Comparison of Three Different Targeted Tissue Factor Fusion Proteins for Inducing Tumor Vessel Thrombosis

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ABSTRACT

Tissue Factor (TF) is a cell membrane receptor protein that is the initiator of the extrinsic pathway of the blood coagulation cascade and normally released from damaged tissues. By substituting the attachment site with a tumor delivery agent, this potent thrombogenic protein in its truncated form (tTF) can be targeted to the tumor where it can initiate clotting, thereby occluding the tumor’s blood supply and causing rapid tumor destruction. To test the therapeutic potential of this vascular targeting approach, three fusion proteins, chTNT-3/tTF, chTV-1/tTF, and RGD/tTF, which target DNA exposed in degenerative areas of tumors, fibronectin on the tumor vascular basement membrane, and αvβ3 on the luminal side of tumor vessels, respectively, were developed and tested for their antitumor effects. Antibody binding and clotting assays demonstrated that each of the fusion proteins retained their antigen binding and thrombogenic activities. In vivo studies in mice bearing established MAD109 lung and Colon 26 carcinomas revealed that all three reagents induced histological evidence of microregional thrombosis and massive cell necrosis. Of interest, the chTNT-1/tTF and RGD/tTF fusion proteins induced thrombosis in small and medium sized tumor vessels, whereas the chTNT-3/tTF induced clotting in relatively larger vessels. Treatment studies showed that chTNT-3/tTF and chTV-1/tTF but not RGD/tTF had a significant inhibition of tumor growth. These studies demonstrate that multiple targets exist which can be used to localize tTF to occlude tumor vessels in two divergently different murine tumor models. To attain a significant antitumor effect, however, these thrombogenic agents had to occlude medium and large vessels within the tumor. Additional studies are warranted to identify maximal conditions for inducing therapeutic vascular coagulation as a new and potent method of cancer therapy.

INTRODUCTION

Rapidly proliferating tumors require an efficient blood supply to meet their nutritional needs in both primary and metastatic disease. Current methods of cancer therapy that focus on the vascular needs of the tumor have relied on the use of antiangiogenic factors which prevent the formation of new blood vessels and inhibit new tumor growth in regions of neovascularization. This approach, however, does little to eliminate areas of existing tumors where mature vessels supply adequate circulation or peripheral regions of tumors that share vascularization with adjacent normal tissues. To address these issues, a new approach has been developed that induces local thrombosis in tumor vessels and subsequent occlusion of blood flow to the tumor. A potential mediator of this event is TF, a cell membrane receptor protein that is the initiator of the extrinsic pathway of the blood coagulation cascade and normally released from damaged tissues or expressed on the surface of activated monocytes and endothelial cells. TF has been developed (4) in which the membrane-binding domain has been deleted, but the surface domain and factor VII-activating capability of the parent protein has been retained. By creating a MAb/tTF fusion protein (MAb/tTF), the MAb moiety in essence creates a new binding domain which targets the thrombogenic capacity of the tTF to the tumor vasculature, thereby enabling the initiation of coagulation and occlusion of blood flow within the tumor after binding to antigen. If proper binding occurs, rapid coagulation will be initiated, and downstream cellular degeneration and destruction will be produced in affected areas of the tumor.

Several advantages of this approach over conventional antitumor therapies have been suggested by Thorpe and Ran (5): (a) the target molecules are directly accessible to antigen, permitting rapid localization of a high percentage of the injected dose; (b) cellular degeneration caused by the occlusion of tumor vessels is microregional, amplifying the effects of therapy; (c) microvascular endothelial cells are a normal, genetically stable cell population, so target antigens remain relatively the same regardless of selective pressures exerted by cytotoxic therapies; and (d) the same target drug can be used for a variety of solid tumors because tumor vessels share common morphological, immunological, and biochemical properties.

