A hybrid fibronectin motif protein as an integrin targeting selective tumor vascular thrombogen

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Abstract

Targeted thrombotic eradication of solid tumors is a novel therapeutic strategy. The feasibility, efficacy, selectivity, and safety are dependent on multiple variables of protein design, molecular assembly, vascular target, and exclusive restriction of function to the tumor vasculature. To advance this strategy, we describe a design of an integrin targeting selective tumor vascular thrombogen. We adopted the fibronectin structural motif of tandem repeating modules with four type III repeat modules of fibronectin followed by two structurally homologous modules of the extracellular domain of tissue factor. This hybrid protein of six tandem modules recognizes integrins and selectively docks and initiates the thrombogenic protease cascade locally on the target cell surfaces. The protein is inactive in blood but is functionally active once assembled on integrin-positive cells. When administered i.v. to tumor-bearing mice, it selectively induces extensive local microthrombosis of the tumor microvasculature. The principles are addressed from the perspective of protein structural design for a class of selective tumor vascular thrombogen proteins that, through interaction with tumor angiogenic endothelium, elicit thrombotic occlusion rather than apoptosis or arrest of angiogenesis. This response can produce local tumor infarction followed by intratumoral ischemia-reperfusion injury, inflammation, and a local host tumor eradicative response. [Mol Cancer Ther 2004;3(7):793–801]

Introduction

There has been a strategic focus on interrupting tumor angiogenesis and inducing local tumor endothelial apoptosis to eliminate the nutrient blood supply to tumors and thereby tumor growth arrest and eradication (1, 2). Support for the general concept may be drawn from the unusual but established clinical application of simple direct physical vascular occlusion of the arterial supply to solid tumors, which has seen some selective, although limited, success. The approach involves embolization of the arterial vessels by physical or chemical means, although this has demonstrated some efficacy only when it has been possible to selectively interrupt the blood supply to the tumor with limited ischemic necrosis of contiguous normal tissue (3, 4). Solid tumors may coopt local capillaries as in skeletal muscle but more frequently induce local angiogenesis to support tumor growth (5, 6). In contrast to arrest of angiogenesis, targeted induction of the coagulation protease cascade for occlusive thrombosis of the tumor vasculature results in local tumor ischemia, infarction, a local inflammatory response, and potential tumor eradication (7, 8). Potentially, this tactic can interrupt the local tumor blood supply at various stages of microvascular development and vasculogenic mimicry with local extension of thrombosis to more established intratumoral vessels that may lack target expression. Similar methods might be applicable in some circumstances to other pathologic states associated with inappropriate local vascularization such as proliferative retinopathy, inflammatory arthritis, and juvenile hemangioma.

To achieve efficient, effective, and highly localized thrombosis, one must target the effector molecules and only there assemble the initiation complex of the coagulation protease cascade. We have adopted as the thrombogenic element of a novel protein tissue factor (TF) the transmembrane cell surface protein that is the major initiator of the thrombogenic cascade. It is the plasmalemma receptor and obligatory cofactor for cell surface assembly and initiation of the coagulation protease cascade (9). Human TF is a glycoprotein of 263 residues, which shares structural homology with the cytokine receptor protein family (10), and is composed of a 219-residue extracellular domain of two fibronectin type III repeat-like modules, a single transmembrane domain, and a short cytoplasmic domain (11, 12). To initiate the coagulation protease cascade, TF binds and facilitates activation of plasma factor VII (FVII) to the serine protease VIIa (FVIIa) and allosterically conforms the catalytic site of the bound FVIIa. TF also serves as the substrate presenting molecule for FVIIa, where it associates with and presents the physiologic substrate zymogens factor X (FX) and factor IX (FIX) for specific proteolytic activation by the FVIIa catalytic site (13, 14). Although the formation of the bimolecular TF:VIIa complex requires only the extracellular domain of TF, the precise localization of the complex on anionic cell membrane microdomains is also required for

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significant gain of function (15). The anionic membrane surface associates with the proteins and enhances the affinity of FVIIa for TF and serves for efficient colocalization of substrates FX and FIX with the requisite three-dimensional alignment necessary for efficient limited proteolytic activation (15-17). This is graphically illustrated in Fig. 1A and B.

We have adopted integrin targeting as a reasonable target for selective tumor vascular thrombogen (STVT). The extracellular matrix glycoprotein fibronectin contains domains that mediate diverse functions including association with cells (18, 19). One well-characterized region that mediates cellular interactions is the central cell binding domain. This is composed of a tandem assembly of multiple homologous modules, mostly type III repeats of ~90 amino acids each. An arginine-glycine-aspartatic acid (RGD) amino acid triad in the 10th type III repeat is a key recognition site for several integrins, including $\alpha_5\beta_1$ and $\alpha_V\beta_3$ (18, 20, 21). Structural contributions from repeat 9 also contribute to the affinity of ligand structure and cellular binding (22, 23).

