Original Article

Tumor Vasculature-targeted Delivery of Tumor Necrosis Factor-α

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BACKGROUND: Recently, considerable efforts have been directed toward antivascular therapy as a new modality to treat human cancers. However, targeting a therapeutic gene of interest to the tumor vasculature with minimal toxicity to other tissues remains the objective of antivascular gene therapy. Tumor necrosis factor-α (TNF-α) is a potent antivascular agent but has limited clinical utility because of significant systemic toxicity. At the maximum tolerated doses of systemic TNF-α, there is no meaningful antitumor activity. Hence, the objective of this study was to deliver TNF-α targeted to tumor vasculature by systemic delivery to examine its antitumor activity. **METHODS:** A hybrid adeno-associated virus phage vector (AAVP) was used that targets tumor endothelium to express TNF-α (AAVP-TNF-α). The activity of AAVP-TNF-α was analyzed in various in vitro and in vivo settings using a human melanoma tumor model. **RESULTS:** In vitro, AAVP-TNF-α infection of human melanoma cells resulted in high levels of TNF-α expression. Systemic administration of targeted AAVP-TNF-α to melanoma xenografts in mice produced the specific delivery of virus to tumor vasculature. In contrast, the nontargeted vector did not target to tumor vasculature. Targeted AAVP delivery resulted in expression of TNF-α, induction of apoptosis in tumor vessels, and significant inhibition of tumor growth. No systemic toxicity to normal organs was observed. **CONCLUSIONS:** Targeted AAVP vectors can be used to deliver TNF-α specifically to tumor vasculature, potentially reducing its systemic toxicity. Because TNF-α is a promising antivascular agent that currently is limited by its toxicity, the current results suggest the potential for clinical translation of this strategy. **Cancer 2009;115:128-39. Published 2008 by the American Cancer Society.***

KEY WORDS: targeted gene therapy, tumor necrosis factor α, adeno-associated virus phage vector, antivascular, human melanoma.

Given the inherent molecular diversity between normal and tumor vasculature, tumor vasculature appears to be an important target for the delivery of antivascular agents.[1] There has been renewed interest

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in the development of new vascular-targeting agents as well as improvements in the delivery of existing agents to damage established tumor blood vessels. Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine with both antivascular and antitumor effects.[2] TNF-α induces tissue factor production and increases vascular permeability and procoagulant activity on endothelial cells.[3-5] Unfortunately, recombinant TNF-α has limited clinical utility because of predictable but severe systemic toxicity. At the maximum tolerated doses, there is no meaningful antitumor activity when TNF-α is delivered systemically. However, in isolated limb and organ perfusions in which the effects of TNF-α are limited to the perfused tissue, TNF-α has activity in the treatment of human primary melanomas, sarcomas, and metastatic hepatic tumors[6-8] with minimal systemic toxicity. Nevertheless, most metastatic tumors are not amenable to treatment in an isolated perfusion setting. Thus, although targeted delivery of TNF-α to tumor vasculature can overcome the systemic toxicity, a systemic route of administration would be very desirable. To develop such a strategy, we have studied the vascular-targeted delivery of TNF-α by using a novel gene therapy approach.

Considering the various limitations of current vector systems, we chose to evaluate a targeted adeno-associated virus phage vector (AAVP), a hybrid prokaryotic/eukaryotic vector,[9] to deliver TNF-α. Recently, a hybrid vector using elements of adeno-associated virus (AAV) and fd-tet phage,[10] an M13-based bacteriophage, was developed.[9] Bacteriophage vectors have no native tropism for mammalian cells; however, they can be engineered to target specific eukaryotic cells by altering the expression of surface peptides. The AAVP vector used in this study targets gene products to tumor vasculature by using an αv integrin ligand (termed RGD-4C) motif.[9] Compared with quiescent, established blood vessels, endothelial cells in angiogenic blood vessels express additional proteins, such as the αvβ3, αvβ5, and α5β1 integrins.[11] Bacteriophage vectors have been used to deliver mammalian genes in their native form; however, the transduction efficiency is extremely low.[12] Hence, there is a need for AAVP, which retains the advantageous features of bacteriophage vectors while increasing transduction efficiency through the use of elements of adeno-associated virus (AAV).

In this report, we describe the first study to our knowledge on the use of targeted AAVP to deliver the therapeutic gene *TNF*-α to tumor vasculature in human melanoma xenografts. Our results demonstrate that, through systemic administration, targeted RGD-4C AAVP-expressing TNF-α (RGD-A-TNF) can be delivered selectively to tumor vasculature with no gene product expression in normal organs. The expression of the gene product resulted in apoptosis/necrosis of tumor vessels and in significant tumor reduction. Thus, AAVP vectors may enable the targeted delivery of antivascular agents to tumor vasculature with no systemic toxicity.

MATERIALS AND METHODS

Cell Culture

M21 human melanoma cells were grown in RPMI 1640 medium containing 10% serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL gentamicin, and 250 μg/mL fungizone.

Construction of a Targeted Adeno-associated Virus Phage Vector-expressing Tumor Necrosis Factor-α

The general design and construction of the AAVP backbone is described elsewhere.[9,13] An AAVP construct that expresses TNF-α was created in 2 steps. First, an 880-base pair NotI/HindIII fragment from pG1Si*TNF* was digested and ligated into a pAAV-e*GFP*/NotI/HindIII vector replacing green fluorescent protein gene (*GFP*) sequences.[14] In the second step, fdMCS/RGDMCS and AAV-TNF-α were digested by PvuII.[9,13] Then, AAV-TNF-α containing inverted terminal repeats were religated into the fdMCS-/RGDMCS PvuII site to obtain the AAVP vectors. Thus, fd-A-TNF is a nontargeted vector, whereas RGD-A-TNF is a targeted vector with binding affinity to αv integrin receptors on the cell surface. The final vector size was 11.7 Kb.

