Tissue factor is an angiogenic-specific receptor for factor VII-targeted immunotherapy and photodynamic therapy

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Abstract Identification of target molecules specific for angiogenic vascular endothelial cells (VEC), the inner layer of pathological neovasculature, is critical for discovery and development of neovascular-targeting therapy for angiogenesis-dependent human diseases, notably cancer, macular degeneration and endometriosis, in which vascular endothelial growth factor (VEGF) plays a central pathophysiological role. Using VEGF-stimulated vascular endothelial cells (VECs) isolated from microvessels, venous and arterial blood vessels as in vitro angiogenic models and unstimulated VECs as a quiescent VEC model, we examined the expression of tissue factor (TF), a membrane-bound receptor on the angiogenic VEC models compared with quiescent VEC controls. We found that TF is specifically expressed on angiogenic VECs in a time-dependent manner in microvessels, venous and arterial vessels. TF-targeted therapeutic agents, including factor VII (fVII)-IgG1 Fc and fVII-conjugated photosensitizer, can selectively bind angiogenic VECs, but not the quiescent VECs. Moreover, fVII-targeted photodynamic therapy can selectively and completely eradicate angiogenic VECs. We conclude that TF is an angiogenic-specific receptor and the target molecule for fVII-targeted therapeutics. This study supports clinical trials of TF-targeted therapeutics for the treatment of angiogenesis-dependent diseases such as cancer, macular degeneration and endometriosis.

Keywords Angiogenesis · VEGF · Tissue factor · Factor VII-targeted therapy · Vascular endothelial cells

Introduction

Angiogenesis, the formation of new blood vessels from existing blood vessels, is a common and critical pathological process in many human diseases [1], including but not limited to cancer, age-related macular degeneration (AMD), endometriosis and rheumatoid arthritis. For example, in cancer, the newly formed blood vessels, usually termed as tumor angiogenic vessels or tumor neovasculature, can be differentiated from normal (resting, quiescent) vessels in normal tissues in morphology (vascular and endothelial shapes), and function (increased permeability) [2, 3]. As such, tumor neovasculature provides not only nutrients and oxygen for cancer cells to proliferate, but also serves as a conduit for cancer cells to metastasize. Thus, the tumor neovasculature is a key target for development of therapeutic agents. The search for biomarkers as target molecules specific for angiogenic vascular endothelial cells (VEC), the inner layer of pathological neovasculature, is promising and critical in
developing novel neovascular-targeting therapies for these common human diseases [1]. One potential approach to eliminating pathological neovascularization involves attacking the subset of vascular endothelial cells that are angiogenic, via a cell-type-specific surface molecule coupled to a cell-killing agent. Based on the in vivo observation of TF expression on tumor vascular endothelial cells in breast cancer tissues from patients [4] and some mouse models of human tumor xenografts [5–7], we have begun developing such an approach using tissue factor (TF), a 47-kDa membrane-bound receptor [4, 8–11], as the target molecule on angiogenic VECs. To assemble this TF-directed treatment, we used, as the starting molecule, the principal ligand of TF, factor VII (fVII) [12]. To eliminate the coagulation activity of zymogenic fVII, a coagulation active site lysine 341 was replaced by an alanine (thereafter containing the mutation of K341A in fVII polypeptides unless specified) using site-directed mutagenesis [5, 13, 14]. Using this strategy, we have pursued two different therapeutic approaches; immunotherapy (by fusing fVII to an IgG1 Fc as an antibody-like immunoconjugate, i.e., fVII/IgG1 Fc, also called ICON) [5, 14] and photodynamic therapy (PDT) (by chemically conjugating fVII peptides with photosensitizers (PS)) fVII/PS, for fVII-targeted PDT, abbreviated as fVII-iPDT) [15, 16] for the treatment of cancers including melanoma [5, 13], prostate [14], head and neck [17], breast [7, 15, 16] and lung [6], as well as age-related macular degeneration (AMD) [18–20] and endometriosis [21] in preclinical animal models and in phase I and II clinical trials for AMD patients (NCT01485588) [22]. In vivo data confirm that the TF-targeted agents ICON and fVII-iPDT can selectively eradicate pathological neovascularization in cancer [13, 23], AMD [18–20] and endometriosis [21]. However, it remains unclear which angiogenic growth factor induces TF expression commonly detected in these pathological angiogenesis-dependent malignancy and non-malignant diseases, whether TF is also selectively expressed by in vitro angiogenic VEC models and whether TF-targeted agents can selectively eradicate in vitro models of angiogenic VECs via targeting TF without harming quiescent VECs. This study is designed to address these questions by identifying a common angiogenic growth factor (for example, VEGF) in these angiogenic-dependent diseases, examining TF expression on in vitro models of angiogenic and quiescent VECs and then testing the selectivity and effectiveness of TF-targeted agents (ICON and fVII-PS) for binding and killing the in vitro angiogenic VECs.

