategies. Thus far, identification and isolation of such molecules has been slow, mainly because endothelial cells undergo dramatic phenotypical changes when grown in culture (14, 15).

We have developed an *in vivo* system using peptides expressed on the surface of bacteriophage to study organ- and tumor-specific vascular homing (16, 17, 20, 21). Random oligonucleotides are individually fused to cDNAs encoding a phage surface protein, generating collections of phage particles displaying unique peptides in as many as 109 permutations (18, 19); in the *in vivo* procedure, phage capable of homing to certain organs or tumors following an intravenous injection are recovered from such phage display peptide libraries. The ability of individual peptides to target a tissue can also be analyzed by this method (16, 17). Furthermore, this system provides an innovative way of identifying endothelial cell surface markers expressed *in vivo* (16, 20, 21).

Unlike earlier antibody work in which tumor specific antigens were selected *in vitro*, our method directly selects molecules capable of homing to tumor vasculature *in vivo*. Thus, in addition to providing novel tools for selective vascular targeting of therapies, this new technology will further our understanding of tumor endothelium specificity and define the role these markers play in angiogenesis. One of the unique and highly attractive features of this method is that it detects the availability of the target receptors in the tissue based not only on expression level, but also on accessibility to a circulating probe.

With the knowledge that solid tumors cannot progress without the generation of new blood vessels, the development of strategies to inhibit this process has been the focus of intense research. There are a variety of approaches not involving gene therapy that are under evaluation for their ability to suppress tumor neovascularization (3, 4, 23, 24, 45-47). They include treatments using recombinant proteins, monoclonal antibodies and small molecules such as TNP-470, a potent angiogenesis inhibitor (25, 74). Current clinical trials with TNP-470 include patients with cervical cancer, pediatric solid tumors, lymphomas, acute leukemias and AIDS-related Kaposi's sarcoma (26, 27). Preliminary results suggest that the use of therapies that destroy angiogenic vasculature could be a

viable approach to avoid drug resistance in cancer therapy (28-30). To date, most tumor treatment strategies utilize systemic administration of the therapeutic agent. Recently though, gene transfer of a cDNA coding for mouse angiostatin into murine fibrosarcoma cells has been shown to suppress primary and metastatic tumor growth in vivo. Implantation of stable clones expressing mouse angiostatin in mice dramatically inhibits primary tumor growth and pulmonary micrometastases. The tumor cells in the dormant micrometastases exhibit a high rate of apoptosis balanced by a high proliferation rate. These studies also provide support for a novel approach for cancer treatment by antiangiogenic gene therapy with specific angiogenesis inhibitors (31).

The antitumoral effects that follow the local delivery of angiostatin have been studied in xenograft murine models. Angiostatin delivery was achieved by a defective adenovirus expressing a secretable angiostatin K3 molecule from the cytomegalovirus promoter (AdK3). In in vitro studies, AdK3 selectively inhibited endothelial cell proliferation; AdK3-infected endothelial cells also showed a marked mitosis arrest. A single intratumoral injection of AdK3 into pre-established rat C6 glioma or human MDA-MB-231 breast carcinoma grown in athymic mice was followed by arrest of tumor growth, which was associated with angiogenesis inhibition. AdK3 therapy also induced a 10-fold increase in apoptotic tumor cells as compared with a control adenovirus (32). Similar results were obtained using a related approach on an RT2 glioma model (33). These data support the feasibility of targeted antiangiogenesis using adenovirus-mediated gene transfer and indicate that gene therapy can potentially expand the horizons of tumor antiangiogenesis therapy: First, because of the possibility of achieving high concentrations of the therapeutic agent in a given area; Second, because the agent can be produced endogenously, and for a sustained period (24, 34).

In addition, gene therapy can also be designed to deliver the therapeutic gene upon systemic administration. However, for those advantages to be fully explored, one major requirement needs to be made feasible: the targeted delivery of genes to angiogenic vasculature.