The primary objective of our laboratory is to explore the use of MAbs to deliver potent cytotoxic agents and/or immune modulators capable of inducing sustained, effective therapy of established solid tumors. In support of this objective, we have constructed and evaluated three novel MAb fusion proteins that selectively block the blood flow to tumors by targeting different antigens. The first fusion protein, chTNT-3/tTF, targets necrotic regions of the tumor in which conserved and abundant intracellular antigens are exposed in degrading cells (6, 7). The second fusion protein, chTV-1/tTF, targets a vessel antigen, fibronectin, which is located in the basement membrane of vessels but only accessible in fenestrated (leaky) tumor endothelium (8). The third fusion protein, RGD/tTF, targets endothelial αvβ3 and αvβ3 integrins exposed in tumor vessels of several tumor types (9–11). Unlike the original studies of Huang et al. (1) which used DNA transfected tumor cells to establish proof of concept, the studies presented here use antigens present in the majority of human tumors as realistic targets for coagulagind immunotherapy. It is expected, therefore, that the generation and testing of these fusion proteins will enable the identification of potential reagents that can be used in patients to treat solid tumors refractory to other forms of cytotoxic therapy.

MATERIALS AND METHODS

Reagents and Cell Lines. The H6pQE60/tTF vector containing the cDNA of the truncated TF was the kind gift of Dr. Phil Thorpe (Southwestern Medical Center, Dallas, TX). The plasmid pEE12 with the Glutamine Synthetase Gene Amplification System was purchased from Lonza Biologics (Slough, United Kingdom). Restriction endonucleases, T4 DNA ligase, and other molecular biology reagents were purchased from New England Biolabs (Beverly, MA). RPMI 1640, MEM nonessential amino acids solution, penicillin-streptomycin solution, ABTS (2,2′-azobenzene-2-carboxylic acid), and factors VII and X and 4-chloro-1-naphthol were purchased from Sigma Chemical Co. (St. Louis, MO). Sheep antihuman TF antibody was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Factor Xa was purchased from Chromogenix (Molndel, Sweden). Hybridoma SFM medium without glutamine was...
purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Characterized and dialyzed fetal bovine sera were obtained from HyClone Laboratories, Inc. (Logan, Utah). The Madison 109 (MAD109) murine lung carcinoma and Colon 26 murine colon carcinoma cell lines were obtained from the National Cancer Institute (Frederick, MD). The Raji Burkitt’s lymphoma cell line was obtained from the American Type Culture Collection (Manassas, VA). BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Immunohistochemical reagents (HRP-conjugated goat antimouse IgG Fc, alkaline phosphatase-conjugated goat antimouse, and HRP-conjugated streptavidin) were purchased from CalTag Laboratories (Burlingame, CA). Finally, a MAb against TF was a generous gift from Dr. Phil Thorpe.

**Construction of chTNT-3/tTF and chTV-1/tTF Expression Vectors.** The expression vectors were constructed using standard techniques described previously (6, 12). The expression vector pEE12/chTNT-3 HC/LC was used as the parent vector. This plasmid contained the cDNA sequences for the human-mouse chimeric TNT-3/HC and TNT-3/LC, both of which were under the control of the cytomegalovirus major immediate early promoter. It also contained the cDNA sequence for the glutamine synthetase gene under the control of the SV40 early promoter. The PCR fragment of tTF was inserted into the NotI site of pEE12/chTNT-3, resulting in the expression vector pEE12/chTNT-3/tTF that encodes a fusion protein consisting of the chimeric light chain and chimeric heavy chain with tTF at the COOH-terminal end. The chTV-1/tTF was constructed in a manner similar to that of chTNT-3/tTF. In this case, the TV-1 variable heavy and light chain regions were shuttled into the chTNT-3/tTF HC vector.

**Expression and Purification of Antibody Fusion Proteins.** Both chTNT-3/tTF and chTV-1/tTF were expressed in NS0 murine myeloma cells according to the manufacturer’s protocol (Lonza Biologics). The highest producing clones were selected and incubated in 8-L stir flasks. The fusion proteins were then purified from clarified cell culture medium by sequential protein A affinity and ion-exchange chromatography. The purity of the fusion proteins was examined by SDS-PAGE and HPLC, using a Beckman HPLC Gold System (Beckman Instruments, Inc., Fullerton, CA) equipped with two 110B solvent pumps, a 210A valve injector, a 166 programmable UV detector, and a 406 analogue interface module. Size exclusion chromatography was performed on a G4000SW column (TosoHaas, Montgomeryville, PA) with 0.1 M PBS (pH 7.2), as the solvent system, eluting at a flow rate of 1 ml/min. The UV absorbance of the HPLC eluate was detected at 280 nm.