Vascular endothelial growth factor (VEGF) plays a central role in angiogenesis-dependent cancer and non-malignant human diseases [24], such as macular degeneration [25], rheumatoid arthritis [26] and endometriosis [27]. Specifically, VEGF stimulates angiogenesis by binding to VEGFR receptors on VECs in the pathological neovascularature (usually micro- or capillary vessels) in those angiogenesis-dependent diseases. In humans, there are three major types of blood vessels, namely micro- or capillary vessels, veins and arteries. Accordingly, VEC, the inner layer of blood vessels, can be isolated from microvessels, venous and arterial vessels. It is previously known that VEGF can induce TF expression on human umbilical vein endothelial cells (HUVEC), a commonly used VEC model in angiogenesis studies. To better mimic pathological angiogenesis, an ideal angiogenic VEC model should be derived from micro- or capillary vessels. This study is designed to investigate whether angiogenic VECs from micro- or capillary vessels express TF upon VEGF stimulation and whether fVII-dependent therapies target non-pathological (quiescent) endothelial cells using VEGF-stimulated and unstimulated VECs as in vitro models. Therefore, in the current study, we examined the expression patterns of TF on in vitro models of angiogenic and quiescent VECs from three types of blood vessels and sought to rigorously assess the validity of the claim that dimeric fVII-IgG1Fc (ICON) in immunotherapy and monomer fVII peptide in PDT are selective and effective in killing angiogenic VECs via TF targeting.

Materials and methods

Cell culture

HMVECs (Lonza), HUVEC (generously provided by the Yale Vascular Biology and Therapeutics (VBT) Program) and HAEC (Lonza) were purchased and grown in growth medium supplemented with heat-inactivated fetal bovine serum (FBS, Sigma) and 1 × penicillin and streptomycin (Gibco), following the manufacturers’ protocols. Lung-derived normal human microvascular endothelial cells (HMVEC-LBI, simplified as HMVEC) were purchased (Lonza). As assayed by the manufacturer, the HMVEC cells were endothelial marker CD31 positive, lymphatic endothelial marker podoplanin negative and muscle cell marker alpha actin negative. These assay results confirmed their origin of blood VEC. It was cultured in Lonza’s growth medium, EBM-2 basal medium supplemented with EGM-2 MV SingleQuot Kit supplements and growth factors (Lonza) and 20% FBS, propagated with the Reagent-Pack Subculture Reagents (Lonza) containing the three solutions, trypsin/EDTA, trypsin neutralizing solution, and HEPES-buffered saline. HUVECs were grown in M199 growth medium supplemented with 20% heat-inactivated FBS (HI-FBS) and 1:100 ECGS solution, as described [15]. HAECs (Lonza) were grown in EGM-2 medium.
supplemented with 10% HI-FBS. Cells are cultured at
37 °C with 5% CO₂. Human breast cancer MDA-MB-231
line was grown in DMEM supplemented with 10% FBS.
CHO-K1 lines were grown in F-12 K (ATCC) medium
with 10% FBS and 0.5 mg/ml G418 (Invitrogen), including
CHO-K1 cell lines stably expressing TF, EPCR or TF + EPCR. Untransfected CHO-K1 cells were used as
negative control for TF and EPCR and for generating a
stable CHO-K1/EPCR line by transfection with a plasmid
encoding EPCR (kind gift from Dr. Ruf).

Generation of in vitro angiogenic and quiescent
VEC models by VEGF stimulation

HMVECs (Lonza), HUVEC (Yale VBT group) and HAEC
(Lonza) were first seeded with 5 × 10³ cells/cm² cultured in
complete growth media with heat-inactivated FBS and
growth factors, either in 6-well microplates (1.25 × 10⁵
cells per well for extraction of cell lysates for Western
blotting), 96-well microplates (1.5 × 10³ cells in 100 µl per
well for cell ELISA and fVII-tPDT) or in 8-chamber slides
(3.5 × 10³ cells in 500 µl per chamber for immunofluorescent
staining and confocal microscopy), until 80–90% con-
fluence is reached. Prior to VEGF stimulation, the VECs
were washed x3 with HEPES-buffered saline to remove
trace of FBS and growth factors (to minimize their potential
effect on induction of TF and EPCR expression) and then
starved in serum-free and growth factors-free EBM-2 basal
medium (Lonza) or endothelial cell serum-free medium
(Invitrogen) overnight. The cells were then exchanged with
half of the same serum-free medium supplemented with
a final concentration of 1 nM VEGF (BD Biosciences), a
concentration that was previously tested for induction of TF
expression on HUVEC [28], for 2, 4, 6, 8 and 24 h as
angiogenic VEC models. The VECs were starved but were
not stimulated with VEGF as quiescent VEC control (0 h).
And then the VECs were used as in vitro angiogenic (1 nM
VEGF stimulation for 4 h as TF reaching the peak expression
at this time point) and quiescent VEC models in experiments
with Western blotting, confocal microscopy, cell ELISA and
fVII-tPDT treatment and mechanism studies.

