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Research Article

Targeting Tumor Endothelial Marker 8 in the Tumor Vasculature of Colorectal Carcinomas in Mice

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Abstract

Tumor endothelial marker 8 (TEM8) is a recently described protein that is preferentially expressed within tumor endothelium. We have developed a fusion protein that targets TEM8 and disrupts tumor vasculature by promoting localized thrombosis. Fusion protein specificity and function were evaluated using Western blot analysis, ELISA, and enzymatic assays. A xenograft model of colorectal carcinoma was used to test the efficacy of targeted and control fusion proteins. Mice treated with the gene encoding anti-TEM8/truncated tissue factor exhibited a 53% reduction in tumor volume when compared with the untreated animals ($P < 0.0001$; $n = 10$) and achieved a 49% increase in tumor growth delay by Kaplan-Meier analysis ($P = 0.0367$; $n = 6$). Immunohistochemistry confirmed tumor endothelial expression of TEM8, fusion protein homing to tumor vasculature, decrease in vessel density, and localized areas of thrombosis. These data support the hypothesis that targeting TEM8 can be an effective approach to influence tumor development by disrupting tumor vasculature. [Cancer Res 2009;69(12):5126–32]

Introduction

Targeted disruption of tumor vasculature is an area of growing interest in cancer biology and therapeutics. Tumors recruit blood vessels to acquire metastatic potential and to relieve the energy-deprived and hypoxic environment created by uncontrolled cell growth. Angiogenesis is characterized by the production of proangiogenic proteins that induce sprouting of existing vessels and the recruitment of circulating endothelial progenitor cells to form new vascular networks (1). These vascular networks are influenced by the tumor microenvironment, causing alterations in the expression of various proteins. Using molecular techniques including suppression subtractive *in situ* hybridization (2) and serial analysis of gene expression (3), several investigators have probed tumor endothelial cells to identify preferentially expressed proteins. Several promising candidate genes have been identified and one such protein is tumor endothelial marker 8 (TEM8; ref. 3).

TEM8 is a type 1 transmembrane protein, the exact physiologic function of which remains unknown. *In vitro* studies indicate that TEM8 has a role in various endothelial cell functions (4–6) and the extracellular domain of TEM8 can bind to extracellular matrix proteins (4, 7) as well as the protective antigen of anthrax toxin (8). Expression profiles for TEM8 include both research and clinical samples that provide strong evidence for the increased expression of TEM8 in tumor endothelial cells (3, 7, 9). In normal adult tissues, TEM8 expression is sporadic and found at lower levels than in tumor endothelium (7, 10). Expression of TEM8 was also found in endothelial cells during murine embryonic development (11) and in some cases of infantile hemangioma (12). Together, these reports suggest that TEM8 expression in adults is elevated in cases of aberrant vessel formation, such as tumor vasculature, and that TEM8 may have therapeutic applications by allowing targeting of tumor endothelium.

In this report, we employ a gene therapy-based approach to target murine TEM8 in the vessels of a xenograft human tumor and to disrupt existing tumor vasculature by promoting thrombosis. The approach involves the synthesis of a gene encoding a novel fusion protein with two domains. The NH_2-terminal domain is a single-chain antibody (scFv) that enables binding to TEM8, whereas the COOH-terminal domain is truncated tissue factor (tTF), which promotes thrombosis. We further compare the results achieved by targeting TEM8 with that of a similar fusion protein that targets the established tumor marker carcinoembryonic antigen (CEA) and a fusion protein with a nonspecific scFv domain. We show that systemic gene delivery using the *Sleeping Beauty* (SB) transposon system results in the production of the fusion proteins within the lung, which can home to the tumor and influence tumor growth. Collectively, our experiments indicate that TEM8 expression is prevalent within tumor vasculature and that targeting this protein has therapeutic potential.

Materials and Methods

Cell Lines

All cell lines were grown in DMEM high-glucose supplemented with 1% penicillin-streptomycin-glutamine and 10% fetal bovine serum at 37°C in 5% CO_2, except the murine hybridoma cell line, HB-8747 (American Type Culture Collection), which required 20% fetal bovine serum.

Creation of the Cell Lines 293T/TEM8 and 293T/CMG2

The 293T cell line was obtained from Dr. Gary Nolan and is derived from human embryonic kidney 293 cells transformed with the large T antigen. The cDNAs for human *TEM8* splice variant 2 and murine *CMG2*^{488} were transduced individually into 293T cells using the Phoenix A packaging cell line to create retrovirus (13). The resulting cell lines were called 293T/TEM8 and 293T/CMG2. Proper expression of TEM8 was shown by Western blot analysis using the SB5 monoclonal antibody and reverse transcription-PCR. Proper expression of CMG2 was shown through reverse transcription-PCR analysis because no antibody to CMG2 was commercially available at the time of cell line creation.

Fusion Protein Construction and Cloning into the SB Transposon

Purified antibody and mRNA from the SB5 monoclonal antibody hybridoma were a kind gift from Dr. Brad St. Croix. We captured the cDNAs of the variable light chain (V_{LC}) and variable heavy chain (V_{HC}) of SB5 using a 5′ rapid amplification of cDNA ends kit (Invitrogen) following the

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manufacturer's recommendations. We modified the cDNA for the V_{LC} using the primers 5′-GCGGCCGCCACCATGGACATGAGGACCCCTGC-3′ and 5′-*CGCCAGATCCGGGCTTGCCGGATCCAGAGGTGGAGCC*TTTCAGCTCCAGCTTGGTCC-3′ and the cDNA for the V_{HC} using the primers 5′-*CGGCAAGCCCGGATCTGGCGAGGATCCACCAAGGGC*CAGATCCAGTTGGTGCAGTCTGA-3′ and 5′-*CCGCCTCCGGAGCCCCCACCCCC*TGCAGAGACAGTGACCAGAGTC-3′. These secondary amplifications added a partial 218 linker (14) to both the 3′ end of the V_{LC} and the 5′ end of the V_{HC} and a partial $(G_4S)_3$ linker (15) to the 3′ end of the V_{HC} (italicized regions).