Adeno-associated Virus Phage Vector Particle Purification

To obtain nontargeted and targeted AAVP particles, DNA was electroporated into MC1061 *Escherichia coli*, and virus particles were purified from the culture

supernatant fluid. The methodology has been described in details previously.[9,13] Large-scale AAVP particles were purified from permissive k91Kan cells. To determine the number of bacterial transducing units (TUs), k91Kan cells were infected with serial dilutions of AAVP particles and plated on Luria-Bertani agar plates containing tetracycline and kanamycin. Then, the numbers of TUs were determined by counting the number of bacterial colonies.

Immunofluorescence Assay

Immunofluorescence (IF) was used to observe internalized viral particles in M21 cells. Briefly, cells grown on 8-well Lab-Tek chamber glass slides (Nunc, Rochester, NY) were infected with AAVP particles by using Dulbecco Modified Eagle Medium containing 10% serum at 37°C for 16 hours. After infection, cells were fixed in 3.7% formaldehyde for 10 minutes, permeabilized by 0.1% saponin (Sigma Chemical Company, St. Louis, Mo) in phosphate-buffered saline (PBS), and blocked with blocking buffer (PBS containing 1% bovine serum albumin, 0.025% sodium azide, and 0.1% saponin) for 15 minutes. Next, cells were incubated with a mouse antibacteriophage antibody for 1 hour followed by a fluorescein isothiocyanate-conjugated antimouse immunoglobulin G antibody for 1 hour. Gaskets were detached and cells were mounted by using Antifade (MP Biomedicals, Solon, Ohio) and were examined under a Zeiss Axiovert fluorescent microscope (Zeiss Inc., Germany).

Adeno-associated Virus Phage Vector Virus Recovery From Infected Cells

M21 cells were infected either with vehicle or with AAVP at 37°C for 3 hours. After this infection was completed, cells were washed with Hank balanced salt solution (Quality Biologicals, Inc., Gaithersburg, Md), and uninfected AAVP particles were inactivated by subtilisin treatment (Sigma Chemical Company) for 15 minutes. Internalized AAVP particles were obtained by treating infected cells with the lysis buffer (2% deoxycholic acid, 2 mM ethylene diamine tetracetic acid, and 10 mM Tris [pH 8.0]) for 1 hour. The number of AAVP particles were counted as TUs using k91Kan cells as described above.

Adeno-associated Virus Phage Vector-mediated Gene Expression

Culture supernatant fluids were collected to measure secretable human TNF-α levels by using an enzyme-linked immunosorbent assay (ELISA) (Invitrogen, Carlsbad, Calif). For tumor tissues, total cell lysates were prepared using lysis buffer (50 mM Tris, pH 7.4; 140 mM NaCl; 0.1% sodium dodecyl sulfate; 1% NP40; and 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Roche, Branchburg, NJ). The lysates were cleared by centrifugation at 13,000 revolutions per minute for 10 minutes. The amount of protein was quantified by using the protein assay reagent (BioRad, Hercules, Calif). The amount of lysate equivalent to 100 μg of total protein was assayed for human TNF-α by ELISA (Biosource, Camarillo, Calif).

In Vivo Adeno-associated Virus Phage Vector Delivery and Detection

All animal experiments were conducted according to protocols approved by the National Institutes of Health Animal Care and Use Committee. Female athymic nude mice were obtained from Jackson Laboratories and housed in the animal facility of the National Cancer Institutes. Human melanoma cells (4×10^6) were inoculated subcutaneously into the right flank of nude mice. Tumor volume (mm^3) was measured in 3 dimensions and calculated as length × width × height × 0.52. When tumor volumes reached approximately 100 to 150 mm^3, 1×10^{11} AAVP particles were administered intravenously into the tail vein. Each cohort had 3 animals. Animals were killed at established time intervals. Resected tumor tissues and normal tissues (liver, lung, kidney, brain, heart, spleen, and skeletal muscle) were snap frozen for further analysis.

To detect the presence of AAVP, 5-μM-thick frozen sections were stained by dual IF staining. Briefly, sections were fixed in 4% paraformaldehyde for 20 minutes and then were treated in acetone for 10 minutes. Permeabilization was done in PBS containing 0.3% Triton-X-100 for 15 minutes. Nonspecific binding was blocked with blocking buffer (5% goat serum and 2.5% bovine serum albumin in PBS) for 1 hour at room temperature. The primary antibodies were applied overnight at 4°C in a 1:1000 dilution of rabbit anti-fd bacteriophage antibody

(Sigma Chemical Company) and a 1:50 dilution of rat antimouse CD31 (BD Biosciences, San Jose, Calif). Next, slides were incubated with the secondary antibodies (Invitrogen): We used 1:200 dilutions each of goat antirabbit Alexa Fluor 594 and goat antirat Alexa Fluor 488 for 45 minutes in the dark. After 2 PBS washes, sections were mounted using Vectashield mounting medium with 4′6-diamidino-2-phenylinodole (Vector Laboratories, Burlingame, Calif). Images were taken using a Zeiss LSM 510 confocal fluorescent microscope.

Caspase-3 Staining

Caspase-3 staining was performed exactly the same as the dual IF staining that was used to detect AAVP particles. Rabbit antiactive caspase-3 antibody was used at 1:200 dilution (BD Biosciences), and rat antimouse CD31 was used at 1:50 dilution (BD Biosciences).

Staining for CD31

CD31 staining was performed as follows. Briefly, sections were fixed in acetone for 10 minutes, then washed in PBS, and nonspecific binding was blocked in 10% goat serum for 1 hour. Sections were incubated with 1:50 diluted rat antimouse CD31 antibody overnight (BD Biosciences). Sections were washed with PBS and incubated with 1:500 diluted donkey antirat biotinylated secondary antibody for 1 hour. Sections were incubated with Vectastain Elite avidin-biotin complex (Vector Laboratories, Burlingame, Calif), and staining was detected by using diaminobenzidine tetrahydrochloride substrate (Dako, Carpinteria, Calif) for 5 minutes and a hematoxylin counterstain.