Immunofluorescent staining and confocal
microscopic imaging

For confocal microscopy, the VECs grown on chamber
glass slides were fixed with 4% paraformaldehyde (PFA)
and then immunostained for TF using mouse mono-
clonal anti-human TF (HTF1) (kind gift from William
Konigsberg) (clone HTF 1) [29], anti-human endothelial
marker CD31 PE conjugate (BD Biosciences) and nuclei
staining dye DAPI (Molecular Probes) for confocal
microscopy.

Protein samples of angiogenic and quiescent VECs
for Western blotting

For Western blotting, protein samples were extracted from
the VECs (HMVEC, HUVEC and HAEC) by TRizol
reagent (Invitrogen) followed by immunoblotting for TF
and GAPDH expression using antihuman TF (HTF1) [29]
and anti-GAPDH (Research Diagnostic Inc) antibodies,
and the band intensities were normalizing to GAPDH using
NIH ImageJ analysis.

Cell ELISA and inhibitory cell ELISA

For cell ELISA, the VECs grown in 96-well plates were
assayed for binding of mfVII/hIgG1 Fc, mfVII/Sp and anti-
TF (HTF1) antibodies followed by anti-mfVII (for mfVII
proteins) and then by corresponding secondary antibody
HRP conjugates (antihuman IgG, anti-mouse IgG or anti-
rat IgG antibodies). For inhibitory cell ELISA, the VECs
were incubated first with anti-HTF or anti-EPCR antibodies
(clone RCR-379, Sigma, as control antibody) prior to
incubation with mfVII/hIgG1 Fc at molar ratios from 0.1:1
to 10:1 (antibody to fVII/IgG1 Fc).

We compared two anti-HTF antibodies, mouse mono-
clonal antihuman TF (HTF1) and goat polyclonal antihu-
man TF (goat anti-TF, American Diagnostica), for their
inhibitory effect on mfVII/IgG1 Fc binding to human
breast cancer MBA-MB-231 cell line, which was known to
express a high level of TF [15, 16]. For fVII blocking
experiments, we first stimulated HMVEC with 1 nM
VEGF for 4 h then fixed the cells. After blocking with 1%
BSA, we incubated the VEGF-stimulated HMVEC with
goat anti-TF (American Diagnostica) or mouse anti-TF
(HTF1) antibodies then added mfVII/hIg Fc. To determine
whether fVII is a ligand for TF, we developed an inhibitory
cell ELISA assay, in which angiogenic VEC (stimulated
with 1 nM VEGF for 4 h) was first fixed by 4%
paraformaldehyde (PFA) and subsequently blocked by
0.1–100 nM of inhibitory anti-HTF (goat anti-HTF) or
anti-EPCR (RCR-379), which blocks binding of EPCR
ligand to EPCR [30]. The procedure was terminated by
incubation with 10 nM mfVII/hIgG Fc (molar ratios
0.01:1–10:1, inhibitory antibody to mfVII/hIgG Fc) and
then with anti-hIgG HRP conjugate.

Production of mfVII/Sp- or mfVII/NLS-SnCe6
conjugates

The procedures have been described in detail [6, 15, 16].
Briefly, the mfVII/Sp protein is composed of murine
fVII(K341A), an S peptide (Sp) tag with a mutation at
D14 N and a polyhistidine tag (His tag) for protein
purification and detection (mfVII(K341A)/Sp(D14 N)/His,
abbreviated as fVII/Sp [15, 16] for simplification). The mfVII/NLS protein is composed of the coding sequences for mfVII(K341A), 2 repeats of the wild-type nuclear localization sequence (NLS) (PKKKRKVG) of SV40 T-antigen [31], a tyrosine residue (requisite for future radiiodination imaging) and a His tag (mfVII(K341A)/NLS/His, abbreviated as fVII/NLS). The fVII/Sp and fVII/NLS proteins were produced using CHO-K1 producer cells and purified using the Ni-NTA affinity resin as described [15, 16]. The procedure for the conjugation of mfVII proteins to photosensitizer SnCe6 was described [15, 16].