In an effort to achieve vector targeting, a few groups have reported on gene therapy-based adenovirus re-targeting strategies that redirect the adenovirus to receptors endogenously present on the cell surface of tumor cells (reviewed in ref. 34). By using a bifunctional conjugates consisting of a blocking antiadenoviral knob Fab linked to basic fibroblast growth factor, gene transduction of target cells in vitro was improved; recombinant adenoviruses encoding either the firefly luciferase reporter gene, or the herpes simplex thymidine kinase gene, demonstrated quantitative enhancement of expression. Moreover, tumor cell lines that were previously refractory to native adenovirus transduction could be successfully transduced by the addition of the conjugate (35). Other bispecific antibodies have been used successfully to target entry of an adenovirus vector into endothelial cells expressing a receptor up-regulated during angiogenesis, such as the construction of Ad vectors which contain modifications to the Ad fiber coat protein that redirect virus binding. Retargeting has been demonstrated using av integrin [AdZ.F(RGD)] or heparan sulfate [AdZ.F(pK7)] cellular receptors. These vectors were constructed by a novel method involving E4 rescue of an E4-deficient Ad with a transfer vector containing both the E4 region and the modified fiber gene. AdZ.F(RGD) increased gene delivery in vitro to endothelial and smooth muscle

cells expressing av integrins. Likewise, AdZ.F(pK7) increased transduction 5- to 500-fold in multiple cell types lacking high levels of Ad fiber receptor, including macrophage, endothelial, smooth muscle, fibroblast, and T cells. In addition, AdZ.F(pK7) significantly increased gene transfer in vivo to vascular smooth muscle cells of the porcine iliac artery following balloon angioplasty. Although binding to the fiber receptor still occurs with these vectors, they demonstrate the feasibility of tissue-specific receptor targeting in cells which express low levels of Ad fiber receptor (36, 37). Heterologous ligands have also been incorporated into the HI loop of the fiber knob, and preliminary results using artificial systems in vitro suggested that this locale possesses properties consistent with its employment in adenovirus retargeting strategies (38).

These studies address the utility of adenoviral retargeting and indicate that practical use of gene therapy approaches can be developed based on our vascular targeting technology. Here we have been exploring the homing ability of angiogenic vasculature-homing peptides by incorporating them as targeting devices for gene delivery systems. Of all the vector systems currently being explored, adenoviruses have the greatest potential to test the principles of cancer gene therapy (34, 39-43). Here we plan to develop a new approach for vector targeting. We will first obtain virus-binding antibodies that can ablate the endogenous tropism of the adenovirus vectors. These antibodies will then be utilized to form molecular conjugates with the homing peptides in order to redirect adenovirus gene transfer upon systemic delivery.

The studies undertaken in this proposal specifically focused on the use of peptide sequences with selective angiogenic vasculature targeting properties. We are now seeking to validate these probes as delivery vehicles in targeting approaches.

Phage capable of homing to tumor angiogenic vasculature were recovered from a phage display peptide library following intravenous administration. Using this strategy, we have isolated several tumor-homing phage. Among those were phage displaying the tripeptide asparagine-glycine-arginine (NGR), glycine-serine-arginine (GSL), and a double cyclic RGD (RGD-4C). We have shown that each of those peptides binds to three different receptors in tumor angiogenic vasculature. Based on our in vivo studies targeting tumors with NGR-, GSL- and RGD-4C-phage, our idea is that such peptides are suitable for the generation of molecular adapters for the targeted delivery of therapies to angiogenic vasculature.

PROPOSAL BODY

The tasks originally approved for this proposal are listed below.

1. To perform in vivo screenings to select phage-displayed peptides that home into human breast cancer xenografts. We will use various phage peptide library configurations and a series of human breast carcinoma cell lines injected in the mammary fat pad of immuno-deficient mice.
2. To test the tumor-homing phage selected in the screenings for their tumor vasculature and tumor stroma binding specificities. Each tumor homing phage

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displayed peptides will be tested individually for their ability to home into tumors *in vivo*. Immuno-staining experiments will be performed to ascertain the location of the target molecules.