**Construction, Expression, and Purification of RGD/tTF.** Overlapping oligonucleotides encoding the RGD peptide sequence CDCRGDCFC (RGD-4C; Ref. 11) were synthesized and allowed to anneal to the tTF sequence. The entire fragment of RGD-tTF was amplified by PCR. The PCR product was digested with the NcoI restriction enzyme and cloned into the H6pQE60/tTF vector, resulting in an expression vector encoding a fusion protein consisting of three sections: (a) the RGD cDNA for targeting tumor vessels; (b) the tTF cDNA for thrombogenic activation; and (c) a 6XHis tag to facilitate purification. The RGD/tTF fusion protein was expressed in *Escherichia coli* strain Top10 and purified by Ni-NTA affinity chromatography according to the manufacturer’s protocol (Qiagen, Valencia, CA). The purified RGD/tTF was analyzed by SDS-PAGE as described above.

The presence of the tTF moiety for each fusion protein was further confirmed by Western blotting analysis. The proteins in the SDS-PAGE gel were transferred to a nitrocellulose membrane (Micron Separations, Inc.) and incubated sequentially with sheep antimouse TF antibody, biotinylated secondary antibody, HRP-conjugated streptavidin, and 4-chloro-1-naphthol to identify those bands containing the tTF moiety.

![Fig. 1. Analysis and identification of TFF constructs by SDS-PAGE and Western blot.](https://cancerres.aacrjournals.org/content/67/21/5047/F1.large.jpg)

![Fig. 2. Antigen-antibody binding studies to demonstrate retention of antibody activity by chTNT-3/tTF to crude DNA (A), chTV-1/tTF to fibronectin (B), and RGD/tTF to αβ integrin (C).](https://cancerres.aacrjournals.org/content/67/21/5047/F2.large.jpg)
A University Animal Care Committee-approved protocol. The tumors were to which 5 nM Factor X was added. The mixture was incubated at room temperature for another 10 min, to which 100 mM EDTA were added to quench the reaction. Next, 2 nM chromogenic substrate Spectozyme Factor Xa were added, and the mixture read at 405 nm in the first 5-min time period.

To verify the clotting abilities of the tTF moiety of these fusion proteins, a clotting activity assay was performed as described by Ruf et al. (13). Briefly, various concentrations of tTF or fusion proteins were mixed with 100 nM Factor VII in Tris-buffered saline buffer and incubated at 37 °C for 30 min, to which 5 nM Factor X was added. The mixture was incubated at room temperature for another 10 min, to which 100 mM EDTA were added to quench the reaction. Next, 2 nM chromogenic substrate Spectozyme Factor Xa were added, and the mixture read at 405 nm in the first 5-min time period.

Experimental. Functional assays to test the targeting moiety of the fusion proteins were conducted based on the availability of the antigen or receptor. In the case of chTNT-3/tTF, antigen-binding studies of the fusion protein were analyzed by ELISA (7) using crude DNA as antigen. For the chTV-1/tTF and RGD/tTF fusion proteins, fibronectin and α5β3 integrin were used, respectively, in ELISA studies as described previously (8, 10).