**In vitro fVII-tPDT for HMVECs**

For fVII-tPDT, HMVECs grown in 96-well plates were incubated with 2 μM fVII/Sp-SnCe6 or fVII/NLS-SnCe6 for 90 min, which was previously optimized as the drug-light interval for fVII-tPDT [16], then were irradiated (635 nm, 36 J/cm²) with a 635-nm fiber-coupled diode laser (BWF2-635-0.1-100-0.22, B&W Tek, Inc.). The fluorescence rate of the laser unit was measured by using a laser power meter (LaserCheck, Coherent, Inc.) prior to carrying out PDT. We used two assays to determine the effect of fVII-tPDT, one was crystal violet assay staining for monolayer membrane loss (overnight after fVII-tPDT) and the other was clonogenic assay for observing longer-term viability (10-14 days after fVII-tPDT), as described [7, 15, 16, 32]. Briefly, HMVEC cells, uninduced or induced by VEGF for 4 h, were washed with 1× HBSS containing 1% BSA, incubated with different concentrations of SnCe6-conjugated mfVII/Sp or mfVII/NLS, or free SnCe6 in the incubation buffer (1× HBSS with 1% BSA and 10 mM CaCl₂), or no treatment in the buffer as controls, and cultured for 90 min at 37 °C. Then, the cells were washed and refilled with the growth medium for laser irradiation at 635 nm for 36 J/cm².

**Crystal violet staining and clonogenic assay**

Crystal violet staining and clonogenic assays after PDT were performed as described [15, 16]. Briefly for clonogenic assay, HMVECs in 96-well plates after PDT were cultured overnight, were transferred to a 60-mm dish and were diluted at tenfold serial dilutions. The cell suspensions were cultured for 12–14 days to allow colony formation, followed by fixation and crystal violet staining at the same condition used above for staining cells directly after PDT. Visible colonies of more than 50 cells were counted, and total cells in each original well, designated as colony formation units # (CFU), were back calculated by colony number times dilution factor. Percent colony formation, in comparison with no-treatment controls, was calculated as (colony # in experimental wells—average colony # in no-treatment wells) × 100%.

**Apoptosis and necrosis assays for mechanisms of in vitro PDT**

Cells cultured in 96-well plate after PDT were immediately washed with the HEPES buffer (10 mM HEPES, pH 7.4, 140 mM saline, 2.5 mM CaCl₂), stained with 1:20 Annexin V-FITC (Invitrogen), for phosphatidylserine, and 1 μg/ml of propidium iodide (PI), for DNA, in the HEPES buffer for 15 min at room temperature, and then washed and refilled with in 1× DPBS. The cells were visualized and photographed immediately under a fluorescent microscope.

**Statistical analyses**

All quantitative assays were done in duplicates to calculate averages and standard deviation (mean ± SD). The half maximal effective concentrations (EC₅₀) of fVII-tPDT and mPDT were calculated by the best-fit linear regression or nonlinear one-phase decay equations using Prism software (GraphPad). P values were calculated using 2-way ANOVA with multiple comparisons test using Prism software (GraphPad), as specified in each figure legend.

**Results**

To test for expression of angiogenic surface receptors, we used VEGF to generate angiogenic VEC models in vitro, and tested for the presence of TF on primary human VECs derived from three major types of mammalian vessels: (1) HMVEC, human microvascular endothelial cells; (2) HUVEC, human umbilical venous endothelial cells; and (3) HAEC, human aortic endothelial cells.

We first examined the time course of induced TF expression on angiogenic and quiescent HMVEC (Fig. 1a), HUVEC (Fig. 1b) and HAEC (Fig. 1c). The Western blotting results in Fig. 1 showed that (1) TF protein, reported MW 47 kDa, was not detected in quiescent VEC (starved and unstimulated) (time point = 0 h, red arrows in TF expression fold chart) from all three VECs; (2) TF was detected in VEGF-treated angiogenic VECs (HMVEC, HUVEC and HAEC) within 2 h of VEGF stimulation; (3) and peaked at 4–6 h, then started decreasing at 6 h post-stimulation.

To confirm the time course of induced TF expression on endothelial cells, we stained VEC for TF and the endothelial marker CD31 and observed the stained VEC under confocal microscope. The results in Fig. 1d showed that TF was not detected on quiescent VEC (0 h). After VEGF stimulation, however, TF expression was detected at
2 h with a peak in fluorescent intensity at 4–6 h followed by a decrease that was still visible at 24 h.