3. To generate a panel of monoclonal antibodies against adenovirus. RBF superimmune mice will be immunized with adenovirus. Hybridomas will be screened using a number of strategies and clones producing antibodies to surface viral antigens will be characterized.
4. To use the reagents described above to produce Fab conjugates that can be used for maximizing the delivery of Adenovirus-based gene vectors to tumors *in vivo*.

In the following pages we report on significant progress on each specific aim during the past year.

We have isolated phage that home into tumors. The distinct phage display 3 classes of peptides (NGR, GSL and RGD-4C). We have shown that phage displaying such peptides bind selectively to angiogenic tumor vessels when injected into tumor-bearing mice (21). The RGD-4C peptide binds to α_v integrins in angiogenic vessels (17, 21). The receptor for NGR-containing peptides in the tumor vasculature is CD13 (22, 78). We have evidence that the receptor for the GSL-containing peptides is the proteoglycan NG2 (49, 93).

The phage methodology that we have developed is appropriate for the search of homing sequences, and it can serve as a model for the selective targeting of angiogenic vasculature. Our published results show that when cytotoxic drugs are coupled to peptide motifs that target tumor vasculature, they appear to be more effective and to cause fewer untoward effects than when the drugs are administered alone. To demonstrate the feasibility of therapeutic tumor targeting, we coupled a tumor-targeting peptide to doxorubicin to see if the peptides could improve the efficacy and/or toxicity profile of a drug. We have shown that this could be accomplished in an impressive manner. Doxorubicin, when coupled to a tumor-targeting peptide, is both less toxic and more effective than free doxorubicin or doxorubicin coupled to an unrelated peptide (see below) (21).

Many existing therapies could be made more effective if site-specific delivery could be achieved, particularly vectors for cancer gene therapy (39-43). The utility of adenovirus vectors for gene therapy is being intensely investigated, and there are a number of genetic approaches that are being explored for the treatment of cancer. These include the transfer of "suicide" genes that convert inactive pro-drugs into cytotoxic compounds, transcomplementation of defective tumor-suppressor genes and the use of oncolytic viruses (34, 75, 76).

As discussed above, vectors that drive the expression of antiangiogenic agents such as endostatin and angiostatin, as well as suicide or pro-apoptotic genes have also shown promise as antitumor agents. In an independent but related line of work, our group has recently reported on novel homing-proapoptotic peptides for cancer treatment (77).

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We found that some cell death receptors require embedded pro-apoptotic peptide sequences (80). These sequences and structurally similar pro-apoptotic antibiotic peptides disrupt negatively charged membranes and thus induce mitochondrial swelling and mitochondria-dependent cell-free apoptosis (81). We integrated this concept with our vasculature targeting technology by introducing homing pro-apoptotic peptides (HPPs) composed of 2 functional domains. The homing domain is designed to guide the HPP to targeted cells and allow its internalization. The pro-apoptotic domain is non-toxic outside of cells but promotes disruption of mitochondrial membranes and subsequent cell death when internalized by target cells. Our prototype peptide is only 21 amino acids long, is selectively toxic to endothelial cells undergoing angiogenesis *in vitro*, and has strong anti-tumor activities in mice. For the homing domain, we used either the cyclic (disulfide bond between cysteines) CNGRC peptide or the double cyclic ACDCRGDCFC peptide, both of which have tumor-homing properties (17, 21) and for which there is evidence of internalization (21, 82, 83). We synthesized this domain from all L-amino acids because of the presumed chiral nature of its receptor interaction. Homing pro-apoptotic peptides represent a new class of anti-cancer agents that can be optimized for maximum therapeutic effect by adjusting properties such as residue placement, domain length, peptide hydrophobicity and hydrophobic moment (84). Beyond this, future HPPs might be designed to disrupt membranes using a completely different type of pro-apoptotic domain such as β -strand/sheet-forming peptides (85). Our results provide a glimpse at the potential of a novel cancer therapy combining two principles of specificity — homing to targeted cells and selective apoptosis of targeted cells (77).