Quantification

Images were captured at ×10 magnification using a Zeiss microscope equipped with a Hamamatsu ORCA-ER fluorescent camera for caspase-3 staining and a Carl Zeiss AxioCam brightfield camera for CD31 staining. For each tumor, 3 different images, 1 from the center of the tumor and 2 from the periphery, were captured at the same settings. Each cohort represents 3 animals; thus, quantification data were analyzed using a total of 9 images. Quantification of staining was performed using Axiovision 4.6 software to obtain the percentage area.

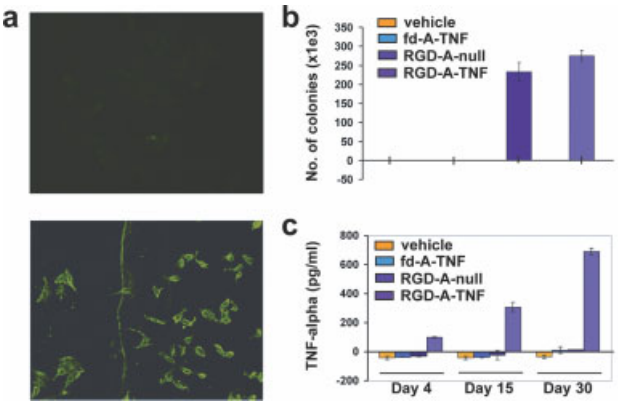

FIGURE 1. Targeted hybrid adeno-associated virus phage vector (AAVP) particles can infect and internalize into mammalian cells. (a) Human melanoma M21 cells were infected with nontargeted AAVP expressing tumor necrosis factor-α (fd-A-TNF) (top) and targeted αV integrin ligand AAVP-expressing TNF-α (RGD-A-TNF) (bottom), and the cells were detected by using an antibacteriophage antibody followed by a fluorescein isothiocyanate-labeled secondary antibody. (b) M21 cells were infected with vehicle, nontargeted fd-A-TNF, RGD-A-null, and RGD-A-TNF for 3 hours in triplicate experiments. After infection, internalized viral particles were recovered in the cellular lysate. The number of AAVP particles was counted as the number of transducing units using k91Kan cells. The y-axis represents the recovered particles as the number of colonies. Error bars indicate the standard error of mean. (c) M21 cells were infected with vehicle, nontargeted fd-A-TNF, RGD-A-null, and RGD-A-TNF. Culture supernatant fluid was analyzed in triplicate experiments to measure TNF-α by enzyme-linked immunosorbent assay at various time points after the infection. Error bars indicate the standard error of mean.

Tumor Growth Analysis

Human melanoma cells (4×10^6) were inoculated subcutaneously into the right flank of nude mice. When tumor volumes reached approximately 50 mm^3, 1×10^{11} AAVP particles were administered systemically through the tail vein. AAVP particle administration was repeated after 7 days. The tumor-bearing mice were followed thereafter by measuring tumor volumes in a blinded fashion. Each treatment cohort had 6 animals.

Statistical Analysis

Groups were compared by using analyses of variance and Tukey comparison post-tests (GraphPad Instat Software, Inc., San Diego, Calif). P values $<.05$ were considered statistically significant.

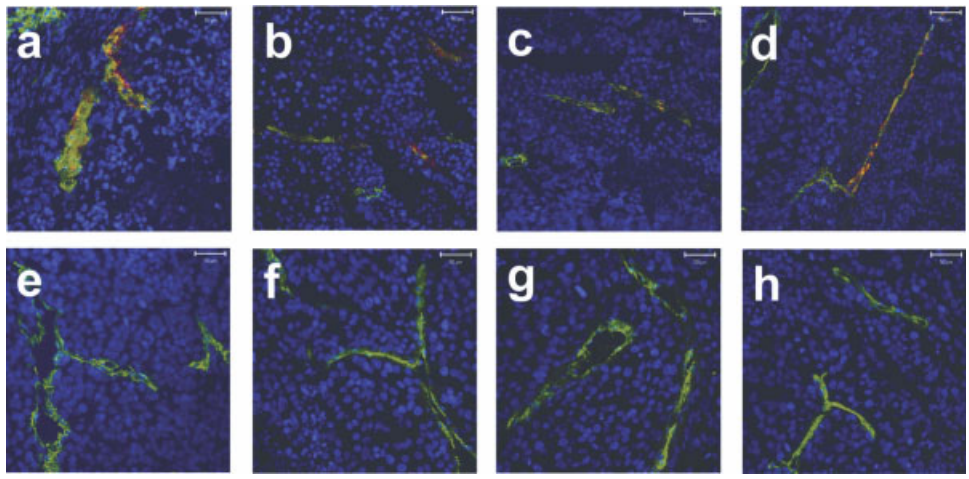

FIGURE 2. The hybrid adeno-associated virus phage vector AAVP can be targeted specifically to tumor vasculature on systemic administration in vivo. Mice bearing human melanoma xenografts were injected systemically with αV integrin ligand AAVP-expressing tumor necrosis factor-α (RGD-A-TNF) (a-d) and with nontargeted fd-A-TNF (e-h) 2 days after injection (a and e), 4 days after injection (b and f), 8 days after injection (c and g), and 10 days after injection (d and h). Frozen tumor biopsies were analyzed for the presence of viral particles by dual immunofluorescence staining. AAVP particles were stained red with bacteriophage antibody (Alexa Fluor 594), blood vessels were stained green with CD31 antibody (Alexa Fluor 488), and nuclei were stained blue with 4′6-diamidino-2-phenylinodole. Scale bar = 50 μM.

RESULTS

Targeted Adeno-associated Virus Phage Vector Particles Can Infect and Internalize Into Mammalian Cells

Both M21 human melanoma cells and the M21 tumor vasculature express αv integrins on their cell surface (data not shown). We observed M21 cell infection with targeted RGD-A-TNF virus (Fig. 1a, bottom). In contrast, nontargeted fd-A-TNF did not infect melanoma cells (Fig. 1a, top). We recovered internalized particles and counted them as TUs using k91 bacterial cells. There was no AAVP internalization by M21 cells infected with either the vehicle or fd-A-TNF (Fig. 1b). In contrast, cells infected with targeted RGD-A-null or RGD-A-TNF showed significant virus internalization, indicating that the addition of the RGD-4C motif results in AAVP internalization in M21 cells.