The cDNA for the V_{LC} and V_{HC} of the anti-CEA monoclonal antibody T84.66 were captured from RNA obtained from the HB-8747 hybridoma (American Type Culture Collection) using Trizol reagent and reverse transcription-PCR. The primers used for the V_{LC} were 5′-GCGGCCGCCACCATGGAGACAGACACACTCCTGC-3′ and 5′-*CGCCAGATCC>GGGCTTGCCGGATCCAGAGGTGGAGCC*TTTTATTTCCAGCTTGGTCC-3′, and the primers used for the V_{HC} were 5′-*CGGCAAGCCCGGATCTGGCGAGGGATCCACCAAGGGC*GAGGTTCAGCTGCAGCAGTC-3′ and 5′-*CCGGAGCCTCCGCCTCCGGAGCCCCCACCCCC*TGAGGAGACGGTGACTGAGG-3′. Italicized portions mark overlapping linker regions.

The cDNA for murine tTF was captured from RNA obtained from brain tissue of a C57Bl/6 mouse using Trizol reagent and reverse transcription-PCR. The primers used were 5′-*GCTCCGGAGGCGGAGGCTCCGGAGGAGGCGGGTCC*ATGGCGATCCTCGTGCGCC-3′, which added a partial $(G_4S)_3$ linker region (italicized) to the 5′ end of tTF and 5′-GCGGCCGC*TCAATGGTGATGGTGATGGTGGTCCACGGCGCTGTTCAGGTCCTCCTCGCTGATCAGCTTCTGCTCGCCAA*AGCAGTAGCTTACTCCTTC-3′, which added a myc-His tag to the 3′ end of tTF (italicized).

Dead-sc was created by joining the V_{LC} of SB5 with the V_{HC} of T84.66. In addition, DNA sequences encoding critical amino acids within the antigen binding domains of each chain were modified. The modifications required two separate PCRs for each variable region and each PCR fragment was joined together using restriction enzyme digestion and ligation. The fragments for the V_{LC} of SB5 were created using the primers 5′-GCGGCCGCCACCATGGACATGAGGACCCCTGC-3′ and 5′-A*AGATCT*GTCCTGACTCGCCTTGC-3′ to make the top fragment and 5′-A*AGATCT*GGGGTCCCATCAAGGTTCC-3′ and 5′-CGCCAGATCCGGGCTTGCCGGATCCAGAGGTGGAGCCTTTCAGCTCCAGCTTGGTCC-3′ to make bottom fragment. The italicized region represents a *Bgl*II site that was used to join the two fragments together. The fragments for the V_{HC} of T84.66 were created using the primers 5′-CGGCAAGCCCGGATCTGGCGAGGGATCCACCAAGGGCGAGGTTCAGCTGCAGCAGTC-3′ and 5′-C*CTCGAG*AATGTTGAAGCCAGAAGC-3′ to make the top fragment and 5′-C*CTCGAG*TTCCAGGGCAAGGCCACT-3′ and 5′-CCGGAGCCTCCGCCTCCGGAGCCCCCACCCCCTGAGGAGACGGTGACTGAGG-3′ to make the bottom fragment. The italicized region represents a *Xho*I site that was used to join the two PCR fragments together.

Gene splicing by overlap extension using PCR (16) was used to join the V_{LC} regions to the V_{HC} regions to create anti-TEM8, anti-CEA, and Dead-sc. Separate gene splicing by overlap extension using the PCR was done to join the individual scFv to tTF using complementary regions of the $(G_4S)_3$ linker. All samples were cloned into the pCR4-TOPO shuttle vector (Invitrogen) and sequenced by Seqwright. Correct clones were placed into the SB backbone pMSZ/CBA.

In vitro Characterization of the Fusion Proteins

The SB vectors pMSZ/CBA-anti-TEM8/tTF, pMSZ/CBA-anti-CEA/tTF, and pMSZ/CBA-Dead-sc/tTF were transfected into 293T cells using calcium phosphate transfections (13). Secreted fusion proteins were purified from the medium using Ni-NTA agarose beads (Qiagen) as per manufacturer's specifications. Eluted samples were concentrated using an Amicon 30 cartridge (Millipore) to a final volume of 200 μL. Cell lysates were obtained by washing cells with ice-cold PBS and lysing the cells with 0.3 mL sample application buffer (62.5 mmol/L Tris, 10% glycerol, 3% SDS). Cell lysate (30 μg) or purified fusion protein (25 μL) was separated on 10% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane. Membranes were probed with a horseradish peroxidase (HRP)-conjugated anti-His antibody (Invitrogen).

To show anti-CEA/tTF binding, an indirect ELISA was done as reported previously with the following changes (17). The Maxisorp microtiter plates (Thermo Fisher Scientific) were coated with 100 μL purified CEA protein (Abcam) at a concentration of 4 μg/mL. Binding of the fusion proteins was detected using a rabbit anti-murine tissue factor polyclonal antibody (American Diagnostica) followed by a goat anti-rabbit HRP-conjugated antibody. Color was developed by adding 150 μL undiluted ABTS (Pierce) to each well for 30 min at room temperature. The reaction was quenched with the addition of 100 μL of 1% SDS. The plate was read at a wavelength of 405 nm on a Molecular Devices plate reader and the results were recorded using SoftMax Pro Software (Molecular Devices).