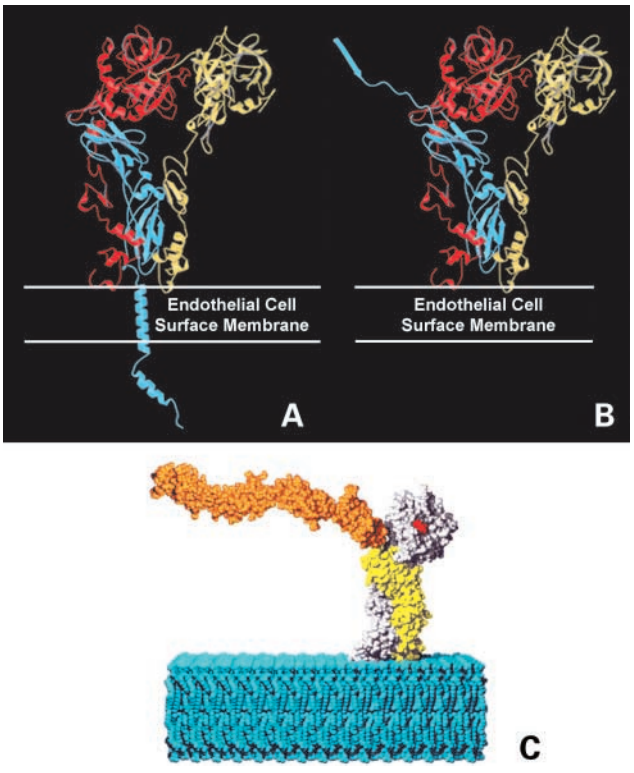

Figure 1. **A,** a model of the ternary complex of TF/FVIIa/FX on cell surface. The TF, FVIIa, and FX are *blue*, *red*, and *yellow*, respectively. The transmembrane structure of native TF ensures proper positioning of both FVIIa and FX onto the cell surface. The interaction of the NH_2-terminal Gla domain of both FVIIa and FX with cell membrane is critical for the full coagulant activity of this complex. **B,** a model of ternary complex of STVT with FVIIa and FX. STVT is a soluble TF fused with a binding structure (*blue arrow*) through its NH_2 terminus. The NH_2 terminus of TF is not involved with binding with either FVIIa or FX, such that fusion constructs will allow a proper docking of this complex onto cell surface in a conformation very similar to its native structure and recover substantial coagulant activity. **C,** a model of Fn-$TF_{3\text{-}218}$ associated with VIIa docked on cell surface. The fibronectin domain is *orange*, the TF extracellular domain is *yellow*, and the VIIa is *white*. The cell membrane is *blue*, and the catalytic site of VIIa is *red*.

Based on current understanding of the molecular interactions and their structural basis for initiation of the thrombogenic protease cascade, and with the goal to create a soluble hybrid human protein that in plasma would be inactive but initiate local occlusive thrombosis only on a selected local vascular surface, we adopted the extracellular domain of TF as one element. The other requirement for targeting was to be achieved by incorporating in the same protein part of the cell binding domain of fibronectin. We created a six-module fibronectin structural homologue in which fibronectin type III repeat modules 8 to 11 were incorporated as the NH_2-terminal aspect followed by the two modules of the extracellular domain of TF to complete the protein. This protein indeed demonstrates both targeting and localized activation properties in a manner comparable with the targeting prodrug concept (24) in which it assembles on the endothelial surface of the tumor microvasculature and only once there functions to initiate and support the thrombogenic cascade (Fig. 1C). This was indeed observed, and synthetic peptides containing the RGD sequence blocked binding and inhibited the local initiation of the thrombogenic cascade. *In vivo* activity of this integrin targeting activatable thrombogen was demonstrated in an *in vivo* tumor thrombosis assay in which tumor vascular thrombosis was rapidly induced by i.v. administration of the protein.

Materials and Methods

Plasmid Construct for Fibronectin-$TF_{3\text{-}218}$

The selected fibronectin nucleotide sequence was acquired by PCR amplification from marathon-ready cDNA of human placental origin (Clontech Laboratories, Palo Alto, CA) using primers 5′-CACCAACAACTTGCATCTGGAGGC-3′ and 5′-AACATTGGGTGGTGTCCACTGGGC-3′ and vent DNA polymerase (New England Biolabs, Beverly, MA). After 35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 75°C, a resultant 1,445-bp fragment was purified and used as the template for another PCR with oligos FN5a 5′-ACCATCACGGATCCGGGGTCGTCGACACCTCCTCCCACTGACCTGCGA-3′ and 5′-GGTACCGGAGGAGCTCGTTACCTGCAGTCTGAACCAGAGG-3′ to amplify a 1,131-bp fragment. The selected TF sequence was amplified from plasmid pTrcHisC-tTF (25) with the oligos 5′-ACGAGCTCCTCCGGTACCACAAATACTGTGGGCAGC-3′ and pTrc-seq 5′-TCTGCGTTCTGATTTAATCT-3′ to generate a 714-bp fragment. The 1,131-bp fibronectin fragment and the 714-bp TF fragment were combined and amplified as a fusion construct by PCR using primers FN5a and pTrc-seq to yield a 1,827-bp fragment. This product was digested with *Hin*dIII and partially digested with *Bam*HI, and the resulting 1,753-bp fragment was ligated into the *Bam*HI and *Hin*dIII sites of the vector pTrcHisC (Invitrogen, Carlsbad, CA). The resulting plasmid (FNTF2)

encodes a protein with a short His6 tag at the NH_2 terminus followed by fibronectin residues 1,237 to 1,600, a five-residue linker peptide, and TF_{3-218} residues at the COOH terminus. Plasmid FNTF2 was transformed into the *Escherichia coli* host strain BL21 (Stratagene, La Jolla, CA) for protein production.