Adeno-associated Virus Phage Vector-mediated Gene Expression

M21 cells were infected with RGD-A-TNF or controls in triplicates, and production of the TNF-α gene product was measured by ELISA at 3 different time points (Fig. 1c). The gene product was secretable and, thus, could be detected in the culture supernatant fluids. Cells infected with RGD-A-TNF revealed secretion of TNF-α, whereas the vehicle, fd-A-TNF, and RGD-A-null had no detectable levels of TNF-α secretion (Fig. 1c). We observed significantly increasing amounts of the gene product starting at Day 4 and continuing until Day 30, which was the maximum time point tested.

In Vivo Adeno-associated Virus Phage Vector Trafficking to Tumor Vasculature

Viral particles were administered systemically through the tail vein in human melanoma-bearing nude mice. Tumor-bearing mice that received nontargeted fd-A-TNF, RGD-A-null, or RGD-A-TNF were euthanized at 2 days, 4 days, 8 days, and 10 days after injection. Frozen sections from harvested tumors were analyzed for the presence of viral particles by dual IF staining. AAVP particles were stained red with bacteriophage antibody, blood vessels were stained green with CD31 antibody, and nuclei were stained blue (Fig. 2). One representative image at each time point from animals that were injected with RGD-A-TNF and nontargeted fd-A-TNF are shown in Figure 2. Animals that received RGD-A-TNF had colocalization of AAVP within the blood vessels of their tumors. (Fig. 2a-d). Animals that were injected with RGD-A-null, displayed

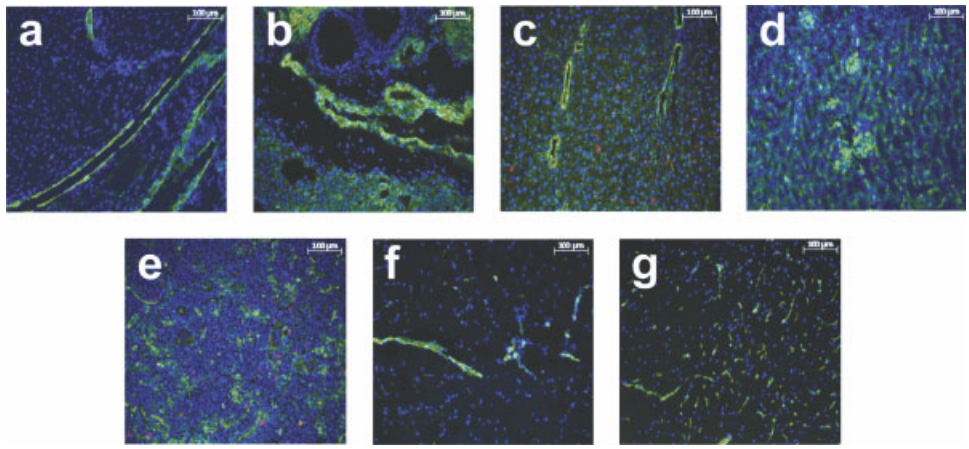

FIGURE 3. The hybrid adeno-associated virus phage vector AAVP does not traffic to normal vasculature on systemic administration in vivo. Mice bearing human melanoma xenografts were injected systemically with αV integrin ligand AAVP-expressing tumor necrosis factor-α (RGD-A-TNF). Normal organs were harvested 4 days after injection, heart (a), lung (b), liver (c), kidney (d), spleen (e), skeletal muscle (f), and brain (g), and frozen sections were analyzed for the presence of viral particles by dual immunofluorescence staining. AAVP particles were stained red with bacteriophage antibody (Alexa Fluor 594), blood vessels were stained green with CD31 antibody (Alexa Fluor 488), and nuclei were stained blue with 4′6-diamidino-2-phenylinodole. Scale bar = 100 μM.

a pattern of vector trafficking to tumor vasculature similar to that displayed by RGD-A-TNF (data not shown). The presence of targeted AAVP was detected at all the time points tested: Day 2 (Fig. 2a), Day 4 (Fig. 2b), Day 8 (Fig. 2c), and Day 10 (Fig. 2d). None of the animals that received nontargeted fd-A-TNF showed the presence of AAVP in tumor vasculature at any time point tested (Fig. 2e-h).

In Vivo Adeno-associated Virus Phage Vector Does Not Traffic to Normal Vasculature

Furthermore, we evaluated whether systemically administered AAVP was localized in normal tissue vasculature. We tested heart, lung, liver, kidney, spleen, skeletal muscle, and brain tissue specimens that were harvested 4 days after tumor-bearing animals received RGD-A-TNF. Frozen sections from normal tissues were analyzed for the presence of viral particles by dual IF staining. AAVP particles were stained red with fd antibody, blood vessels were stained green with CD31 antibody, and nuclei were stained blue (Fig. 3). Figure 3 shows 1 representative image each of tissue from the heart (Fig. 3a), lung (Fig. 3b), liver (Fig. 3c), kidney (Fig. 3d), spleen (Fig. 3e), skeletal muscle (Fig. 3f), and brain (Fig. 3g). The presence of AAVP was not observed in the animals that received RGD-A-TNF in tissues from the heart, lung, kidney, skeletal muscle, or brain. Although we observed the presence of some AAVP particles in the liver and spleen (Fig. 3c and e), the AAVP particles did not colocalize with blood vessels from these normal tissues.

In Vivo Expression of Gene Product by Adeno-associated Virus Phage Vector

To determine whether the animals that received RGD-A-TNF expressed the gene product in their tumors, we analyzed tumor tissues on Days 4, 8, and 10 in triplicate experiments for TNF-α protein levels by ELISA (Fig. 4a). Mice that received RGD-A-TNF had significant tumor expression of TNF-α at Day 4, Day 8, and Day 10. In contrast, mice that received nontargeted fd-A-TNF or RGD-A-null did not have tumor expression of TNF-α. Figure 4 shows individual tumors from each cohort.