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Treatment Studies in Mouse Tumor Models. Groups of 6-week-old female BALB/c mice were injected s.c. in the left flank with a 0.2-ml inoculum containing 5 × 10⁶ of MAD109 lung or Colon 26 colon carcinoma cells under a University Animal Care Committee-approved protocol. The tumors were grown for 7 days until they reached ~0.5 cm in diameter. In the first treatment study, groups of MAD109-bearing mice (n = 6–8) were injected i.v. daily × 5 with 10 μg of RGD/tTF or 20 or 40 μg of chTV-1/tTF using a 0.1-ml inoculum. In the second study, groups MAD109-bearing mice (n = 6–8) were injected i.v. at 3-day intervals × 3 with 2.5 or 10 μg of chTNT-3/tTF using a 0.1-ml inoculum. In the third treatment study, groups of Colon 26-bearing mice (n = 6–8) were injected i.v. daily × 5 with RGD/tTF (10 μg) or chTV-1/tTF (40 μg) using a 0.1-ml inoculum. Other groups of Colon 26-bearing mice were also injected with chTNT-3/tTF (2.5 or 10 μg) at 3-day intervals × 3. In addition, a combination treatment study was performed by injecting chTV-2/tTF (20 μg) and RGD/tTF (5 μg) daily × 5 followed by chTNT-3/tTF (2.5 μg) at 3-day intervals × 3. In all treatment studies, control groups of mice were injected with PBS or chTNT-3 (10 μg). Tumors were assessed every other day by caliper measurement in three dimensions. Tumor volumes were calculated according to the formula: width × length × height.

Immunohistochemical Localization of tTF Fusion Proteins in Tumor. Tumors from treated mice were removed and snap frozen in liquid nitrogen. Cryostat sections of the tissues were cut and stained immunohistochemically for the presence of tTF fusion proteins. chTNT-3/tTF and chTV-1/tTF were detected using HRP-conjugated goat antihuman IgG Fc, followed by development with the colorimetric agent, 3,3’-diaminobenzidine. RGD/tTF was detected using a biotinylated MAb against TF followed by HRP-conjugated streptavidin and development with 3,3’-diaminobenzidine. Slides were observed under the microscope, and fields of interest were recorded using a digital camera.

Histological Analyses. To assess the extent and location of thrombosis in tTF fusion protein-treated mice, tumor and normal organs (heart, lung, liver, and kidney) were collected at 12, 24, 48, and 72 h after injection, fixed in 10% buffered neutral formalin overnight, embedded in 2% paraffin, sectioned, and stained with H&E. Thrombosis of vessels was assessed as either total or incomplete depending on the extent of closely packed erythrocytes, blurring of the vessel outline, and the presence of aggregated platelets and fibrin deposition.

RESULTS

Construction, Expression, and Purification of Fusion Proteins. The pEE12 expression vectors have been successfully used by our laboratory for the expression of other chimeric antibodies and fusion proteins (12, 14). Positive clones expressing the fusion proteins were selected by ELISA and cloned by limiting dilution methods. The highest yielding clone was selected by a 24-h assay (10⁶ cells/1 ml/24 h). Selected clones were then grown in 8-liter stir flasks for large scale production. By these methods, the chTNT-3/tTF and chTV-1/tTF produced 24 and 36 mg/liter, respectively, after purification.

Fig. 3. Induction of thrombosis by fusion proteins to demonstrate retention of TF-clotting activity by chTNT-3/tTF (A), chTV-1/tTF (B), and RGD/tTF (C) using the Spectozyme Fxa assay.

Functional Studies of chTNT-3/tTF, chTV-1/tTF, and RGD/tTF Fusion Proteins. Functional assays to test the targeting moiety of the fusion proteins were conducted based on the availability of the antigen or receptor. In the case of chTNT-3/tTF, antigen-binding studies of the fusion protein were analyzed by ELISA (7) using crude DNA as antigen. For the chTV-1/tTF and RGD/tTF fusion proteins, fibronectin and α5β3 integrin were used, respectively, in ELISA studies as described previously (8, 10).