Anti-TEM8/tTF binding specificity was shown via Western blotting. Transfected medium was passed through a 0.2 μm filter and concentrated 10 times using Amicon 30 cartridges (Millipore). The concentrated medium from each group was used to probe separate polyvinylidene difluoride membranes containing 30 μg cell lysate from cells expressing either TEM8 or CMG2 in adjacent lanes. Fusion protein binding was detected using a rabbit anti-murine tissue factor antibody followed by a goat anti-rabbit HRP-conjugated secondary antibody (Abcam). The parental monoclonal antibody, SB5, was diluted 1:1,000 and used to probe another blot with the same cell lysates. A goat anti-mouse HRP-conjugated secondary antibody (Abcam) was used for detection of SB5.

Tissue Factor Activity of the Fusion Proteins

Purified fusion proteins were passed through a PD-10 desalting column (GE Healthcare) to exchange their buffer to 50 mmol/L Tris (pH 9.0). Lyophilized recombinant murine factor VII (R&D Systems), recombinant murine tissue factor (R&D Systems), thermolysin (R&D Systems), and fluorogenic substrate (R&D Systems) were reconstituted and run in a tissue factor activity assay according to the manufacturer's recommendations. The results were read on a kinetic setting at excitation and emission wavelengths of 380 and 460 nm using a Molecular Devices plate reader and the results were captured using SoftMax Pro Software.

Animal Studies

All animal experiments used 7-week-old athymic nude mice purchased from The Jackson Laboratory and maintained under standard specific pathogen-free conditions. All procedures performed on the mice were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

Tumor growth curve. On day 0, mice received intramuscular injections of 1×10^6 HT-29 human colorectal carcinoma cells in the left hind limb. The total volume was 25 μL consisting of a 1:1 ratio of sterile PBS and Matrigel (Becton Dickinson). On day 7, the mice were divided into treatment groups ($n = 6$ for Dead-sc/tTF and anti-CEA/tTF and $n = 10$ for untreated and anti-TEM8/tTF) and received either no therapy or weekly tail vein injections. The DNA injections (40 μg) consisted of a 5:1 molar ratio of a transposon plasmid carrying one of the fusion proteins and the transposase expression plasmid (pCMV-SB16) complexed with the linear polymer polyethylenimine in 200 μL (Polyplus Transfection). Tumor volumes were measured biweekly with a caliper along a *X-Y-Z* axis and mice were sacrificed on day 30. Tumor and lung tissues were harvested for immunohistochemistry and Western blot analysis.

Tumor growth delay. Mice in this experiment followed the same protocol as the tumor growth curve, except that animals continued receiving weekly injections until their tumor volume surpassed 1.3 cm^3 ($n = 6$ for each group; anti-CEA/tTF had one censored animal).

Circulating concentration of the anti-TEM8/tTF fusion protein. Plasma from non-tumor-bearing animals either untreated or treated with the anti-TEM8/tTF transposon plasmid as described above was examined using a capture ELISA. Untreated animals or animals receiving injections on days 7 and 14 had their plasma examined on days 0, 10, and 17. The capture ELISA was done by coating the wells of a Maxisorp microtiter plate with 100 μL of a 5 μg/mL anti-His antibody (Roche). The wells were exposed to either 150 μL plasma or a serially diluted sample of a COOH-terminal His-tagged recombinant murine tissue factor protein in PBS-0.05% Tween 20. The fusion proteins or the recombinant murine tissue factor were detected and quantified using the same procedure described in the anti-CEA/tTF binding assay ($n = 6$ for untreated and anti-TEM8/tTF).

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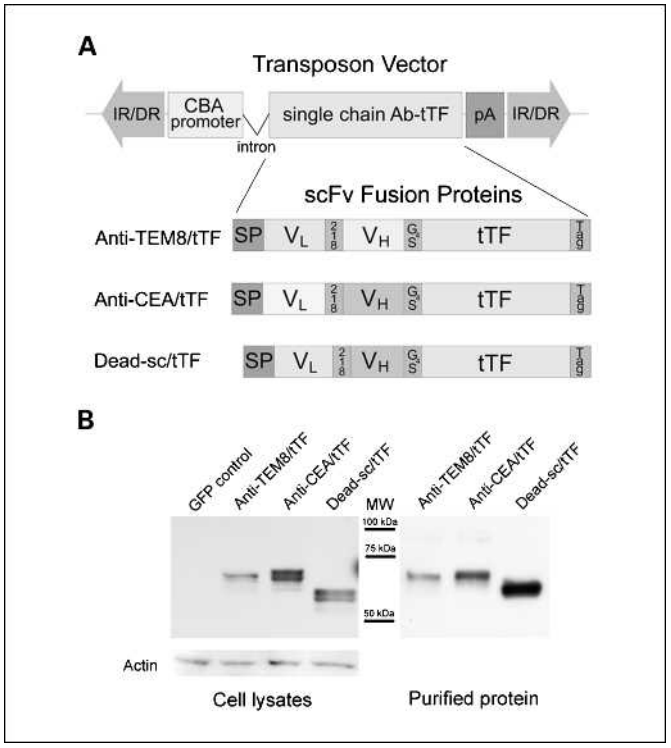

Figure 1. Fusion protein constructs. *A,* schematic diagram of the expression cassettes for the three fusion proteins anti-TEM8/tTF, anti-CEA/tTF, and Dead-sc/tTF flanked by inverted repeat/direct repeat elements (*IR/DR*). *B,* Western blot analysis showing expression and secretion of the fusion proteins with each expression vector. A molecular weight (*MW*) ladder is shown between the blots.