Here we proposed to extend our findings with drug conjugates and targeted pro-apoptotic peptides, by developing molecular adaptors that can be used to target gene therapy vectors to tumor vasculature *in vivo* following intravenous administration. Our hypothesis is that the homing peptides we have isolated can be incorporated into targeted gene transfer methodologies that will increase the efficiency and decrease the harmful effects of delivering genes into normal cells. Specifically we will compare the properties of various tumor-targeting peptides for their ability to target adenovirus vectors when combined as molecular adaptors to peptides that bind to the viral gene therapy vectors.

Our results suggest that it may be possible to use av-, CD13-, and NG2-directed peptides (RGD-4C, CNGRC, and GSL) to target genes to angiogenic vasculature and from there, into the target tissue. Peptides may have advantages in this regard, especially when compared to antibody-based approaches, because they are smaller, more likely to diffuse efficiently within the tumor, and less likely to be immunogenic. Targeted delivery of gene therapy vectors, especially adenoviral ones, may also lead to reduced immunogenicity against the virus, one of the major complicating factors that hamper the success and efficiency of gene therapy-based approaches to date.

Targeting of tumor vasculature with phage

We reasoned that *in vivo* selection could be used to target endothelial markers on angiogenic tumor vessels. We have tested this hypothesis and have successfully targeted the blood vessels in several human tumor types. Injection of phage libraries

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into the circulation of mice bearing human breast carcinoma xenografts followed by recovery of phage from the tumors led to the identification of a number of peptide motifs that selectively directed the phage into the tumors. Different libraries yielded different peptides, but three main motifs emerged. One of the motifs contained the sequence RGD (arginine-glycine-aspartic acid; (53)). The RGD sequence was embedded in a peptide structure that, as we have previously shown, binds selectively to α_v integrins (54, 17). As the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are known markers of angiogenic vessels (12, 55, 91), we had previously tested phage carrying this motif, CDCRGDCFC (termed RGD-4C), for tumor targeting. This phage homes into tumors in a highly selective manner and its homing is specifically inhibited by the cognate peptide (17, 21). One of the two new peptides that accumulated in tumors was derived from a library with the structure CX3CX3CX3C (21). This peptide, CNGRCVSGCAGRC, contained the NGR (asparagine-glycine-arginine) motif, which has been identified previously as a cell adhesion motif (56, 57). We tested two other peptides that contain the NGR motif, but are otherwise entirely different from the CNGRCVSGCAGRC peptide. One of them is a linear peptide, NGRAHA (56), and the other a cyclic one, CVLNGRMEC. The CNGRCVSGCAGRC-phage and both of the other NGR-displaying phage homed into the tumors. The tumor homing was not dependent on the tumor type or on species; the phage accumulated selectively in the human breast carcinoma used in the selection, as well as in a human Kaposi's sarcoma and a mouse melanoma (data not shown). We synthesized the minimal cyclic NGR peptide from the CNGRCVSGCAGRC-phage and found that this peptide (CNGRC), when co-injected with the phage, inhibited the accumulation of CNGRCVSGCAGRC-phage and of the two other NGR-displaying phage in breast carcinoma xenografts.

The RGD-4C homes selectively to the breast cancer tumor and its homing is readily inhibited by the free RGD-4C peptide (17). The tumor homing of RGD-4C phage was not inhibited by the CNGRC peptide, even when the peptide was used in amounts 10-fold greater than those that inhibited the homing of the NGR phage. The tumor homing of the NGR phage was also partially inhibited by the RGD-4C peptide, but this peptide was 5-10 times less potent than the CNGRC peptide. Thus, the two peptides displaying RGD and NGR appear to bind to different, albeit perhaps somehow related, receptor sites in tumor vasculature. An unrelated cyclic peptide, GACVFSIAHECGA had no effect in the tumor homing ability of either phage.