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Fig. 4. Histological sections of MAD109 lung carcinoma removed from mice treated with chTNT-3/tTF (10 μg) demonstrating thrombosis of large tumor vessels and massive necrosis at 24 h [A; arrow points to blood vessel (H&E, ×200)] and 72 h [B; arrow points to necrotic cells (H&E, ×160)].
The synthesized oligonucleotides of the RGD sequence were inserted into the pGEx60/tTF vector, resulting in an expression vector encoding a fusion protein consisting of 6Xhis, RGD, and tTF. The resulting RGD/tTF fusion protein was expressed in E. coli and yielded ~4 mg/liter after purification. SDS-PAGE analysis demonstrated that the three fusion proteins were properly assembled as shown in Fig. 1A. The molecular weights of the light and heavy chain chTNT-3 plus tTF and chTV-1 plus tTF were at $M_\text{r} \approx 30,000$ and $88,000$, respectively, and the molecular weights of RGD/tTF and 6Xhis/tTF were $M_\text{r} \approx 38,000$ and $35,000$, respectively. The presence of the tTF moiety of the three fusion proteins was identified by Western blotting (Fig. 1B). A contaminating $M_\text{r} 31,000$ band in the RGD/tTF preparation is a by-product of the Ni-NTA affinity chromatography purification process as shown previously by Stone et al. (4). The purity of the mammalian constructs was confirmed by HPLC, which showed that the chTNT-3/tTF and chTV-1/tTF each had a main peak with a retention time of ~685 s.

**Functional Analysis Studies.** In the case of chTNT-3/tTF, crude DNA was used as the antigen for coating the ELISA plates (Fig. 2A). The dose response binding to crude DNA was similar to that seen for the chTNT-3 parent antibody. ELISA studies demonstrated that the chTV-1/tTF bound fibronectin as the parent antibody (Fig. 2B), and RGD/tTF bound $\alpha_\text{v}\beta_3$ integrin as expected (Fig. 2C).

As shown in Fig. 3, the TF moieties of the three fusion proteins were indirectly measured by the Spectrozyme FXa assay. The half-maximal activities of the three fusion proteins were observed at a concentration of ~100 nmol of protein, which is comparable with the activity of free tTF.

**Histological Studies.** In the chTNT-3/tTF group, 80% of the tumor blood vessels were thrombosed 12 h after the first injection of 10 $\mu$g. In addition, a few of the large vessels of the tumor showed RBC extravasation indicative of vessel injury. By 24 h, almost all of the blood vessels in the tumor were thrombosed and completely occluded, and tumor cells around the vessels appear damaged (Fig. 4A). By 48 h, advanced degeneration and necrosis were observed throughout the tumor, and by 72 h, extensive necrosis and cytolysis were obvious in the central regions (Fig. 4B). By contrast, no apparent abnormality was observed in normal organs (heart, lung, liver, and kidney) of the treated mice at autopsy or by histological analysis at 24 and 72 h after the first injection.

In the chTV-1/tTF-treated group, 80% of the tumor vessels were thrombosed, which generated massive tumor necrosis distal to the site.
of occlusion. Most of the thrombosed vessels, however, were small and medium in size, but some larger vessels were also affected (Fig. 5C). In the RGD/tTF-treated group, ~40% of the tumor vessels were thrombosed, and most of them were either capillaries or small vessels of the tumor (Fig. 5B). Compared with the chTNT-3/tTF- (Fig. 5D) and chTV-1/tTF-treated groups (Fig. 5C), RGD/tTF-treated mice had less completely thrombosed vessels and only minimal areas of tumor degeneration (Fig. 5B).

Histological analyses at the rims of the tumors showed that chTNT-3/tTF (Fig. 6D) treatment produced the most complete thrombosis of tumor vessels (80%) and necrosis of tumor among the three fusion proteins. By contrast, treatment with chTV-1/tTF (Fig. 6C) caused mostly incomplete thrombosis of tumor vessels (60%) and less tumor necrosis, whereas the RGD/tTF treatment produced thrombosis in only 20% of tumor blood vessels at the rims (Fig. 6B), with little accompanying tumor necrosis when compared with tumor sections obtained from PBS-treated control mice (Fig. 6A).

**Localization Studies.** Immunohistochemical studies of sections from the treated mice showed that RGD/tTF localized after i.v. administration to capillaries and small vessels of the tumor (Fig. 7B), whereas chTV-1/tTF cells localized to both small and medium sized vessels of tumor (Fig. 7C), consistent with the thrombogenic activity of these fusion proteins noted above. By contrast, chTNT-3/tTF localized to relatively larger tumor vessels and nuclei of necrotic tumor cells and degenerating endothelial cells after i.v. administration (Fig. 7D).