Immunohistochemistry

Tumors were surgically removed from each mouse and placed in 4% paraformaldehyde overnight. The samples were paraffin-embedded and cut into 5 μm sections. Slides were deparaffinized and rehydrated to undergo either immunofluorescent analysis or H&E staining. Immunofluorescent analysis used three retrieval processes that were dependent on compatibility with the primary antibodies. Studies using the anti-CEA (Abcam) and anti-CD31 (Santa Cruz Biotechnology) antibodies underwent a proteinase K (DAKO) digestion for 2 min. Immunofluorescent staining with anti-TEM8 (Abcam) and anti-murine plasmalemma vesicle protein-1 (PV-1; Pharmingen) underwent a 20 min incubation at 95°C with Target Retrieval Solution (DAKO) followed by a 20 min incubation at room temperature in the same solution. Immunofluorescent staining with anti-TEM8 (Abcam), anti-murine tissue factor (American Diagnostica), pentaHis 488 (Qiagen), or anti-CD34 (Abcam) antibodies underwent a 0.01 mol/L citrate (pH 6.0) retrieval for 7 min at 98°C with microwave followed by a cool time of 20 min at room temperature. All samples were washed with TBS and blocked with normal horse serum (Vector Laboratories). Primary antibodies were incubated on slides overnight at 4°C. Primary antibody concentrations were as follows: anti-TEM8 (1:75), anti-CD34 (1:100), anti-PV-1 (1:10), anti-murine tissue factor (1:100), anti-CEA (1:100), anti-CD31 (1:50), and pentaHis 488 (1:100). The slides were washed with TBS and the secondary antibodies used were Alexa Fluor donkey anti-rabbit 488 (Invitrogen) for anti-TEM8, anti-CEA, and anti-murine tissue factor antibodies and Alexa Fluor donkey anti-rat 594 (Invitrogen) for anti-CD34, anti-PV-1, and anti-CD31. Immunofluorescent staining was evaluated on either a Leica DM2500 fluorescent microscope (Leica Microsystems) with an Optronics Camera and Magnifier Software (Optronics) or an Olympus 1×81-DSU spinning disk confocal microscope with Slidebook software (Olympus).

Vessel Density Quantification

Vessels were illuminated with the anti-PV-1 antibody and visualized at ×100 magnification to find highly vascular areas in four separate regions per slide. A ×200 magnification was used to count the areas with the greatest amount of vascular density and the average of the four areas was recorded. Areas positive for PV-1 that were distinct from other areas positive for PV-1 were considered a separate microvessel and counted as such.

Targeted Thrombosis

Localized thrombosis was visualized using H&E staining and light microscopy, and ×100 and ×200 magnification were used to find and capture images of thrombosis events in tumor and lung tissue. Thrombosis events were considered genuine if areas of RBC accumulation were surrounded by large areas of necrosis and tissue fragmentation. Images were captured using a Leica DM2500 fluorescent microscope (Leica Microsystems).

Western Blot of Tumor and Lung Tissue

Murine lung and tumor tissue were cut into smaller pieces and washed in sterile PBS. The tissue was centrifuged and resuspended into sample application buffer containing a protease inhibitor cocktail (Roche). The samples were homogenized using a Dounce homogenizer cooled to 4°C and incubated overnight at 4°C on a rotary shaker. The resulting lysate was passed through a 30-gauge needle and cleared via centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was boiled for 5 min, and 100 μg were run on a 10% SDS-PAGE gel. The separated sample was transferred to a polyvinylidene difluoride membrane and probed with a murine primary HRP-conjugated His antibody (Invitrogen).

Western Blot Stripping and Equal Loading

Blots were stripped by incubation at 50°C with stripping buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol] for 30 min and washed with PBS-0.05% Tween 20. Equal loading was shown by probing membranes with an anti-actin antibody (Abcam) for 2 h at room temperature followed by washing with PBS-0.05% Tween 20 and detection with a goat anti-mouse HRP-conjugated secondary antibody (Abcam).

Statistical Analysis

Data collected were analyzed using GraphPad Prism software. Comparisons made between the means of each group were analyzed using one-way

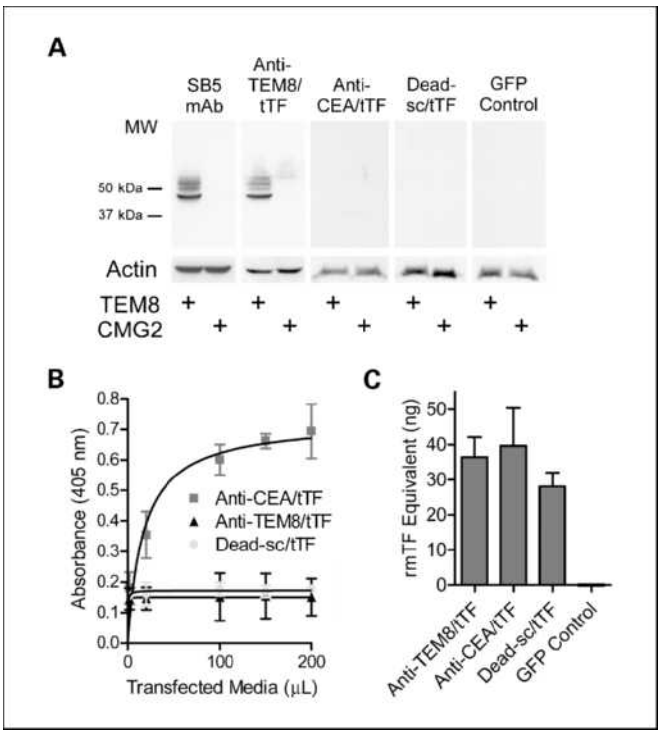

Figure 2. Fusion protein specificity and tTF activity. *A,* anti-TEM8/tTF fusion protein as well as the original SB5 monoclonal antibody recognizes TEM8 but not CMG2. *B,* anti-CEA/tTF fusion protein binds to CEA. $P = 0.0015$, ANOVA. *C,* tissue factor activity of the fusion proteins purified from medium. $P = 0.0011$, ANOVA. Results in *B* and *C* are from three separate experiments. *rmTF,* recombinant murine tissue factor.