Proteins

The soluble extracellular domain of TF_{1-218} was expressed in *E. coli*, purified, and folded according to an established procedure (25). Human FX was purified from plasma (26) followed by immunoaffinity chromatography over immobilized anti-FVII monoclonal antibody F21-4.2 to reduce FVII contamination (27). FVII was affinity purified with a calcium-dependent antibody to the glutamic acid (Gla) domain and followed by a Mono-Q ion exchange chromatography that resulted in activation of FVII to FVIIa. The fibronectin-TF_{3-218} (Fn-TF_{3-218}) protein was expressed in *E. coli* and folded. Briefly, BL21 strain bacteria harboring the expression vector was pelleted from cultures 5 hours after isopropyl-1-thio-B-D-galactopyranoside induction and lysed using lysozyme. Inclusion bodies were isolated by repeated sonication and centrifugation. The resultant inclusion bodies were resuspended in Ni-NTA affinity purification buffer containing 6 mol/L guanidium chloride by sonication and affinity purified on a Ni-NTA column. The purified fractions were combined, and DTT was added to 50 mmol/L final concentration and incubated at room temperature overnight. Folding of the protein was performed in 50 mmol/L Tris, 2 mol/L urea, 0.5 mmol/L oxidized glutathione (0.5 mmol/L), and 2.5 mmol/L reduced glutathione for 4 days at 4C. The folded soluble fraction was collected and subjected to size exclusion chromatography.

Cell Binding Assay and Western Blot Analysis

Serial concentrations of Fn-TF or TF_{1-218} were incubated with integrin-positive Chinese hamster ovary (CHO) K1 cells. The bound and unbound proteins were separated by centrifugation of the cells. The cell-bound Fn-TF_{3-218} and TF_{1-218} were quantitated by Western blot analysis. Immunoreactivity of Fn-TF and TF_{1-218} was analyzed by Western blot, where serial concentrations of protein were electrophoretically separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline. A cocktail of three monoclonal antibodies to conformation-dependent epitopes of human TF at 1 μg/mL was incubated with the membranes for 1 hour at 37°C (28). Enzyme-conjugated rabbit anti-mouse IgG antibody was used as the second antibody, and the blots were visualized by enhanced chemiluminescence (Amersham-Pharmacia, Piscataway, NJ). Band intensity was quantitated by scanning laser densitometry and compared with that of TF_{1-218} standards.

Amidolytic Assay

The amidolytic activity for peptidyl substrate of FVIIa in complex with Fn-TF_{3-218} was analyzed by hydrolysis of chromozym tissue plasminogen activator (Boehringer-Mannheim, Mannheim, Germany) and compared with that of TF_{1-218} (29). Serial concentrations of TF_{1-218} or Fn-TF_{3-218} were incubated with FVIIa at room temperature for 15 minutes. Chromozym tissue plasminogen activator was added to 1 mmol/L. The initial rates of hydrolysis were determined at 406 nm with a kinetic microtiter plate reader for data processing. The final concentration of FVIIa was 5 nmol/L and Ca^{2+} was 5 mmol/L.

FXa Generation Assay

The functional proteolytic activity of both Fn-TF_{3-218}:VIIa and TF_{1-218}:VIIa complexes for FX conversion to FXa was determined by a functional assay (30) using Spectrozyme FXa reagent. Briefly, serial concentrations of Fn-TF_{3-218} and TF_{1-218} were preincubated with FVIIa at 75 nmol/L for 5 minutes at 37°C in 5 mmol/L $CaCl_2$, TBS. The reaction was initiated by addition of substrate FX at 1.5 μmol/L. After 10 minutes at 37°C, the reaction was arrested with EDTA to 0.1 mol/L. The FXa generated was quantitated from FXa chromogenic substrate Spectrozyme FXa (American Diagnostica, Greenwich, CT) at 50 mmol/L at 405 nmol/L.

Coagulation Assay

Coagulation assays were performed using an established procedure with modifications to accommodate the binding of Fn-TF_{3-218} to cells (15, 31). Platelet depleted, pooled citrated human plasma was used. Cells were dislodged with trypsin-free cell dissociation buffer (Life Technologies, Inc., Carlsbad, CA), washed twice with TBS, and counted. Cell viability was <90%. Serial concentrations of Fn-TF_{3-218} were incubated with 1×10^5 cells in 100 μL TBS containing 10 mmol/L Ca^{2+} and 5 mmol/L Mg^{2+} for 15 minutes at 37°C. The assays were initiated by addition of 100 μL plasma at 37°C, and clotting times were recorded.

In Vivo Tumor Vascular Thrombosis

The activity of the recombinant Fn-TF_{3-218} protein *in vivo* was assayed in BALB/c mice bearing syngeneic CT26 colon carcinoma tumors. BALB/c mice ages 4 to 6 weeks from the Scripps breeding colony were inoculated s.c. on the back with 500,000 CT26 tumor cells per site. Assay was performed ~10 days later when the tumors reached ~6 mm in diameter through bolus i.v. injection of the indicated proteins associated with FVIIa via tail vein. Mice were sacrificed 20 minutes after protein administration, and tumor and organ specimens were collected for histologic analysis. These procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at the Scripps Research Institute. The work was conducted within the Scripps Research Institute facilities, which are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Scripps Research Institute maintains an assurance with the Public Health Services, is registered with the U.S. Department of Agriculture, and is in compliance with all regulations relating to animal care and welfare.