We also analyzed normal tissues from 2 animals that received RGD-A-TNF for gene expression, because we had observed the presence of AAVP in the liver and spleen of these animals (Fig. 4b). Both animals had significant levels of TNF-α in tumor tissues, but no gene expression was observed in any of the normal tissue tested, including liver and spleen (Fig. 4b). Thus, despite the presence of AAVP particles in liver and spleen, AAVP-mediated gene expression was very specific to tumor.

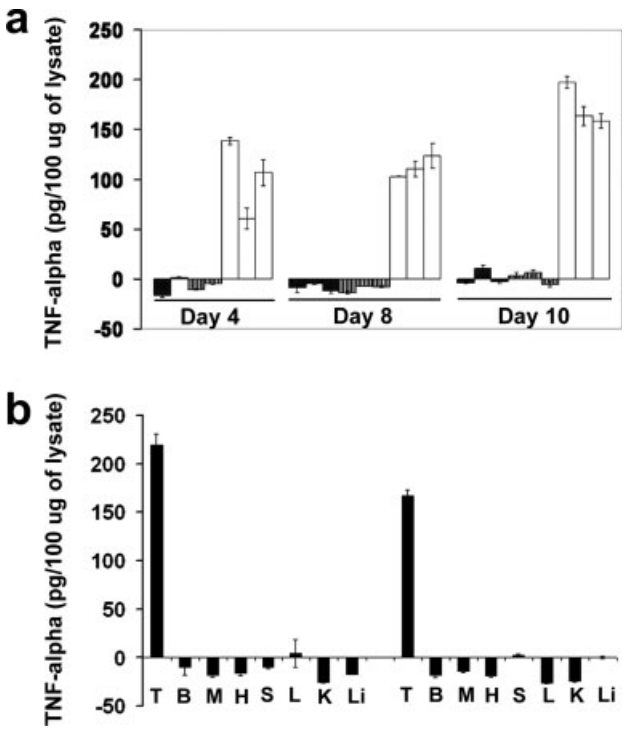

FIGURE 4. The hybrid adeno-associated virus phage vector AAVP can express a specific gene product in tumor tissues but not in normal tissues in vivo. Frozen sections of tumor tissues and normal tissues from animals with human melanoma xenografts that received AAVP were used to extract total protein. One hundred micrograms of total protein were used to measure tumor necrosis factor-α (TNF-α) protein levels using enzyme-linked immunosorbent assays in triplicate. Error bars indicate the standard error of mean. (a) TNF-α levels from frozen sections of tumor tissues from animals that received nontargeted fd-A-TNF (dotted histogram), αV integrin ligand AAVP (RGD-A-null) (histogram with vertical lines), or αV integrin ligand AAVP-expressing TNF-α (RGD-A-TNF) (open histogram) for different time points are shown. The y-axis represents TNF-α levels in picograms in 100 μg of total protein tested. (b) TNF-α levels from frozen sections of tumor tissues (T) and normal tissues that were harvested 4 days after injection; brain (B), skeletal muscle (M.), heart (H), spleen (S), lung (L), kidney (K) and liver (Li) from 2 animals that received RGD-A-TNF are shown. The y-axis represents TNF-α levels in picograms in 100 μg of total protein tested.

Induction of Apoptosis/Necrosis by Adeno-associated Virus Phage Vector

To observe the effect of TNF-α expression, we stained tumor sections from animals that received either RGD-A-null or RGD-A-TNF for an apoptotic marker, caspase-3, using dual IF staining (Fig. 5). We used tumor sections from Day 4. Beyond this time point, M21 tumors revealed some spontaneous necrosis, which made interpretations across various cohorts difficult. Caspase-3 staining indicates cells undergoing apoptosis. Caspase-3 was stained red with caspase-3 antibody, blood vessels were stained green with CD31 antibody, and nuclei were stained blue. Animals that received RGD-A-TNF had colocalization of caspase-3 staining with the blood vessels (Fig. 5d-f). Tumor cells surrounding the blood vessels also were positive for caspase-3 staining in these animals. Animals that were injected with RGD-A-null did not have caspase-3 staining in or around the blood vessels (Fig. 5a-c). In Figure 5, 3 representative fields are shown. We observed a significant increase in caspase-3 staining in animals that received RGD-A-TNF compared with animals that received RGD-A-null (Fig. 5g) ($P = .0100$).

We also stained tumor tissue sections for CD31, a vascular endothelial cell marker, by immunohistochemical staining to observe vascular morphology (Fig. 6). Animals that received either nontargeted fd-A-TNF (Fig. 6a) or RGD-A-null (Fig. 6b) had normal vascular patterns in their tumors. In contrast, animals that received RGD-A-TNF had very few vessels (Fig. 6c). At higher magnification, we observed normal vascular architecture in animals that were injected with nontargeted fd-A-TNF (Fig. 6d) or RGD-A-null (Fig. 6e). However, animals that received RGD-A-TNF had a loss of normal vascular architecture, indicating vascular damage (Fig. 6f). We also observed necrosis of surrounding tumor tissue in animals that received RGD-A-TNF (Fig. 6f). We observed a significant decrease in CD31 staining in animals that received RGD-A-TNF compared with animals that received either fd-A-TNF or RGD-A-null ($P = .0031$) (Fig. 6g).