Immunostaining of tissues for phage also showed that the NGR phage homing selectively homes into tumors. In one set of experiments, phage was allowed to circulate for 3-5 minutes, followed by perfusion. In the second set of experiments, tissues were analyzed 24 hours after phage injection. At this time point, there is almost no phage left in the circulation, and perfusion is no longer needed (17, 21). Strong phage staining was seen in tumor vasculature but not in normal endothelia. Testing of the CNGRCVSGCAGRC-phage homing to MDA-MB-435 cell-derived breast carcinoma xenografts and KS1767 cell-derived Kaposi's sarcoma xenografts gave a similar result. The two other NGR phage, NGRAHA and CVLNGRMEC, also showed strong tumor staining (data not shown). In both tumor types, phage was clearly detected in the tumor vessels in the 3-5 minute time frame. The phage appeared to have accumulated and the staining was spread outside the blood vessels and into the tumors at 24 hours.

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Increased permeability of tumor blood vessels (23, 48) may account for the spreading of phage proteins into the parenchyma of tumors. Receptor-mediated internalization by angiogenic endothelial cells (21, 82, 83), and subsequent transfer to tumor tissue may also play a role.

The CNGRCVSGCAGRC-phage yielded the largest difference between phage staining in tumor tissues compared to normal tissues among all of the tumor-homing peptides analyzed. Several control organs were also studied and gave very low or no immunostaining, confirming the specificity of the NGR motifs for tumor vessels (21). Spleen and liver contained detectable phage; the uptake by the reticuloendothelial system is a general property of the phage particle and independent of the peptide it displays (16, 17). These immunostaining results with the NGR phage are similar to observations made with the RGD-4C phage (17). The time frame for the phage staining experiments presented was based on our previous experiments on kinetic of phage clearance after intravenous administration (17, 21, 79). We have more recently also examined the intermediate time points (1, 3, 8, 12h), and observed similar results.

Control phage were injected in tumor-bearing mice and showed no homing to tumors. Those controls include (i) phage without insert, (ii) unselected phage library mixtures, (iii) phage selected and shown to home to other normal vascular beds, and (iv) phage displaying peptides that are unrelated to NGR. In addition, an insertless phage with a different selective marker - ampicillin instead of tetracyclin (79) - was co-injected with the NGR phage at the same input to assess specificity within the same tumor-bearing animal. Plating the phage recovered from the tissues on tetracyclin and ampicillin plates showed that over 10-fold more NGR phage than ampicillin phage accumulated in the tumor. In contrast, slightly more ampicillin phage than the NGR phage was recovered from other control organs tested. Moreover, co-injection of CNGRC-phage with a 10-fold excess of phage particles engineered to be non-infective did not affect tumor homing, whereas this procedure decreased the accumulation of phage in trapping organs that are members of the reticuloendothelial system, such as the spleen and the liver (17).

The accumulation of the NGR phage into tumors is not a result of increased tumor vascularization in comparison to normal tissues. There is an extensive body of literature to the effect that tumors do not contain more blood vessels than normal organs (23). In fact, quite the opposite is observed; it is well established that solid tumors are less vascularized than many normal tissues. In a tumor, approximately 100 tumor cells can be supported by one endothelial cell. In contrast, every cell within normal tissue lives adjacent to an endothelial cell in a capillary blood vessel, or in certain cases, lies between two capillary blood vessels (23). Therefore, the accumulation of the NGR phage in tumor vasculature is specific and reflects NGR-phage binding to tumor vasculature, and not simply phage trapping.

These experiments make an important point because they demonstrate that it is possible to develop probes that target angiogenic vasculature, a common feature in all solid tumors. Moreover, targeting tumor vasculature, unlike conventional tumor targeting, possesses an intrinsic amplification mechanism; it has been estimated that 100 tumor cells should die for each destroyed endothelial cell in tumor blood vessels. Finally,

because tumor endothelial cells are diploid and nonmalignant, they are unlikely to lose a cell surface target receptor or acquire resistance to therapy through mutation and clonal evolution (28-30).