Histologic and Immunohistochemical Analysis

Histologic analysis was performed on formalin-fixed sections. The immunohistochemical staining was performed on unfixed frozen 5 μm thick sections on poly-L-lysine slides. For endothelial identification, biotinylated rat

anti-mouse CD31 monoclonal antibody (MEC 13.3) was used with Texas red–conjugated strepavidin as the secondary reporting reagent. For Fn-TF_{3-218} identification, a cocktail of three monoclonal antibodies (TF8-5G9, TF9-6B4, and TF9C3) to conformation-dependent epitopes of human TF (28, 32) was used at 1 μg/mL. The reaction was visualized with FITC-conjugated anti-mouse IgG. The slides were analyzed by laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Model of Ternary Structure of TF:VIIa:X

The ternary structure model is based on the crystal structure of TF:VIIa (33). A Gla-deleted FX structure (34) is the primary source for the FX model and for FX docked on the TF:VIIa complex using Insight II software.

Results

Proteins

A DNA fragment encoding human fibronectin type III repeats 8 to 11 was amplified by PCR and fused to a DNA fragment encoding the TF_{3-218} extracellular domain residues. Both TF_{1-218} and Fn-TF_{3-218} proteins accumulated in *E. coli* inclusion bodies. The protein was purified and folded. SDS-PAGE analysis of purified TF_{1-218} and of Fn-TF_{3-218} (Fig. 2A) is shown as well as Western blot analysis with anti-TF monoclonal antibodies to conformational native epitopes on a replica gel (Fig. 2B).

Amidolytic Activity of Fn-TF_{3-218}:VIIa Is Comparable with That of TF_{1-218}:VIIa

The functional activity of Fn-TF_{3-218} to serve as the requisite cofactor for FVIIa amidolytic activity as compared with TF_{1-218} was analyzed (Fig. 2C). Little, if any, difference is discernable between the two protein complexes, indicative that the functional integrity of the TF domain expressed within Fn-TF_{3-218} is not adversely affected by hybridization with the fibronectin modules, and the subtle contacts that are responsible for the allosteric induction of FVIIa amidolytic activity are preserved.

Proteolytic Activity of Fn-TF_{3-218}:VIIa Toward FX Is Not Affected by NH_2-Terminal Fusion

The effect of the fused docking structure on the proteolytic activity of Fn-TF_{3-218}:VIIa toward FX was investigated by a linked functional FXa generation assay (30). The proteolytic activity of soluble Fn-TF_{3-218}:VIIa for the FX substrate increases with the concentration of Fn-TF_{3-218} similar to that observed with TF_{1-218}, indicating that the fused fibronectin modules do not interfere with the recognition of FX by the hybrid Fn-TF_{3-218}:VIIa protein (Fig. 2D).

Binding of Fn-TF_{3-218} to Integrin Expressing CHO K1 Cells

Fn-TF_{3-218} protein bound to integrin-positive CHO K1 cells increased incrementally with increasing concentration of Fn-TF_{3-218}. This was in contrast to control TF_{1-218}, which had no demonstrable association with CHO K1 cells (Fig. 3A). The most highly expressed integrins on the CHO cells are of the β1 family (VLA), and there is dynamic regulation of integrin β1-mediated adhesion. The binding of soluble Fn-TF_{3-218} to these CHO K1 cells is consistent with the known relatively low-affinity binding of fibronectin to β1 integrins (35).