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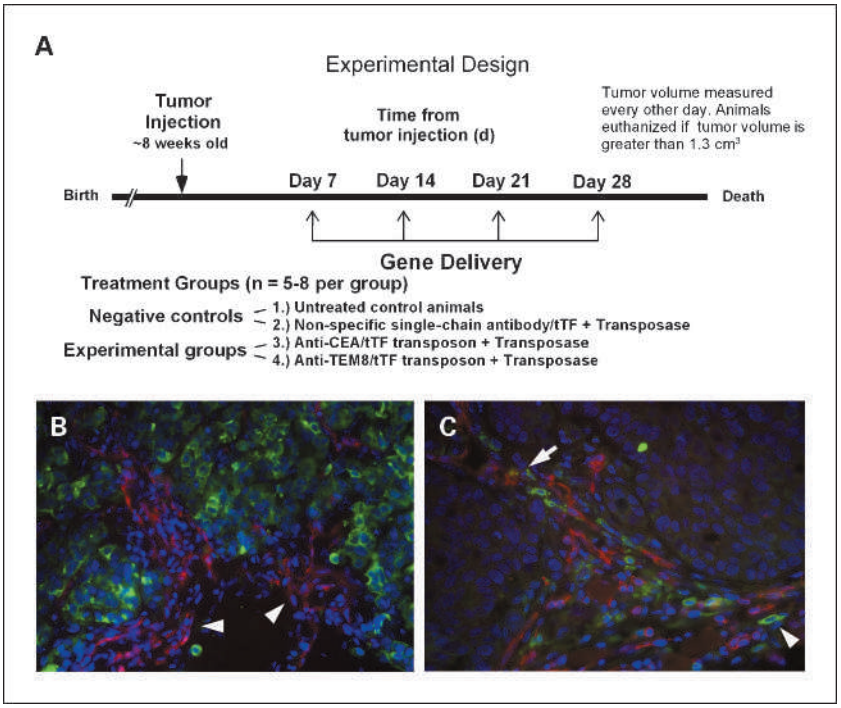

Figure 3. Xenograft model and tumor immunohistochemistry. *A,* schematic diagram of the xenograft model illustrating timing of tumor implantation and gene delivery. *B,* immunohistochemistry shows CEA expression (*green*) compared with CD31 vessel staining (*red, arrowheads*), illustrating that CEA expression is limited to tumor cells. *C,* TEM8 expression (*green, arrow* and *arrowhead*) in comparison with PV-1 vessel staining (*red*) illustrates that TEM8 expression is localized to the tumor vasculature.

ANOVA. For the tumor growth curve experiment, a two-way ANOVA was used when the mean tumor growth between each of the treatment groups was compared followed by a Bonferroni post-test to compare individual time points between each of the groups. For the tumor growth delay experiment, data were analyzed using a Kaplan-Meier curve and the mean survival for each group was compared using a log-rank test. Data were shown as mean ± SE. The threshold for statistical significance was $P < 0.05$.

Results

***In vitro* activities of the fusion proteins.** The SB transposons (Fig. 1*A*) were initially examined for their ability to produce functional fusion proteins in a cell culture system. To verify expression and secretion, 293T cells were transiently transfected with transposon vectors carrying the gene for each of the various fusion proteins or the green fluorescent protein. The cell culture medium was purified by nickel column chromatography and the eluted fractions as well as crude cell lysates were examined by Western blot analysis (Fig. 1*B*). The blot yielded immunoreactive bands of ~65 kDa for the anti-TEM8/tTF protein, ~68 kDa for the anti-CEA/tTF protein, and ~60 kDa for the Dead-sc/tTF protein. Crude cell lysates expressing green fluorescent protein did not produce immunoreactive bands. These results are consistent with the predicted sizes for each fusion protein and confirm proper processing and secretion.

The activity of the fusion proteins was shown using medium from transiently transfected cells. Figure 2 shows the binding capabilities of each fusion protein as well as the tissue factor activity. To show TEM8 affinity, cell lysates from 293T cells expressing either TEM8 or CMG2 were probed with medium containing the various fusion proteins or the SB5 monoclonal antibody using Western blot analysis. The SB5 monoclonal antibody and the anti-TEM8/tTF fusion protein detected a band of 42 kDa, which corresponds to the predicted size of the nonglycosylated TEM8 protein. The series of faint bands above the major form likely represents glycosylated forms. Neither the SB5 monoclonal antibody nor the anti-TEM8 fusion protein detected a band in CMG2-expressing cells in the adjacent lane (Fig. 2*A*). Dead-sc/tTF, anti-CEA/tTF, and control medium did not produce immunoreactive bands.

The binding capability of the anti-CEA/tTF fusion protein was shown using an indirect ELISA assay. Concentration-dependent binding was observed with the anti-CEA/tTF protein, whereas Dead-sc/tTF and anti-TEM8/tTF did not bind (Fig. 2*B*). These results confirmed that the anti-TEM8/tTF and anti-CEA/tTF scFv domains retained their binding abilities. Dead-sc/tTF did not recognize either TEM8 or CEA in the same assays, confirming the predicted loss of affinity for either antigen.