Receptors for NGR, RGD-4C and GSL tumor vasculature-homing phage

The fact that the receptors for our tumor-homing phage are known represent a major advantage because one is able to evaluate the presence of the receptors in human tumors using antibodies in addition to phage. This information is obviously crucial in terms of the development of realistic therapy targeting strategies that can be applicable to cancer patients.

Several lines of evidence have implicated the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, the receptors for the RGD-4C tumor-homing phage, in the angiogenic process. It has been shown that αv integrins are selectively expressed in angiogenic vasculature in human tumors but not selectively expressed in normal vasculature (17, 21, 55). Moreover, αv integrin antagonists have been shown to block the growth of neovessels (12, 13, 64); in these experiments, endothelial cell apoptosis was identified as the explanation for the inhibition of angiogenesis (12, 13). Concordant with these findings, it appears that two distinct cytokine-induced pathways that lead to angiogenesis depend on specific αv integrins. Angiogenesis initiated by bFGF can be inhibited by an anti- $\alpha v\beta 3$ blocking antibody, whereas VEGF-mediated angiogenesis can be prevented by a blocking antibody against $\alpha v\beta 5$. The integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ have been reported to be preferentially displayed in different types of ocular neovascular disease in humans (55, 91).

As for the characterization of the NGR receptor - CD13 - we have evidence that NGR-containing phage co-localizes with CD13/APN in human tumors where it binds to tumor blood vessels but not to resting vessels in normal tissues. We also have immunohistochemical evidence also that CNGRC-phage binds to human tumor vessels in tissue sections but not to normal vessels. A negative control phage with no insert (fd phage) did not bind to normal or tumor tissue sections. These findings prompted us to evaluate expression of CD13/APN in cell lines, in normal vasculature and in the vasculature of tumors and other angiogenic tissues.

Flow cytometry and immunohistochemistry showed that CD13/APN is expressed in a number of tumor cells and HUVECs. CD13/APN was not detected in the vasculature of normal organs of mouse and human tissues. Immunohistochemistry also enabled us to analyze the distribution of CD13/APN in tumor cells, tumor vasculature, and normal vasculature. Our studies clearly demonstrate that CD13/APN, the NGR receptor in tumor vessels, is specifically expressed in angiogenic endothelial cells and pericytes of both human and mouse tissue.

Confocal microscopy showed that CD13/APN expression is confined to the endothelial cells and pericytes in breast carcinoma human tissue sections. Similar staining patterns were obtained with the NGR phage and the anti-CD13 antibodies. In each case, reactivity could be detected in the vasculature of the tumors but not in that of normal tissues. Unquestionable direct evidence for the identity of the NGR receptor as CD13 derived from recent experiments involving studies using CNGRC phage and anti-CD13 antibodies. We have shown that the staining with anti-CD13 antibodies in human tumor tissue sections can be abrogated by pre-incubation of the tumor section with

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NGR-phage but not a control phage (78). We have also demonstrated that the homing of the CNGRC phage into tumors *in vivo* is inhibited by co-injection with anti-CD13 antibodies, but not control antibodies. In some of the experiments, the tumors were generated by injecting MDA-MD-435 breast carcinoma, a cell line that does not express CD13. Conversely, Hodgkin's lymphoma, C8161 melanoma, and Kaposi's sarcoma cell lines express high levels of CD13. The targeting of the CNGRC-phage or the CNGRC-dox conjugate *in vivo* is equally efficient, whether or not the tumor cells express CD13/NGR receptor.