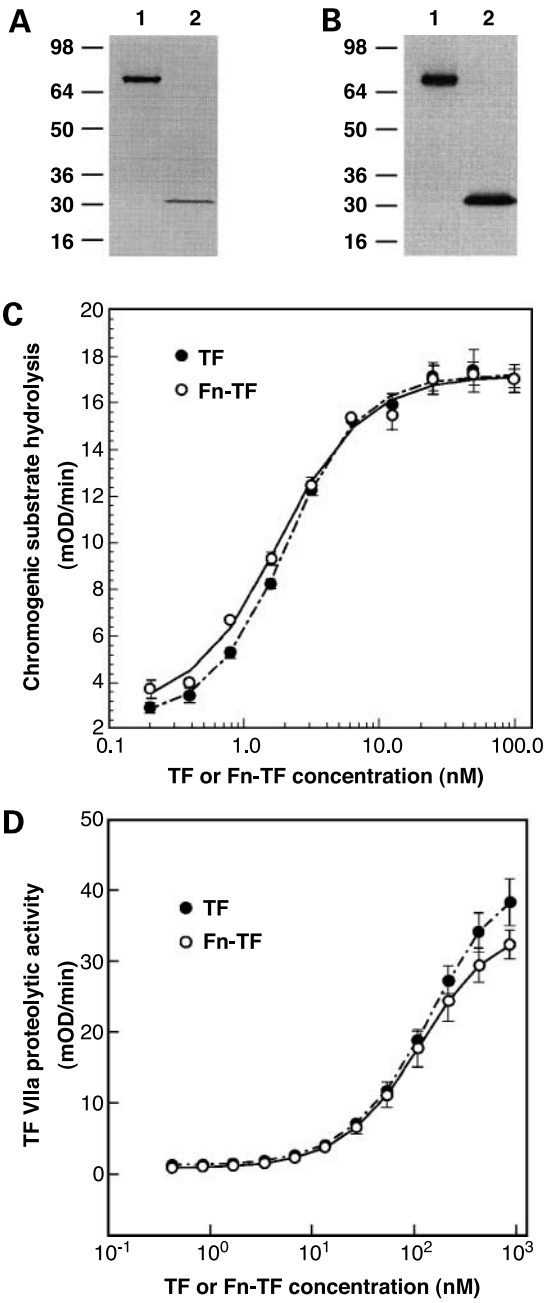

Figure 2. **A,** a silver-stained gel of purified Fn-TF_{3-218} (*lane 1*) and TF_{1-218} (*lane 2*). Only a single band was observed in both samples, indicating the purity of the preparations. **B,** a Western blot analysis of a replica gel of **A**, purified Fn-TF_{3-218} and TF_{1-218} in *lanes 1* and *2*, respectively. respectively. **C,** ability of Fn-TF_{3-218} to act as cofactor for enhancement of VIIa amidolytic activity. **D,** the effect of the fused docking structure on the proteolytic activity of Fn-TF_{3-218}:VIIa in comparison with TF_{1-218}:VIIa was compared using a linked functional assay.

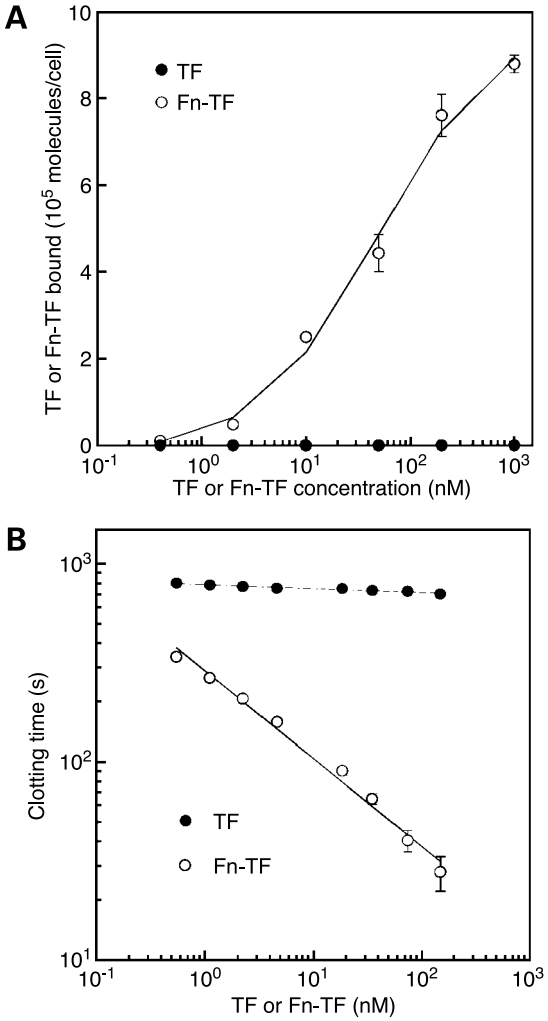

Figure 3. **A,** binding of Fn-$TF_{3\text{-}218}$ to integrin expressing cells. The amount of Fn-$TF_{3\text{-}218}$ bound increases as the Fn-$TF_{3\text{-}218}$ concentration increases, in contrast to soluble TF, which shows no appreciable association with CHO K1 cells. **B,** induction of localized coagulation at cell surface is studied by a cell-mediated coagulation assay of Fn-$TF_{3\text{-}218}$ using cells expressing integrins (CHO K1).

Induction of Coagulation by Fn-$TF_{3\text{-}218}$ Association with the Cell Surface

There is no endogenous TF expression by CHO K1 cells that is detectable by Northern blot, anti-TF antibody, or plasma coagulation assay (data not shown). Thus, they represent a suitable cell line to investigate the functional activity of Fn-$TF_{3\text{-}218}$ on an integrin-positive cell surface. Fn-$TF_{3\text{-}218}$ bound to CHO K1 cells and induced activation of the coagulation cascade in a plasma clotting assay. The coagulation time decreased with increasing Fn-$TF_{3\text{-}218}$ in contrast to $TF_{1\text{-}218}$, which lacked this property (Fig. 3B). This indicates that cell surface–associated Fn-$TF_{3\text{-}218}$ is able to assemble a functional initiation complex with substrates FX and FIX to initiate the protease cascade (Fig. 1C). In reactions with CHO K1 cells in the absence of Fn-$TF_{3\text{-}218}$, there was no acceleration of the plasma clotting time (>600 seconds), whereas at estimated association of 100,000 Fn-$TF_{3\text{-}218}$ molecules per cell, the clotting time was accelerated to 20 seconds. This is $\sim 10^4$ functional acceleration.

Inhibition of Coagulant Activity of Fn-$TF_{3\text{-}218}$ by RGD Peptide

The coagulant activity conferred by Fn-$TF_{3\text{-}218}$ in the presence of CHO K1 cells was fully abolished by the presence of RGDS peptide, inferred to result from inhibition of association of the fibronectin component of the Fn-$TF_{3\text{-}218}$ protein with cell surface integrins (Fig. 4). There is no effect of the peptide on TF^+ CHO cells. This supports that enhancement of function is mediated through cell surface docking of Fn-$TF_{3\text{-}218}$ and functional positioning of the TF structure of Fn-$TF_{3\text{-}218}$ on anionic microdomain sites of the cell to facilitate functional orientation of Fn-$TF_{3\text{-}218}$:VIIa complex and presentation of FX and FIX.