Inhibition of Tumor Growth in Adeno-associated Virus Phage Vector-treated Animals

We analyzed the effect of RGD-A-TNF on the growth of subcutaneous M21 melanoma xenografts grown in nude mice. Tumor bearing mice were treated systemically at Day 0 and Day 7 with either vehicle, or nontargeted fd-A-TNF, RGD-A-null, or RGD-A-TNF (Fig. 7). Tumor growth was monitored for 27 days. On Day 27, which was the final measured time point for the different cohorts, the vehicle-treated group had mean (± standard deviation) tumor volume of 743 ± 383 mm^3, the nontargeted fd-A-TNF group had mean tumor volume of 613 ± 155 mm^3, the RGD-A-

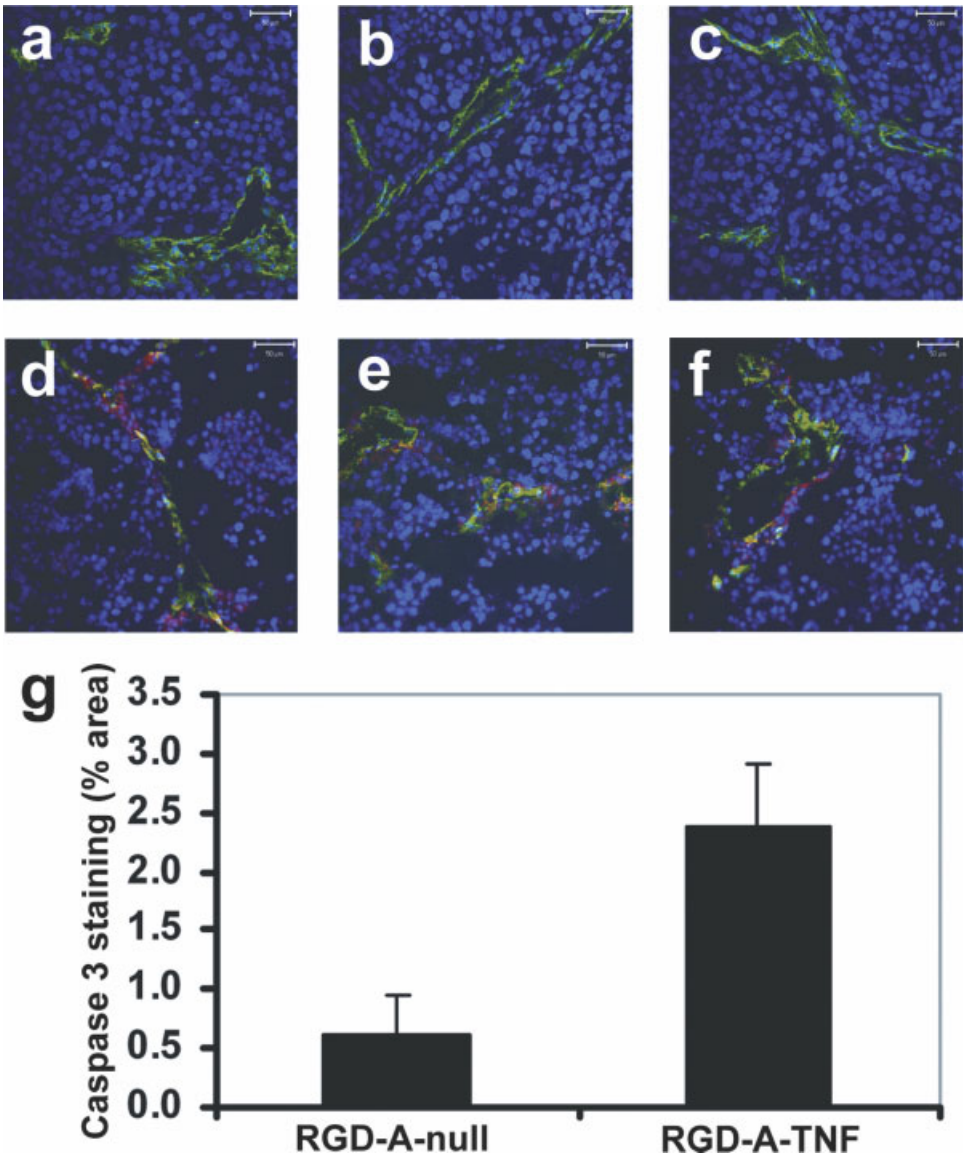

FIGURE 5. Targeted αV integrin ligand adeno-associated virus phage vector-expressing tumor necrosis factor-α (RGD-A-TNF) induces apoptosis in tumor vessels in vivo. Day 4 tumor sections from animals that received either RGD-A-null (n = 3) (a-c) or RGD-A-TNF (n = 3) (d-f) were stained for an apoptotic marker, caspase-3, by using dual immunofluorescence staining. Caspase-3 was stained red with caspase-3 antibody (Alexa Fluor 594), blood vessels were stained green with CD31 antibody (Alexa Fluor 488), and nuclei were stained blue with 4′6-diamidino-2-phenylinodole. Three representative fields are shown. Scale bar = 50 μM. Caspase-3 staining was quantified and is represented as the percentage area (g). Error bars indicate the standard error of mean.

null group had mean tumor volume of 622 ± 141 mm^3, and the RGD-A-TNF cohort had mean tumor volume of 358 ± 98 mm^3 ($P = .048$) (Fig. 7). The reduction in tumor volume in the RGD-A-TNF cohort was statistically significant starting on Day 20.

DISCUSSION

In the current study, we analyzed the selective delivery of antivascular therapy to tumor vasculature using a new, targeted AAVP vector. We investigated the fate of the AAVP vector that expressed the antivascular agent human TNF-α in mammalian cells in vitro and in vivo. The AAVP vector is a chimera between an adeno-associated virus and a single-stranded bacteriophage. Bacteriophage-based vectors have many of the desirable properties of both animal viral and nonviral systems without some of the drawbacks. They lack intrinsic tropism for mammalian cells; however, they have a long history of safe delivery to humans to treat