Activity of the tTF domain for each fusion protein was verified using an assay mimicking the activation of the coagulation cascade (Fig. 2*C*). For these experiments, purified fusion proteins underwent buffer exchange due to fetal bovine serum and dye in the medium causing false-positive reactions. The results from this assay show that the tTF domain from each fusion protein is active and yields quantifiable tissue factor activity when compared with mock-transfected cells. Together, these experiments show that the fusion proteins are expressed, secreted, and maintained their predicted antigen recognition and tissue factor activity.

Tumor model and expression of TEM8 and CEA. A xenograft model of colorectal cancer using nude mice and the human colorectal carcinoma cell line HT-29 was chosen because these cells express the tumor antigen CEA, and TEM8 expression was originally reported in colorectal carcinoma samples (3). Tumor injections were optimized so that untreated tumors would reach the size of ~1.3 cm^3 in 30 days (Fig. 3*A*). We verified the expression patterns of both CEA and TEM8 using immunohistochemistry. CEA expression localized to tumor cells (Fig. 3*B*), whereas TEM8 expression localized to tumor vasculature (Fig. 3*C, arrow*). These results are consistent with the reported patterns for both proteins; however, it is important to note that TEM8 expression did not always colocalize with the murine endothelial marker PV-1 (*arrowhead*). A finding further evaluated in subsequent experiments.

Gene delivery and effect of fusion proteins. Previous work in our laboratory has shown that nonviral gene delivery using systemic injections of DNA/polyethylenimine complexes results in predominant gene expression within the lung (18). A Western blot

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of lung tissue following gene delivery confirmed expression of the fusion proteins within the lung (Fig. 4*A*). A capture ELISA was used to show that the anti-TEM8/tTF protein could be detected in plasma following serial rounds of gene delivery (Fig. 4*B*). The peak concentration of 425 ng/mL is far less than that achieved by viral vectors expressing similar fusion proteins *in vivo* (19) and ~30 times less than that used when similar fusion proteins are delivered by direct protein injection (20–22). Given the small amounts of the fusion protein present within the circulation, we modified the experimental design to include weekly rounds of gene delivery to enhance protein expression.

The effects of gene delivery were analyzed first in the tumor growth curve experiment (Fig. 4*C*). By day 18, the anti-TEM8/tTF group showed a statistically significant reduction in tumor size ($P < 0.05$) that only improved by day 30 ($P < 0.001$) when compared with the untreated control. The anti-CEA/tTF group also had a reduced tumor size, yet the reduction did not reach statistical significance ($P < 0.01$) until day 30. In these experiments, 15% of the anti-TEM8/tTF group displayed a complete regression of tumor volume and was the only group to display such an effect. In the tumor growth delay experiment (Fig. 4*D*), the anti-TEM8/tTF plasmid-treated group had a median tumor growth delay of 42.5 days ($P = 0.0367$), whereas the other groups fell within 28 ± 2 days. These results translated to the anti-TEM8/tTF transposon construct achieving a tumor growth inhibition of ~55% and a tumor growth delay of 49% when compared with the untreated control.

Fusion protein analysis in tumors. Tumors from gene-treated animals were assessed for evidence of fusion protein targeting, tumor vessel density, and evidence of thrombosis. We examined tumor crude cell lysates from untreated animals and anti-TEM8/tTF-treated animals for the presence of the fusion protein (Fig. 5*A*). After identifying a band corresponding to anti-TEM8/tTF, we examined all groups for tumor localization of the fusion proteins via immunohistochemistry. Both anti-TEM8/tTF and anti-CEA/tTF fusion proteins localized in the tumor in a manner predicted by their antigen recognition (Fig. 5*B, arrowheads*). Dead-sc/tTF and untreated controls showed no staining. These results suggest that the anti-TEM8/tTF and anti-CEA/tTF proteins are able to home to the tumor and bind their respective antigens.

Evidence of localized thrombosis induced by the tTF domain of the fusion proteins were examined by H&E staining of lung and tumor sections (Fig. 5*C*). Major thrombotic events were only seen in the tumor sections of the anti-TEM8/tTF and anti-CEA/tTF groups and were visible to the unaided eye. Localized thrombosis was not observed in the tumors of either untreated or Dead-sc/tTF-treated animals. No animals in any group perished during peak fusion protein expression and the lungs from these animals showed no evidence of localized thrombosis or thromboembolism. These results suggest the tTF domain on anti-TEM8/tTF and anti-CEA/tTF is able to activate the coagulation cascade inside tumor vessels.

Tumor vessel density was examined in all groups using PV-1 staining. A 45% ($P = 0.005$) reduction in vessel density was observed in animals receiving the anti-TEM8/tTF fusion protein when compared with Dead-sc/tTF, anti-CEA/tTF, and untreated controls (Fig. 5*D*).

TEM8 expression on CD34$^+$ cells. Immunohistochemistry revealed that TEM8 protein expression did not always colocalize with the endothelial marker PV-1. However, TEM8 protein was always associated with tumor vasculature. This observation led us to investigate other populations of cells associated with tumor vasculature that might be TEM8 positive. We examined tumor sections