**Treatment Studies in Mouse Tumor Models.** MAD109-bearing BALB/c mice were treated with each of the three tTF fusion proteins at different dose levels when the tumors grew to ~0.5 cm in diameter. Twenty-four h after the first dose, tumors of mice treated with chTV-1/tTF (20 and 40 μg) and chTNT-3/tTF (2.5 and 10 μg) turned black, whereas less marked color changes were noted in the RGD/tTF-treated mice. By contrast, no coloration change was observed in the PBS- and chTNT-3-treated control groups. Average tumor volumes of the chTV-1/tTF- (Fig. 8) and chTNT-3/tTF (Fig. 9)-treated groups on the 12th day after the first injections were <50% of the tumors in the PBS- and chTNT-3-treated control groups. However, the RGD/tTF-treated mice, which received five consecutive doses, showed no significant inhibition of tumor growth (Fig. 8).

Similar treatment results with each of the three fusion proteins were observed in the Colon 26 tumor model (Fig. 10). Both the groups treated with chTNT-3/tTF (10 μg) and chTV-1/tTF (40 μg) displayed...
a significant inhibition of tumor growth, but neither groups treated with RGD/tTF (10 μg) nor chTNT-3 (2.5 μg) displayed tumor inhibition. Treatment with a combination of the three fusion proteins [chTNT-3/tTF (2.5 μg) + chTV-1/tTF (20 μg) + RGD/tTF (5 μg)] demonstrated more significant reduction of tumor growth, compared with chTNT-3/tTF (10 μg) or chTV-1/tTF (40 μg) alone (Fig. 10). In all of the above experiments, complete tumor regression in the treated mice was not observed. Histological examination of the heart, liver, lung, and kidney from the treated mice did not show any evidence of thrombosis.

DISCUSSION

In this study, three genetically engineered fusion proteins have been successfully generated and produced in high yield. Both the targeting and thrombogenic moieties of the fusion proteins were shown to retain binding activity to antigen and coagulation properties by in vitro assays, respectively. In vivo, all three fusion proteins were found to produce significant thrombosis in tumor vessels. Interestingly, each displayed different features of thrombosis according to their targeting properties. Thrombosis caused by RGD/tTF mainly occurred in capillaries and small vessels, whereas thrombosis caused by chTV-1/tTF occurred in small and medium vessels. By contrast, thrombosis caused by chTNT-3/tTF appeared principally in relatively larger vessels. A comparative histological analysis of thrombosis and subsequent degeneration within tumors showed that chTNT-3/tTF caused the most extensive amount of vessel occlusion with widespread degeneration and necrosis followed by chTV-1/tTF and RGD/tTF, which caused relatively less complete thrombosis and tumor necrosis. Additional histological studies of the thrombosed vessels at the rims of tumor confirmed that chTNT-3/tTF and chTV-1/tTF caused greater thrombosis and subsequent degeneration in tumors than RGD/tTF. A cause-and-effect relationship between the occlusion of tumor vessels and degeneration and necrosis of tumor cells was clearly seen in chTNT-3/tTF- and chTV-1/tTF-treated tumor-bearing mice. In both animal tumor models (MAD109 and Colon 26), chTNT-3/tTF and chTV-1/tTF showed a significant inhibition of tumor growth but no apparent dose response effect. Because the amount and severity of tumor vessel thrombosis induced by tTF fusion proteins were found to depend on the distribution of their targets in the tumor, tTF fusion protein-induced tumor vessel thrombosis appears to be more of a threshold event. Compared with the Colon 26 tumor model, the Mad109 lung carcinoma model was more sensitive to coaguligand therapy possibly

Fig. 7. Immunohistochemical localization of tTF fusion proteins in MAD109 tumors analyzed by immunohistochemical staining at 48 h after injection with PBS control (A), RGD/tTF (10 μg; B), chTV-1/tTF (10 μg; C), and chTNT-3/tTF (10 μg; D). Arrows point to blood vessels (×160).
impressive results obtained with this fusion protein. Because of these results, it was found that the antitumor effects induced by these fusion proteins were determined not only by the specificity of the delivery moiety but also by the population and distribution of their target (antigens or receptors).