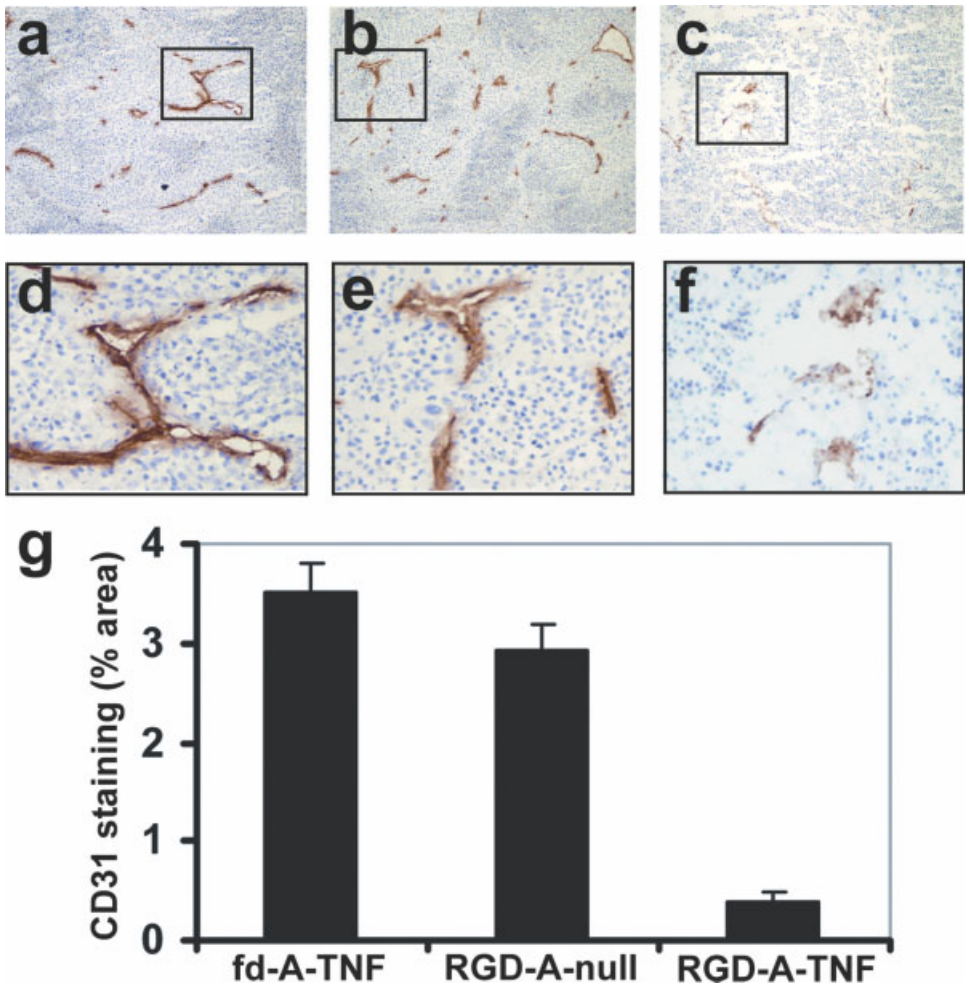

FIGURE 6. Targeted αV integrin ligand adeno-associated virus phage vector-expressing tumor necrosis factor-α (RGD-A-TNF) induces vascular damage of tumor vessels in vivo. Day 4 tumor sections from animals that received either nontargeted fd-A-TNF (n = 3) (a and d), RGD-A-null (n = 3) (b and e), or RGD-A-TNF (n = 3) (c and f) were stained for CD31, a vascular endothelial cell marker, by immunohistochemical staining. The blood vessels stained brown with anti-CD31-specific antibody (original magnification, ×100 in a-c, ×200 in d-f). The higher magnification highlights an area demonstrating vessel morphology. CD31 staining was quantified and is represented as the percentage area (g). Error bars indicate the standard error of mean.

bacterial infections.[15] In addition, they can be produced at high titers in bacterial culture and remain stable under a variety of conditions.[16]

The phage-display library of random peptides was developed previously to select peptides that specifically target vascular beds in vivo.[17] This strategy revealed vascular-specific ligands, which allowed experimental tissue-specific targeting of tumor blood vessels[18] as well as normal blood vessels to create a molecular map of the human vasculature.[19-21] It has been demonstrated that the ligands carrying an RGD-4C motif sequence (CDCRGDCFC) can be internalized by cells that express αv integrins through receptor-mediated endocytosis. Only targeted AAVP constructs that display an RGD-4C motif in their major coat protein subunit bind to cells and are internalized efficiently, whereas constructs with scrambled or mutated motifs are not.[9] Cells that lack the integrin receptor cannot bind or internalize these ligands.[22,23]

Bacteriophage vectors have been used previously to target mammalian cell surface receptors for gene delivery by other groups using either a specific ligand or an antibody. Larocca et al attached fibroblast growth factor 2 (FGF2) to the bacteriophage coat protein and observed long-term transgene expression in FGF receptor-expressing cells.[12,24] Bacteriophage that carries an antibody directed to the cell surface receptor ErbB2 can infect and

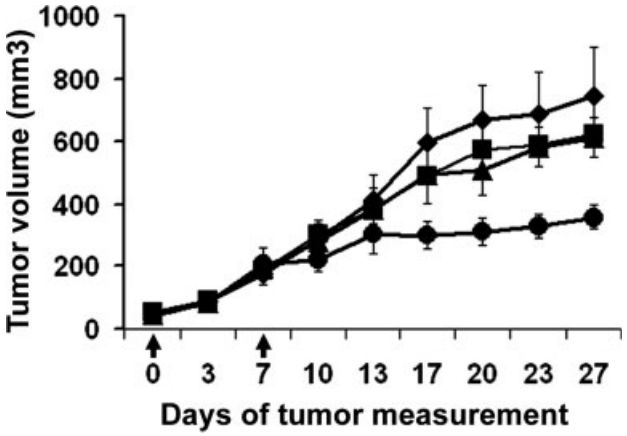

FIGURE 7. Inhibition of tumor growth in adeno-associated virus phage vector (AAVP)-treated animals. Nude mice with subcutaneously implanted human melanoma tumors were treated twice (on Day 0 and Day 7) with AAVP systemically through tail vein injection, and tumor volumes were measured at different time points. The tumor volumes (in mm^3) were plotted against the different days post-treatment. The animals that were treated with targeted αV integrin ligand (RGD-4C) AAVP-expressing tumor necrosis factor-α (RGD-A-TNF) (circles) had a statistically significant reduction in tumor volumes ($P = .048$) compared with animals that were treated either with vehicle (diamonds), or nontargeted fd-A-TNF (triangles), or RGD-A-null (squares).