Next, we examined the binding (specificity) of fVII therapeutic agents to angiogenic VECs. To generate angiogenic VEC with maximal TF expression, VECs were incubated with 1 nM VEGF for 4 h based on the results in Fig. 1. Confocal imaging confirmed that TF was expressed on angiogenic HMVEC (Fig. 2a), HUVEC (Fig. 2b) and HAEC (Fig. 2c) after 4 h stimulation with VEGF, but not on resting VECs (Fig. 2b, d, f). Taken the results in Figs. 1 and 2, we conclude that TF is selectively expressed on angiogenic VEC, which can be induced on VECs from all three types of blood vessels (vein, microvessels and artery).

To assess the binding specificity of murine fVII/human IgG1 Fc (mouse ICON) and murine fVII/Sp (simplified as mfVII in Fig. 2) to angiogenic VECs, 4 h VEGF-stimulated

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**Fig. 1** TF is an angiogenic-specific receptor on endothelial cells, a–c. Representative imaging of Western blots and expression fold changes (normalized to GAPDH) for TF in HMVEC (a), HUVEC (b) and HAEC (c) before (0 h) and after VEGF stimulation (2–24 h). d Representative confocal imaging of TF (green) and endothelial marker CD31 (red) expression on HMVEC before (0 h) and after VEGF stimulation (2–24 h). Cell nuclei were counterstained by DAPI (blue). Scale bars: 20 µm. *p* values were calculated by 2-way ANOVA with multiple comparisons test. Data in a–c are presented as Mean ± SD and representative of two independent experiments. (Color figure online)
VECs (Fig. 2g, i, k) and unstimulated VECs (Fig. 2h, i, j) were analyzed by cell ELISA. To ensure that cell membrane-bound mfVII/hIgG1 Fc was intact, we separately used anti-mfVII and antihuman IgG Fc antibody horseradish peroxidase conjugates for detection of mfVII/hIgG1 Fc in the cell ELISA. The cell ELISA results in Fig. 2g, h, i are summarized as follows. (1) A mouse monoclonal anti-TF antibody (clone HTF1) demonstrated that angiogenic VECs expressed TF (HMVEC in Fig. 2g, HUVEC in Fig. 2i and HAEC in Fig. 2k), whereas quiescent VECs do not express TF (Fig. 2h, j, l). The TF results by cell ELISA are consistent with the Western blotting results (Fig. 1) and confocal imaging results (Fig. 2a, b, c, d, e, f). (2) Similar to observations made with the anti-TF antibody, fVII agents, either in dimeric (mfVII/hIgG1 Fc) or monomeric form (mfVII), selectively bound all three angiogenic VEC types (Fig. 2g, i, k). TF antibody to angiogenic VECs (g, i, k), but not to quiescent VECs (h, j, l), HMVEC (g, h), HUVEC (i, j) and HAEC (k, l). Scale bars in a–f: 20 μm. p values were calculated by 2-way ANOVA with multiple comparisons test. Data in g–l are presented as Mean ± SD and representative of two independent experiments.

To confirm that selective binding of fVII agents to angiogenic VECs is mediated by TF, we carried out experiments that directly evaluated the ability of anti-TF antibodies to inhibit fVII binding to TF on angiogenic VECs. Two antihuman TF antibodies, mouse monoclonal antibody HTF1 and a goat polyclonal antibody (goat anti-HTF) (American Diagnostica), which were known to be able to block TF coagulation [29, 33], were compared for their relative inhibition of fVII binding to TF. Initial studies established that both anti-TF antibodies (p > 0.05) similarly bind to a breast cancer line (MDA-MB-231) that expresses high levels of TF [16] (Fig. 3a). Subsequently,
goat anti-HTF was found to display a significantly stronger effect than HTF1 on inhibiting the binding of mfVII/hIgG1 Fc to MDA-MB-231 cancer cells (p < 0.001) (Fig. 3b). These observations suggested using goat anti-HTF as an inhibitory antibody for the experiments described in Fig. 3c, d, e.

Fig. 3 Selective binding of fVII agents to angiogenic VECs is mediated by TF. a–b. Choice of anti-TF antibodies for inhibiting the binding of fVII agent (mfVII/hIgG1 Fc) to TF. MDA-MB-231 is a human breast cancer line with high level of TF expression and is used here as a TF-expressing cell model. Two antihuman TF antibodies, goat anti-HTF and mouse anti-HTF (Clone HTF1), display similar binding to the MDA-MB-231 cells (a) but goat anti-TF antibody shows stronger inhibitory effect than monoclonal HTF1 in blocking mfVII/hIgG1 Fc binding to cancer cell TF (b). c–e Selective binding of mfVII/hIgG1 Fc to angiogenic HMVEC (c), HUVEC (d) and HAEC (e) can be completely blocked by goat anti-HTF, but not by control antibody. f Representative Western blots using mfVII/hIgG1 Fc and hfVII/hIgG1 Fc to immune-precipitate their cognate receptor TF (f). The negative control was the untransfected CHO-K1 cells. Human IgG was an isotype control. p values were calculated by 2-way ANOVA with multiple comparisons test. Data in a–e are presented as Mean ± SD and representative of two independent experiments.