Thorpe and Ran (5) have suggested that for optimal effects, tTF needs to be targeted to the luminal surface of the tumor endothelium, preferably in all regions of the tumor mass. Nonluminal markers may not yield effective targets for coagulants, probably because platelet activation, assembly of coagulation factors, or both occur most efficiently on the luminal side. Because RGD receptors are located on the luminal side of tumor vessels (15, 16), the mechanism of vessel thrombosis with the reagent is readily understood. However, chTV-1 targets fibronectin situated on the basement membrane of vessels (8), which is located in the abluminal side of blood vessels. Despite this, chTV-1/tTF also caused a strong thrombosis of tumor vessels and showed a significant therapeutic effect. To explain this occurrence, an alternative view postulated by Nilsson et al. (17) should be considered. These authors reasoned that fenestrations causing leakiness of tumor blood vessels allow the extravasation of Factor VIIa, which might bind to the chTV-1/tTF anchored at high density on fibronectin, fostering the conversion of Factor X into Factor Xa in the perivascular space immediately around the blood vessels and facilitating the diffusion of Factor Xa and the blood clotting cascade. Alternatively, fibrin deposition could start in the perivascular space and propagate back into the luminal aspects of tumor blood vessels.

Likewise, the mechanism by which chTNT-3/tTF, which targets necrosis, produces extensive thrombosis and tumor regression is not obvious. Previous studies (18) by our laboratory have shown that chTNT-3 binds DNA and accumulates in degenerating and necrotic areas of tumors. Two mechanisms may explain the thrombogenic activity of chTNT-3/tTF in these studies. First, coagulation and fibrin deposition may begin in the perivascular space before diffusing into blood vessels via vascular fenestrations (19) by a mechanism similar to the one mentioned above. Alternatively, extracellular DNA may accumulate on the endothelial cell surface or the basement membrane providing a suitable target for the chTNT-3/tTF to induce coagulation.

because of the fact that it is a much faster growing tumor, making it more susceptible to vessel thrombosis.

By contrast, RGD/tTF, which localized to capillaries and small vessels, caused little tumor damage in vivo and did not inhibit tumor growth. This may be explained by the fact that the receptor for RGD, \( \alpha_\beta_3 \), is mainly associated with endothelial cells undergoing angiogenesis known to occur principally in newly formed capillaries and small sized vessels. As a consequence, RGD/tTF was less damaging to larger sized, mature vessels, which when blocked, are able to induce more widespread tumor destruction. The restricted distribution of RGD receptors and their relative low affinity for ligand compared with antigen–antibody interactions may therefore explain the less
and thrombosis of tumor vessels via luminal or abluminal routes. In the immunohistochemical sections shown in Fig. 7D, the extensive amount of chTNT-3/tTF accumulating in necrotic areas is apparent, lending support for this explanation. It should be noted that one advantage of targeting necrosis is that as areas of degeneration are produced by treatment, chTNT-3/tTF will have new sites to bind on the administration of subsequent doses, thereby extending the destructive effects of this thrombogenic agent in the tumor.

A comparison of the three targeting approaches to deliver the tTF to the tumor site demonstrated that chTNT-3 and chTV-1 were found to be the most effective vehicles. Although RGD/tTF alone did not display significant antitumor effects on its own, its use in combination with the other fusion proteins was found to produce additive effects, consistent with the fact that different vessels were targeted by each of the fusion proteins. In summary, we have shown that tTF, when targeted to diversely different target sites, can cause extensive thrombosis in tumor vessels, leading to effective antitumor therapy in two experimental solid tumor models of the mouse. Additional studies are warranted to optimize the effects of these thrombogenic agents, perhaps by previous sensitization with other agents or drugs.

REFERENCES

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