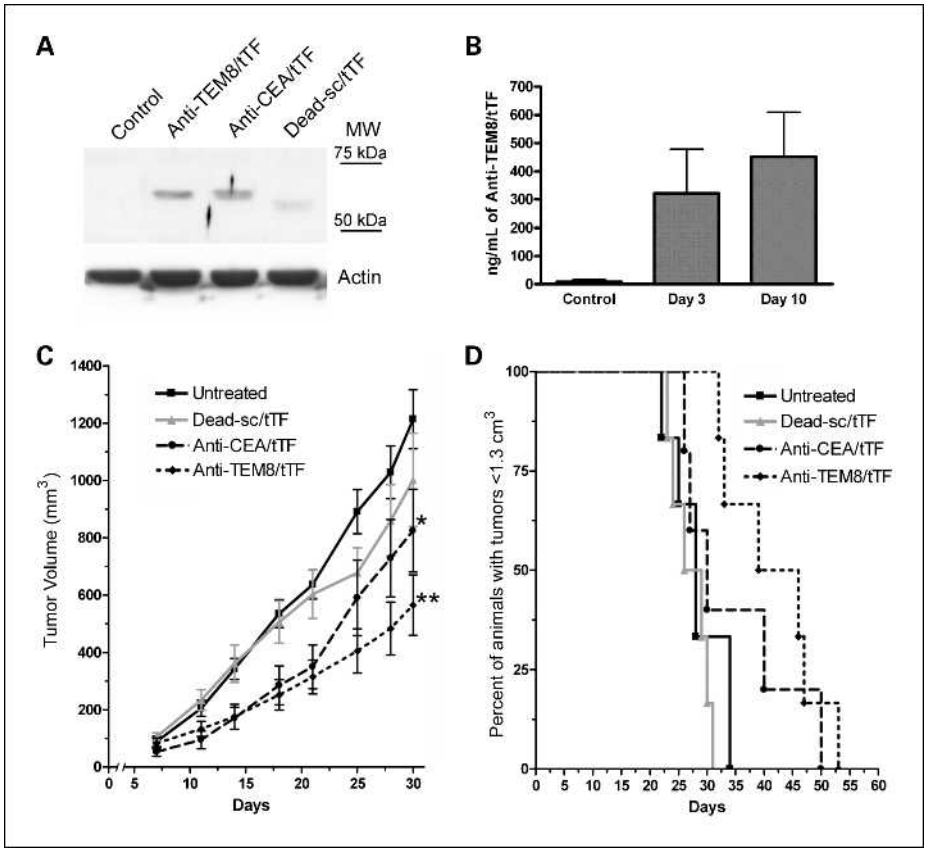

Figure 4. *In vivo* fusion protein expression and antitumor responses. *A,* Western blot analysis shows fusion protein expression in crude lysates of lung tissue. *B,* circulating anti-TEM8/tTF plasma levels were determined by ELISA following two rounds of gene delivery. Results of two separate experiments. *C,* tumor growth curve. Tumor volumes were measured biweekly and graphed as a function of time. All animals were sacrificed at day 30 regardless of tumor size. Data from two separate experiments. *, $P < 0.01$; **, $P < 0.001$. *D,* tumor growth delay. Data from two separate experiments.

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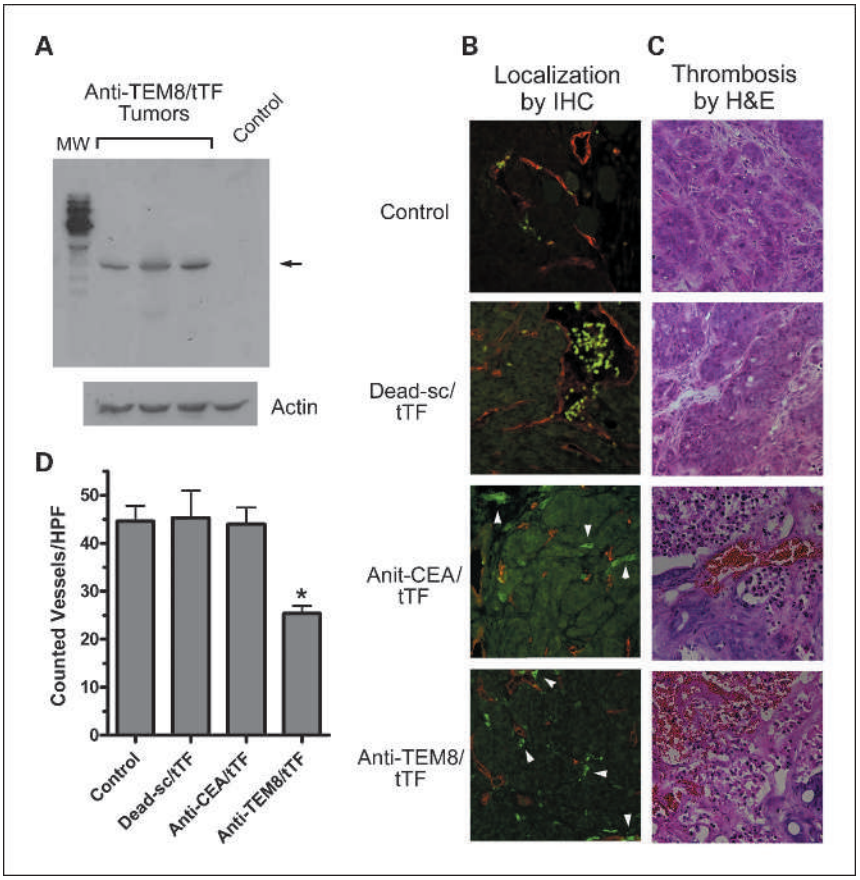

Figure 5. Fusion protein homing and effects on tumor vasculature. *A,* Western blot analysis of crude lysates from tumor tissue detected the anti-TEM8/tTF fusion protein. *B,* immunohistochemistry (*IHC*) of tumor tissue confirmed the presence of the anti-TEM8/tTF and anti-CEA/tTF fusion proteins in a location similar to the TEM8 and CEA staining shown in Fig. 3 (*arrowheads*). Autofluorescent erythrocytes are seen within the vessels in some panels. *C,* H&E staining of tumors shows areas of localized thrombosis in the anti-TEM8/tTF-treated and anti-CEA/tTF-treated groups. Magnification, ×200. *D,* tumor vessel density. *, $P = 0.005$. *HPF,* high-power field.

by confocal microscopy for colocalization of TEM8 with other cellular markers including pericytes (nerve/glial antigen 2), fibroblasts (smooth muscle actin and fibroblast-specific protein 1), leukocytes (protein tyrosine phosphatase, receptor type, C), and hematopoietic stem cells (CD34). We observed that occasionally TEM8-positive cells colocalized with the endothelial marker PV-1 (Fig. 6*A*, *arrowheads*), whereas other populations of TEM8-positive cells were $CD34^+$ (Fig. 6*B, arrowheads*). All of the other remaining markers mentioned above were negative for colocalization.