Comparison of the Functional Activity of Fn-$TF_{3\text{-}218}$ with That of the Native TF

The activity of Fn-$TF_{3\text{-}218}$ was compared with recombinant full-length TF using a CHO K1 cell line transfected and stably expressing TF at 5×10^5 per cell. Based on Fn-$TF_{3\text{-}218}$ binding to CHO K1 cells, 75 nmol/L Fn-$TF_{3\text{-}218}$ incubated with 10^5 CHO K1 cells in a 100 μL volume resulted in an estimated 5×10^5 Fn-$TF_{3\text{-}218}$ molecules bound per cell. Coagulant activity of 10^5 CHO K1 cells with 5×10^5 Fn-$TF_{3\text{-}218}$ bound per cell was compared with that of the 10×10^5 CHO K1 cells each expressing 5×10^5 native

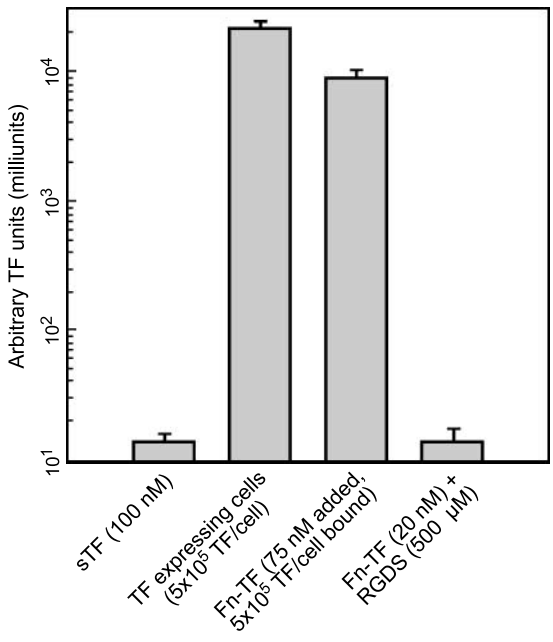

Figure 4. Comparison of the thrombogenic activity of Fn-$TF_{3\text{-}218}$ thrombogen with that of the native TF and inhibition of Fn-$TF_{3\text{-}218}$ thrombogen activity by RGDS peptide. At 5×10^5 Fn-$TF_{3\text{-}218}$ per cell bound, Fn-$TF_{3\text{-}218}$ has recovered an estimated 10% thrombogenic activity of that of native TF expressed at same number per cell.

TF (Fig. 4). The coagulant activity of Fn-TF_{3-218} detected is ~10% of that of the native cell surface TF. Therefore, compared with TF_{1-218}, cell surface targeting of Fn-TF_{3-218} results in an ~10^4 increase in coagulant activity.

In Vivo Tumor Thrombosis

The selective thrombogenic function of Fn-TF_{3-218} was assessed *in vivo* for its ability to selectively thrombose tumor microvasculature using the murine CT26 syngeneic colon carcinoma model. When 20 g of Fn-TF_{3-218} were administered via tail vein, there was very rapid thrombosis of tumor vasculature (Fig. 5A). The appropriate plasma half-life was 20 minutes determined by quantitative Western blotting. In contrast, there were no observed effects or tumor microvascular thrombosis in mice to which TF_{1-218} was administered (Fig. 5B). The number of thrombosed vessels in Fn-TF_{3-218} and TF_{1-218} treated tumors was compared (Fig. 5C). The Fn-TF_{3-218} protein was present in thrombosed tumor vessels that are outlined by