These observations suggested using goat anti-HTF as an inhibitory antibody for the experiments described in Fig. 3c, d, e.
To determine whether fVII is a ligand for TF on in vitro angiogenic VEC models, we developed an inhibitory cell ELISA assay in which angiogenic VEC were first incubated with various nanomolar concentrations of inhibitory goat anti-HTF for blocking TF or anti-EPCR (RCR-379) for blocking EPCR [30] as a control antibody, followed by incubation with 10 nM mfVII/hlgG1 Fc (to generate molar ratios 0.01:1–10:1, inhibitory antibody to mfVII/hlgG Fc). The binding of fVII/IgG1Fc was detected with anti-hlgG HRP conjugate. We found that goat anti-HTF completely (at ratios of 1:1 and 10:1) or partially (at ratios of 0.01:1 and 0.1:1) inhibit the binding of mfVII/hlgG Fc to all three angiogenic VECs (See Fig. 3c, d, e; HMVEC in Fig. 3c, HUVEC in Fig. 3d and HAEC in Fig. 3e). By contrast, control antibody did not inhibit fVII binding to angiogenic VECs, demonstrating that on angiogenic VECs, fVII acts via binding to TF. To further verify the specificity of fVII agents to TF versus the endothelial protein C receptor (EPCR) [34], we carried out immunoprecipitation–Western blotting (IP–WB) analysis using stable CHO-K1 cell lines expressing TF, EPCR, TF and EPCR and the control was untransfected CHO-K1 cell lines expressing TF, EPCR, TF and EPCR [35]. The results showed that mouse and human ICON could pull down TF in the presence and absence of EPCR (Fig. 3f), demonstrating that fVII agents (fVII/IgG1 Fc, ICON) specifically bind to TF.

To determine whether fVII agents are selective and effective in killing angiogenic VECs, we tested fVII-tPDT using photosensitizer SnCe6-conjugated monomeric mfVII peptides. We first confirmed that like goat anti-TF antibody (Fig. 4a), PS-conjugated mfVII/Sp (Fig. 4b) and mfVII/NLS (Fig. 4c) selectively binds to angiogenic HMVECs. Then, we tested the effectiveness and selectivity of fVII-tPDT for in vitro killing of angiogenic HMVEC using the following assays:

1. The first assay was designed to observe immediate effect of fVII-tPDT by using crystal violet staining and photography under microscope for observation of cellular morphology overnight after fVII-tPDT treatment. We found that fVII-tPDT selectively and effectively killed angiogenic HMVEC (see VEGF-stimulated in Fig. 4d), whereas it had no obvious effects on unstimulated, quiescent HMVEC (normal VEC control). In contrast, nontargeted PDT (ntPDT) under the same conditions as fVII-tPDT except using free SnCe6 had no effect on killing angiogenic and quiescent HMVEC (Fig. 4d).

2. The second test for the selectivity and effectiveness of fVII-tPDT to angiogenic VECs was a clonogenic assay, which is designed for observing longer-term cell viability and proliferation. To target TF for photodynamic therapy, we made two mfVII peptides, namely mfVII/Sp and mfVII/NLS. Both are composed of single-chain peptide of mature murine factor VII with an active site mutation (K341A) followed by a 14-amino acid residue S tag (Sp) and a 13-aa nuclear localization sequence (NLS) [6, 15], respectively. We showed that two weeks after treatment with fVII-tPDT using mfVII-SnCe6 conjugates, angiogenic HMVEC did not form any colonies (See Fig. 4e), indicating that fVII-tPDT was able to completely eradicate angiogenic VECs. In contrast, ntPDT-treated angiogenic HMVEC formed similar numbers of colonies as untreated control cells (p value not significant).

3. Using the half-maximally effective concentration (EC50) as an indicator, based on clonogenic assay results, we found that the EC50 of SnCe6 in fVII-SnCe6 tPDT was 0.031 μM for angiogenic HMVEC, whereas it had no observable killing effect on normal quiescent VEC (see Fig. 4f). Taken together, the results in Fig. 4d, e, f suggested that the fVII-tPDT is selective and effective in eradicating angiogenic VECs.