Discussion

Since its discovery, TEM8 has emerged as a protein that is preferentially expressed in tumor endothelium. Differentially expressed proteins are invaluable in medicine and basic research because they can be used to deliver therapeutic molecules to expressing cells. Recently, several reports suggested that targeting TEM8 *in vivo* may have antitumor effects, but these articles either nonspecifically targeted TEM8 using parts of the anthrax toxin or prevented TEM8 from binding a physiologic partner (23–25). Demonstrating specificity for TEM8 is crucial because TEM8 and CMG2 differ greatly in their expression profiles yet share >60% homology in their integrin-binding domain and are both functional anthrax toxin receptors (8, 26). To date, the only other TEM8-targeted therapy proven capable of differentiating TEM8 from CMG2 is a mutated version of protective antigen that has not been tested *in vivo* (27). Our study establishes that specific targeting of TEM8 is a powerful approach to disrupt tumor vasculature.

The reduction in tumor volume and delay in tumor growth observed in our studies are consistent with similar studies that treated tumors with fusion proteins delivering tTF as a vascular disrupting agent (20–22). Our study differs from these previous reports because we used a transposon-based gene delivery system instead of delivering purified protein, and our effector molecule is murine tTF as opposed to human tTF. Given our peak protein expression is 30 times less than that obtained following direct protein injection, we did not observe immediate necrosis of the tumor as reported previously (21). The previous studies and a recent clinical trial in humans suggest that using tTF as an effector molecule is indeed safe (20–22, 28). Our study did not directly assess the safety of the tTF domain; however, we did not observe an increase in unexplained death in animals receiving the tTF domain, nor was there evidence for nonspecific thrombosis. Delivery of purified fusion proteins, such as anti-TEM8/tTF, at higher concentrations may generate a more potent antitumor response but could lead to off-target thrombosis. The safety of tTF containing fusion proteins will depend on generating efficient methods to produce large quantities of these fusion proteins and to test whether higher dosing leads to nonspecific thrombotic events.

Another interesting observation of our study was the decrease in tumor vessel density with the anti-TEM8/tTF protein. A decrease in tumor vessel density is a common theme in studies targeting TEM8. The mere binding of TEM8 or preventing TEM8 from binding its physiologic partner may be enough to decrease tumor vessel density. For strategies using anthrax toxin components, the outcome may be explained by protective antigen forming a pore in cell membranes that disrupts metabolic activity and causes cell swelling (29). In the case of therapeutic molecules that either bind TEM8 or block interaction with a physiologic partner, their effect may be explained

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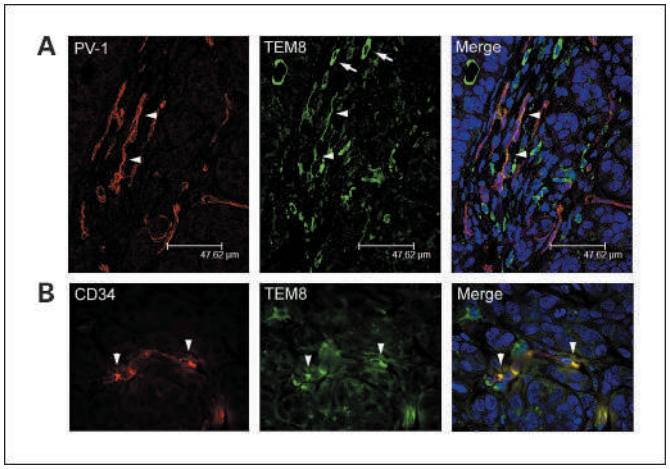

Figure 6. Colocalization of TEM8 with PV-1 and CD34. Confocal microscopy was used to characterize the localization of TEM8 in the tumor microenvironment. *A,* colocalization of TEM8 (*green*) and PV-1 (*red*; *arrowheads*). However, lack of colocalization could also be observed (*arrows*). *B,* colocalization of TEM8 (*green*) with the hematopoietic stem cell marker CD34 (*red*; *arrowheads*).

by a possible role for TEM8 in the vascular endothelial growth factor pathway (12). Therefore, the strategy used in this report may have unintentionally had a two-pronged effect that led to the decrease in vessel density. One effect prevented vessel growth by interfering with TEM8 function, whereas the other effect caused vessel disruption by thrombosis. Which of these potential mechanisms are most important in decreasing vessel density could be determined in future experiments.

Finally, this study provides evidence that TEM8 expression is not limited to endothelial cells in tumor vessels because colocalization was observed with $CD34^+$ cells. This finding supports the recent observations of another group reporting TEM8 expression in stimulated endothelial progenitor cells (30). These observations suggest that endothelial progenitor cells provide aid to distressed tumors by aiding the development of new vessels (31, 32). This is a controversial topic within the literature because the magnitude of support provided by endothelial progenitor cells during tumor angiogenesis may be different in mice and humans (33). Regardless of the exact cell types that express TEM8, our studies show that targeting this protein can influence tumor growth and progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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