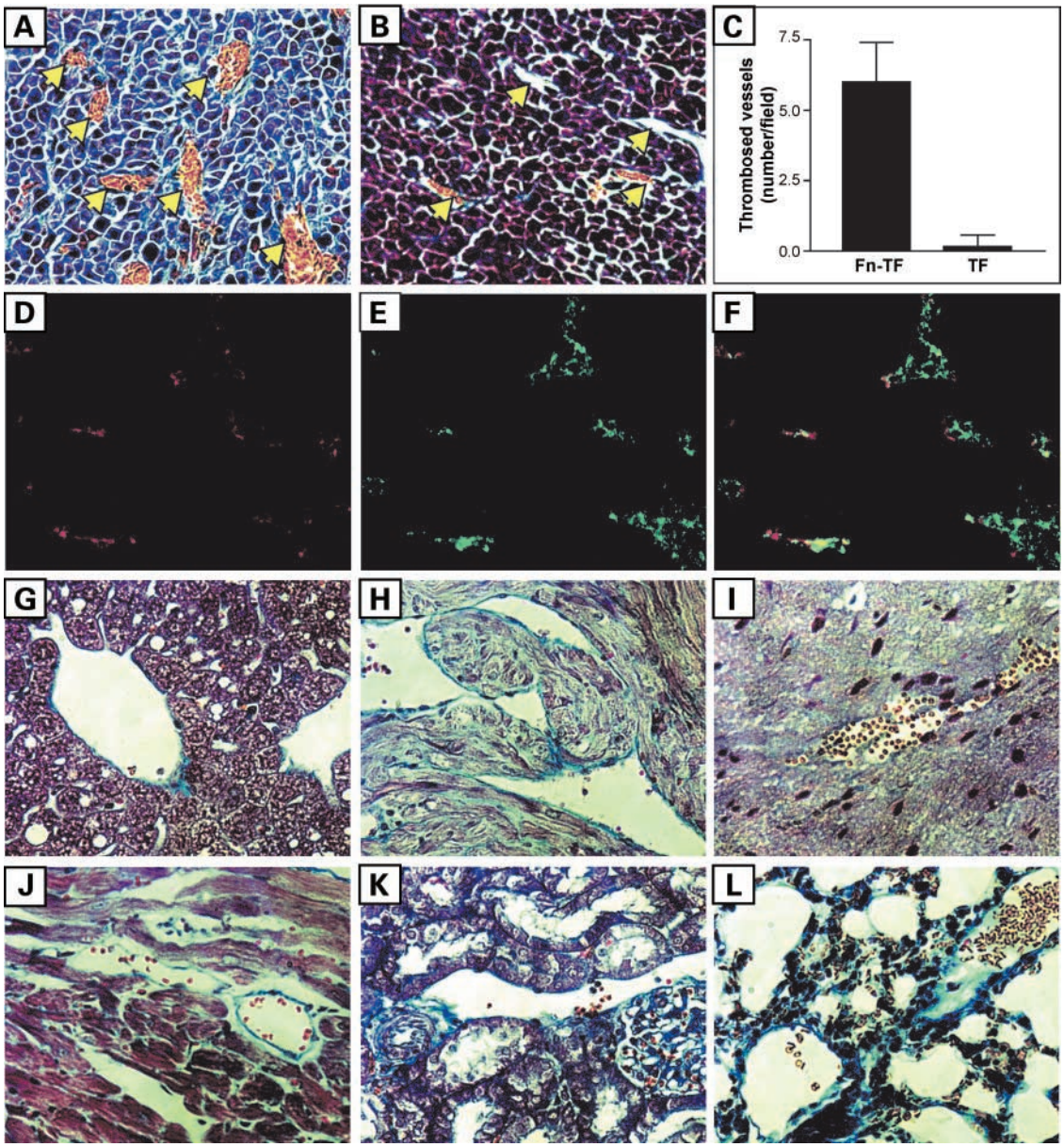

Figure 5. *In vivo* thrombosis of tumor vasculature. Administration of the thrombogen caused thrombosis in tumor vasculature but not in normal organs. **A,** tumor containing thrombotic vessels caused by integrin targeting thrombogen (vessels are indicated by *yellow arrowheads*, ×40). **B,** a control tumor treated with TF_{1-218} (vessels are indicated by *arrows*, ×40). **C,** quantitative analysis of thrombosed vessels in Fn-TF_{3-218} treated ($n = 6$) versus control TF_{1-218} treated ($n = 6$) tumors (magnification at ×40, $P < 0.001$). **D** to **F,** representative immunohistochemical analysis of thrombosed tumors with anti-CD31 antibody (*red*), anti-human TF antibody (*green*), and the merged image of **D** and **E,** respectively. **G** to **L,** representative Carstair staining of organ sections from FN-TF_{3-218} treated tumor-bearing mice from liver, heart, brain, muscle, kidney, and lung, respectively (×40).

CD31-positive endothelial cells (Fig. 5D-F). Despite the presence of integrins on tumor cell surface, integrin targeting thrombogen binding to tumor cells was not evident. There were no discernable gross untoward effects observed for the mice, nor were there demonstrable vascular thrombosis elsewhere in the tissues of Fn-TF_{3-218} treated CT26-bearing mice (Fig. 5F-L). These data indicate that Fn-TF_{3-218} does selectively target tumor microvascular integrins and the thrombogenic cascade resulting in highly selective local tumor thrombosis.

Discussion

Integrin targeting of therapeutic modalities has been an attractive approach in development of prospective cancer therapeutics (1, 36, 37). The concept has been to target such molecules selectively to tumor vasculature and thereby interrupt angiogenesis and deliver drugs or viral vectors to the tumor. The conceptual extension of this strategy has been to deliver, in an inactive prodrug form, the initiating molecule of the thrombogenic cascade to the tumor vasculature in a manner such that there is local complementation for induction of occlusive microvascular thrombosis of the tumor (7, 8, 38, 39). In the present design of a prospective endothelial surface integrin targeting STVT, we created a novel hybrid protein using as the structural theme the tandem repeating modular structure of the cell binding domain of fibronectin (22). This protein incorporates four type III repeat modules of fibronectin containing the integrin binding RGD ligand followed by the two relatively homologous modules of the extracellular domain of TF. We find that a potent and effective STVT results, and it can be expressed as a soluble protein capable of circulating in an inactive state in plasma *in vivo* and localizing to tumor microvasculature. This Fn-TF_{3-218} protein possesses fully functional binding of FVIIa and provision of necessary cofactor function demonstrated by both amidolytic and proteolytic activities of the bound FVIIa using substrate FX comparable. Association of Fn-TF_{3-218} to integrin expressing cells through its fibronectin modules was observed compared with TF_{1-218}, and this association resulted in a $\sim 10^4$ increased initiation of plasma coagulation. These data support the design premise that the NH_2 terminus of TF is tolerant of incorporation into a homologous fibronectin domain and that the hybrid Fn-TF_{3-218} can provide the desired targeting and thrombogenic cascade docking functions.