To better understand its mechanism of action of fVII-tPDT, we first treated angiogenic and resting HMVEC with...
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(a) Goat anti-TF

(b) fVII/Sp-SnCe6

(c) fVII/NLS-SnCe6

(d) VEGF-stimulated

(e) ntPDT

(f) Untreated control

(g) VEGF-stimulated HMVECs

(h) Unstimulated HMVECs

(i) fVII/NLS-IPDT

(j) ntPDT

(k) Annexin V-FITC/PI

(l) Phase contrast

(m) EC50 in fVII-SnCe6=0.031 μM

(n) Springer
fVII-tPDT (mfVII/NLS-SnCe6) or ntPDT (SnCe6) then immediately tested the VEC for apoptosis using Annexin V-FITC and for necrosis using propidium iodide (PI). We found that fVII-tPDT treatment induced immediate apoptosis and necrosis in angiogenic HMVECs (see Fig. 4g), similar to its mechanism in killing human lung cancer cells [6]. Importantly, fVII-tPDT did not induce detectable apoptosis and necrosis in quiescent HMVEC, whereas ntPDT had no effect in angiogenic and quiescent HMVEC (Fig. 4g).

Discussion

Identification of specific anti-angiogenic target molecules in endothelial is crucial in developing anti-angiogenesis therapies. Toward this end, a major effort has focused on targeting VEGF and VEGFRs. Elevated levels of VEGFRs are detected on tumor VECs in many types of solid cancers [36]. Unfortunately, VEGFRs are not angiogenic-specific endothelial receptors since they are also expressed on normal endothelium in many organs [37, 38]. Nevertheless, this study defines the peak expression of TF on angiogenic VECs (4 h VEGF induction), which may be used as an optimized time point for generating in vitro VEC models for identification of more novel target molecules by revealing the differences in gene expression (for example, mRNAs and micro-RNAs) between angiogenic and quiescent VECs.

In this report, we show that, unlike VEGFRs, TF is a specific receptor for angiogenic VECs in vitro. It appears that TF is also a unique pathological angiogenic endothelial cell surface receptor in vivo because of its selective expression on angiogenic VECs in vivo in tumor vasculature [4–7, 9, 14], ocular [18] and endometriotic [21] neovascularization from patients or animal models. In addition to TF on angiogenic VEC, TF is overexpressed on many cancer cells including solid cancer and leukemia [39]. Therefore, TF appears to be a common yet specific biomarker and target molecule for both the pathological neovascularization and cancer cells [6, 7, 16, 39].

Consequently, the use of fVII as targeting vehicle enabled our group to develop two TF-targeted therapeutics, namely fVII/IgG1 Fc (ICON) for immunotherapy and fVII/PS for tPDT, which display selectivity, effectiveness and safety in the treatment of lung and breast cancer (regardless of their drug resistance and other membrane targets) [5–7, 13–17, 23], macular degeneration [18–20] and/or endometriosis [21] in preclinical mouse, rat and pig models and in phase I and II clinical trials for AMD patients (NCT01485588) [22]. In this study, we showed that both mfVII/IgG1 Fc and hfVII/IgG1 Fc bind to human TF, which is only expressed by angiogenic VECs upon VEGF stimulation. Thus, TF-targeted ICON and fVII-tPDT should not cause side effects to quiescent vessels, including microvessels, venous and arterial vessels, while they can selectively target and effectively eradicate angiogenic VECs via TF targeting. Nevertheless, caution should be taken as VEGF and other chemokines (IL-1β, TNF-α, etc.) may potentially induce TF expression on normal resting VECs. They should be monitored before and during the treatment of fVII agents, particularly when fVII agents are administered systemically. In the case of cancer, Drs. Yang and Rosenberg et al. [40] reported that 76 of 113 patients with metastatic renal cell carcinoma had a baseline VEGF level below the lower limit of detection (40 pg/ml). After anti-VEGF antibody bevacizumab (Avastin) therapy, the plasma VEGF levels increased to a range of 150–250 pg/ml. Similarly, a very recent study [41] reported that the plasma baseline levels of VEGF in 60 patients (Hongkong) with colorectal cancer metastases were about 10 pg/ml, and they were increased to 40 pg/ml after anti-VEGF antibody bevacizumab (Avastin) therapy [41]. Importantly, the group found that increased VEGF was in a complex with the neutralizing antibody that prevents VEGF from binding to VEGF receptors. In the cases of AMD and PCV (polypoidal choroidal vasculopathy), Tong et al. [42] reported that the aqueous humor level of VEGF was about 400 pg/ml in eyes with PCV and about 700 pg/ml in eyes with choroidal neovascularization of AMD. A Japanese group also reported aqueous humor level of VEGF was higher in AMD and PCV than control eyes. But they did not find the significant difference of VEGF levels between AMD and PCV. Nevertheless, the levels of VEGF reported in those published studies were at 100-fold lower than the concentration (1 nM VEGF = 38 ng/ml, MW 38 kDa) that we tested in this study. It remains to investigate the lowest VEGF concentration that is able to induce endothelial TF expression. If the blood VEGF level in patients is detected high (enough to induce TF expression on normal endothelial cells), anti-VEGF inhibitors may be administered to neutralize and clear VEGF from circulation, if any, prior to fVII-targeted therapies. Regardless of the VEGF levels in AMD and PCV, fVII-tPDT may be used for the treatment of these ocular diseases as long as TF expression is detected in the neovascularization lesions.