express a transgene in receptor-positive cells.[25] However, the transduction efficiency for these vectors is very low (≈1%-9%), although multivalent phagemid particles have been used.[12,16,25,26] Thus, the use of a native, single-strand bacteriophage has very limited clinical utility as a therapeutic agent. In contrast, the use of targeted AAVP in our study was able to circumvent this problem, because AAVP-transduced cells generate functional AAV particles with reasonable transduction efficiency.[9,13]

RGD-targeted adenovirus infects cells independent of the coxsackie-adenovirus-receptor and has been used effectively to transfer genes to human melanomas.[27] A significant reduction in tumor size has been demonstrated with targeted interleukin-2 gene delivery by adenovirus injected directly into the tumor.[27] However, intratumoral delivery limits the clinical use of this vector, because not all tumors are accessible to direct tumor injection. Recently, RGD and other targeted motifs have been used for tumor-targeted delivery of recombinant TNF-α.[28-31] Among these, NGR-*TNF*, an aminopeptidase N (CD13) ligand that targets activated blood vessels in tumors, looks very promising. In this molecule, TNF-α is fused with the C terminus of CNGRCG peptide. It has demonstrated very little systemic toxicity as well as efficient antitumor activity in several animal models.[29,30] However, published reports on the tumor-targeted delivery of TNF-α have used only recombinant protein. The delivery of recombinant protein has its own limitations.[32]

Targeted AAVP vectors can localize and express gene products specifically in tumor vasculature when they are administered systemically[9] for reporter and suicide transgenes and, in the current study, for antivascular genes. This vector can be used effectively in both immunodeficient and immunocompetent mice.[9] We chose to evaluate the human TNF-α gene in the AAVP backbone, although TNF-α demonstrates species preference for toxicity and efficacy.[33-35] The primary objective in the development of AAVP-expressing human TNF-α was a careful evaluation of vector toxicity and efficacy to translate the treatment for use in human clinical trials.

A single administration of targeted AAVP in melanoma-bearing animals resulted in the presence of AAVP in the tumor for up to 10 days in vivo. We did not observe AAVP trafficking to normal tissues, such as heart, lung, kidney, skeletal muscle, or brain. Although we observed the presence of AAVP in liver and splenic tissues, AAVP did not colocalize with blood vessels in these organs. More important, the presence of AAVP in the liver and the spleen did not lead to the expression of TNF-α in these organs. It is possible that AAVP particles can be cleared nonspecifically by hepatic cells or taken up by reticuloendothelial cells in the spleen. However, this did not result in an undesirable gene transduction of either the liver or the spleen, as evidenced by the absence of gene product. TNF-α demonstrates species preference for toxicity; therefore, the evaluation of human TNF-α in a mouse model can underestimate the potential toxicities.[33,35] However, in the current study, none of the control tissues had any detectable levels of TNF-α, indicating that there was no expression of the gene in the normal tissues. We observed significant levels of TNF-α protein in tumor tissues on Day 4 after intravenous injection of RGD-A-TNF. The systemic delivery of AAVP that expresses a gene product of interest may have wider clinical applications. This is especially relevant for antitumor/antivascular agents that are quite effective but are associated with and limited by a wide range of systemic toxicities.

TNF-α has demonstrated potent antitumor activity in animal models.[36] Despite some observed toxicity in preclinical models, its potent activity against human tumor xenografts led to clinical trials in humans. However, the results were a small number of minimal responses with extensive hemodynamic side effects and hepatic toxicity.[36] Those trial results were sufficiently disappointing that studies of systemically delivered, single-agent TNF-α were discontinued. To avoid systemic toxicity, currently, TNF-α is used clinically only in isolated organ perfusion for human melanoma and soft tissue sarcoma.[37] In this setting, the combination of TNF-α with conventional antineoplastic agents (eg, melphalan, doxorubicin, paclitaxel, actinomycin-D, cisplatin) strikingly increases the tumor response rates.[38] Thus, to make use of the antitumor effects of TNF-α, it is important to have tumor-targeted delivery of the agent. To our knowledge, this is the first report demonstrating the use of targeted AAVP to deliver TNF-α selectively to tumor vasculature.

We observed that the localization of RGD-A-TNF to tumor endothelium resulted in the expression of TNF-α after the systemic delivery of AAVP. The expression of TNF-α resulted in tumor vessel apoptosis as well as tumor necrosis. Finally, mice bearing melanoma xenografts had significant reductions in tumor volume after the systemic delivery of RGD-A-TNF without any evidence of systemic toxicity. The organs obtained from these animals revealed no evidence of toxicity at the tissue level. Thus, the data indicate that TNF-α may be used in clinical settings beyond isolated organ perfusions as an antivascular agent if it is delivered selectively by a vector such as AAVP. This study provides evidence that targeted AAVP administered systemically can be used as a method to deliver TNF-α selectively and effectively to tumor vasculature.

Although we observed significant reductions in tumor volume, RGD-A-TNF should be delivered in combination with other antitumor agents to test its long-term efficacy. We have undertaken studies to determine the effect of RGD-A-TNF on primary tumor growth and metastases in both immunodeficient and immunocompetent mice through the use of different tumor models.

In summary, AAVP vectors can be used to deliver antivascular products directly to the tumor vasculature. Systemically delivered, targeted AAVP transduces tumor vascular endothelium with no detectable transgene expression in normal organs. To our knowledge, this is the first study to demonstrate that AAVP can be used successfully to deliver antivascular therapy to established tumors. The use of targeted AAVP may allow for the selective delivery of agents with antivascular properties specifically to tumor blood vessels. A class of such vectors may be an important addition to the arsenal of therapies for patients with cancer. Translational trials using large animal models of human disease currently are ongoing to further explore the potential efficacy of this experimental approach.

Conflict of Interest Disclosures

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