During the past decade, 2 independent lines of research have lead to compelling evidence that the proteoglycan NG2 is upregulated in angiogenic vasculature. Elegant immunohistochemical studies have shown that NG2 is highly expressed in pericytes (49, 93). In collaboration with Dr. William Stallcup at the Burnham Institute, we have shown that NG2 not only is overexpressed, but that it plays an important role in tumor and retinal neovascularization. An interesting connection between NG2 and our GSL tumor homing peptide came to light because we found that *in vitro* panning on immobilized NG2 yielded GSL-containing phage. GSL-phage, as well as other novel NG2-binding peptides isolated *in vitro*, mediate selective phage homing to retinal neovascularization. Experiments using wild-type and NG2-null mice bearing tumors further demonstrated that NG2 serves as a target for tumor-vasculature homing phage (49). We have *in vitro* and *in vivo* data strongly indicating that GSL-phage specifically binds to NG2.

Proteoglycans have been shown to function in the binding and entry of many viruses into cells (59). Proteoglycans have also been shown to mediate gene transfer into cultured cells by methods relying on polylysine or cationic liposomes (59). These observations suggest that NG2 might be a suitable receptor for the uptake of targeted gene therapy vectors *in vivo*. We plan to explore this possibility using GSL-containing molecular adaptors.

Molecular adaptors for targeted delivery of genes to angiogenic vasculature Selection of adenovirus-binding antibodies

In order to establish molecular adaptors with dual specificities (containing a tumor vasculature-homing moiety and an adenovirus-binding moiety), we have generated monoclonal antibodies against adenovirus. We were successful in isolating distinct monoclonal antibodies that interact with adenovirus. We have determined the affinity and specificity for each of the antibodies by performing ELISA assays and Biacore studies. Clones 3B2 and 1C5 bind to adenovirus fiber knob, as determined by ELISA, using a recombinant fiber knob fusion protein.

We have further characterized the anti-adenovirus antibodies by investigating whether the antibodies can block adenovirus infectivity. The antibody 1C5 was the most effective in this type of assay. We have also tested the reactivity of multiple clones for their ability to recognize adenoviral proteins by Western Blot analysis and immunofluorescence of infected cells. Both clones 1C5 and 3B2 reacted strongly with adenovirus-infected cells.

The next step is to conjugate the best antibodies to our tumor-homing peptides and evaluate their potential as gene therapy targeting. *In vitro*, we were able to show -

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using two different kinds of antibody conjugates - that impressive targeting effects can be achieved. As a prototype, we have used a peptide that recognizes membrane dipeptidase. This peptide contains the motif GFE. MDP is a protease that is highly expressed on the surface of certain tumor cell lines. Increased gene expression using a promoter gene was observed when 436 breast carcinoma monolayers were incubated with adenovirus in the presence of the conjugate. Using chemical conjugates, we could also detect a Fc-dependent targeting effect using antibody conjugate made with the 1C5 monoclonal antibody.

KEY RESEARCH ACCOMPLISHMENTS

We have performed *in vivo* screenings and have selected phage-displayed peptides that home into human breast cancer xenografts.

We have characterized the tumor-homing phage selected in the screenings for their selectivity in homing to tumor vasculature.

We have characterized the receptors for tumor homing peptides in angiogenic vasculature.

We have generated and characterized a panel of monoclonal antibodies against adenovirus.

REPORT OUTCOMES

A manuscript is in preparation to report the results of this project.

CONCLUSION

The ability to target genes to malignant tumors has been a long-standing goal in medical oncology. Unfortunately, to date, there are only a few selected situations in which targeted delivery is actually feasible. Tumor targeting approaches tend to be either highly invasive or suffer from a lack of specificity and incomplete tissue penetration. Several lines of research have recently converged to explore vascular targeting by taking advantage of the differences between the newly formed vessels in tumors and the mature vessels in normal tissues.

Our approach is particularly novel because it directly selects *in vivo* for circulating probes capable of preferential homing into tumors. We have now uncovered new markers in the vasculature of tumor vasculature, providing a new means for selective targeting of therapies and new insights into endothelial tissue specificities. During the remaining of the grant duration, we plan to use the reagents described above to produce Fab conjugates that can be used for maximizing the delivery of Adenovirus-based gene vectors to tumors *in vivo*.

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APPENDICES

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