The three-dimensional structure of human TF (40, 41) and of the functional bimolecular TF:VIIa complex (33) has provided valuable insight into the molecular basis of TF initiation of the thrombogenic cascade and insight and detail for design of proteins that can retain this property. The TF extracellular domain makes extensive contact with VIIa. In the complex, FVIIa adopts an extended conformation with the NH_2-terminal γ-carboxylated Gla domain oriented to the anionic cell surface and the protease domain positioned distally. Both epidermal growth factor domains of FVIIa bind TF. Extensive mutagenesis study of both TF and FVIIa reveal that the TF contact interfaces with the FVIIa Gla domain and that epidermal growth factor-I domain accounts for most of the binding energy (27). In contrast, the interaction between the NH_2-terminal module of TF and the protease domain of FVIIa is energetically much weaker; rather, it appears to be critically important for inducing allosteric activation of the FVIIa catalytic site (42-44). For design of functional thrombogens, the extracellular domain of TF must retain the precise complex structure necessary for the high-affinity assembly of FVIIa with TF for induction of function. This has been preserved in the Fn-TF_{3-218} structure, testifying to the preservation of specific interactions with FVIIa and with substrate FX although incorporated into a larger protein.

The first solutions to the three-dimensional atomic structures of the ternary complexes of FX/Xa and FIX/IXa with TF:VIIa have recently emerged (45, 46). These complexes are relatively stable even with their product active enzymes FXa and FIXa. Because FVIIa, FX, FXa, FIX, and FIXa are highly homologous, it is accepted that the substrate proteins will adopt a similar extended structure on docking as observed for the TF:VIIa complex, and this is compatible with structural solutions for free FIX and FX (34, 47). The substrates FX and FIX associate with cells, binding to anionic membrane surfaces (48), and thus may facilitate association and assembly of the Fn-TF_{3-218} protein in complex with VIIa. The charge-dependent phospholipid surface interaction of both FVIIa and the substrates is critical to the function of this protease complex. The importance of surfaces such as anionic phospholipid membrane for the assembly and function of this protease complex has been extensively characterized. Activation of macromolecular substrates is poor in the absence of such anionic surface (15). The cleavage of small peptidyl substrates of FVIIa is largely unaffected by deletion of its Gla domain, suggesting that membrane association is not critical for the proper activation of the catalytic triad of the protease domain. However, in the TF:VIIa complex, efficient proteolysis is not simply determined by function of the active catalytic site but requires an extensive recognition and association between protease complex and protein substrate as well as their proper assembly on the cell surface. Proteolysis of macromolecular substrate is severely reduced with Gla-deleted FVIIa when associated with TF. Phospholipid-bound FX was shown to be preferentially activated (15), indicating the preferred recognition of membrane-associated substrate is also an important requirement in modulation of the TF:VIIa:X(IX) complex by membrane surfaces. Consequently, the proper assembly of the TF:VIIa:X(IX) complex on the cell surface is required for significant enhancement of the activity of this complex $\sim 10^4$ fold. The fusion of a docking structure to the NH_2 terminus of TF does not affect recognition and activation of substrates as determined by functional assays. The binding of the docking structure to its cell surface target will localize the Fn-TF_{3-218}:VIIa to the vascular cell surface expressing integrins and thereby recruit cell surface–associated FX(IX). The Gla domain of FVIIa and

FX(IX) can mediate the association of the Fn-TF_{3-218}:VIIa:X(IX) complex with the cell membrane and mimic a conformation that is similar to the TF:VIIa:X(IX) complex. Data included in this study show that formation of Fn-TF_{3-218}:VIIa complex on cell surfaces can restore as much as 10% of the function activity of the native complex and ~10^4 greater than in the absence of an integrin-positive cell surface. Depending on the distance between the binding site of the docking structure to the cell membrane, the activity of the complex may be adjusted through the introduction of a flexible linker to allow the complex to touch down onto the cell membrane. Cooperation of a membrane binding region in the COOH terminus of the TF domain may also increase the membrane association of the thrombogen:VIIa:X(IX) complex, thus further improving its activity. VLA is the major integrin expressed on the CHO cell surface. It has not been determined which type expressed integrin supported coagulant activity of this particular thrombogen. Apparently, most integrin subtypes will support the coagulant activity of the Fn-TF_{3-218} thrombogen probably with different potencies. Integrins, especially $\alpha_v\beta_3$ and $\alpha_v\beta_1$, are reported to be expressed on angiogenic tumor vessels (36, 37). Inhibition of $\alpha_v\beta_3$ integrin with LM609 antibody was shown to inhibit angiogenesis and tumor growth (37). This Fn-TF_{3-218} thrombogen caused rapid thrombosis of tumor vessels consistent with accessible integrin presence in tumor neovasculatures.

Positional heterogeneity of the vascular endothelium has been increasingly recognized. Angiogenesis in undesired locations is involved in whole range of disease states. Tumor vasculature is anatomically and physiologically distinct from normal vessels. The value of site-specific induction of thrombosis in tumor vasculature as a potential treatment for solid tumors is promising. A growing list of proteins are discovered enriched on tumor vasculatures, such as endoglin (49, 50), $\alpha_v\beta_3$ integrin (37), vascular endothelial growth factor receptor, prostate-specific membrane antigen (8, 38), and TEM1/endosialin (51), as well as other targetable biochemical entities such as a class of novel glycosaminoglycans recognized by vascular endothelial growth factor heparin binding domain (39), therefore providing targets to construct a variety of STVTs that will specifically target and thrombose tumor vessels (52).

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