In conclusion, this study identifies that TF is an angiogenic-specific endothelial biomarker and verifies that TF is the therapeutic target specific for fVII agents. This study also elucidated for the first time that the specificity of fVII agents for angiogenic VEC is mediated via binding TF. Thus, this study not only identifies TF as an angiogenic endothelial receptor specific for fVII agents, but also helps predict therapeutic efficacy and potential side effects of these fVII-targeted agents. As such, supports use of TF-targeted therapeutics in clinical trials of several
angiogenesis-dependent common human diseases, notably cancer, macular degeneration, endometriosis and rheumatoid arthritis, in which VEGF plays a central role in pathological angiogenesis.

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Compliance with ethical standards

Conflict of interest Z.H. is co-inventor of US patents on neovascular-targeted immunoconjugates (ICON) and is co-inventor of two US patent applications on “Factor VII conjugates for selectively treating neovascularization disorders.” W.R. is currently serving as a consultant for Iconic Therapeutics, a startup biotech based on ICON patents. Other authors declare no conflict of interest.

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References

1. Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1(1):27–31
2. McDonald DM, Choyke PL (2003) Imaging of angiogenesis: from microscope to clinic. Nat Med 9(6):713–725
3. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407(6801):249–257
4. Contrino J, Hair G, Kreutzer DL, Rickles FR (1996) In situ detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease. Nat Med 2(2):209–215
5. Hu Z, Sun Y, Garen A (1999) Targeting tumor vasculature endothelial cells and tumor cells for immunotherapy of human melanoma in a mouse xenograft model. Proc Natl Acad Sci USA 96(14):8161–8166
6. Cheng J, Xu J, Duanmu J, Zhou H, Booth CJ, Hu Z (2011) Effective treatment of human lung cancer by targeting tissue factor with a factor VII-targeted photodynamic therapy. Curr Cancer Drug Targets 11(9):1069–1081
7. Duanmu J, Cheng J, Xu J, Booth CJ, Hu Z (2011) Effective treatment of chemoresistant breast cancer in vitro and in vivo by a factor VII-targeted photodynamic therapy. Br J Cancer 104(9):1401–1409. doi:10.1038/bjc.2011.88
8. Konigsberg WH, Nemerson Y (1988) Molecular cloning of the cDNA for human tissue factor. Cell 52(5):639–640. doi:10.1016/0092-8674(88)90400-X
9. Folkman J (1996) Tumor angiogenesis and tissue factor. Nat Med 2(2):167–168
10. Morrissey JH, Fakhrai H, Edgington TS (1987) Molecular cloning of the cDNA for tissue factor, the cellular receptor for the initiation of the coagulation protease cascade. Cell 50(1):129–135
11. Spicer EK, Horton R, Bloem L, Bach R, Williams KR, Guha A, Kraus J, Lin TC, Nemerson Y, Konigsberg WH (1987) Isolation of cDNA clones coding for human tissue factor: primary structure of the protein and cDNA. Proc Natl Acad Sci USA 84(15):5148–5152
12. Banner DW, D’Arcy A, Chene C, Winkler FK, Guha A, Konigsberg WH, Nemerson Y, Kirchhofer D (1996) The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. Nature 380(6569):41–46. doi:10.1038/38041a0
13. Hu Z, Garen A (2000) Intraplural injection of adenoviral vectors encoding tumor-targeted immunoconjugates for cancer immunotherapy. Proc Natl Acad Sci USA 97(16):9221–9225
14. Hu Z, Garen A (2001) Targeting tissue factor on tumor vascular endothelial cells and tumor cells for immunotherapy in mouse models of prostatic cancer. Proc Natl Acad Sci USA 98(21):12180–12185
15. Hu Z, Rao B, Chen S, Duanmu J (2010) Targeting tissue factor on tumour cells and angiogenic vascular endothelial cells by factor VII-targeted verteporfin photodynamic therapy for breast cancer in vitro and in vivo in mice. BMC Cancer 10:235. doi:10.1186/1471-2407-10-235
16. Hu Z, Rao B, Chen S, Duanmu J (2011) Selective and effective killing of angiogenic vascular endothelial cells and cancer cells by targeting tissue factor using a factor VII-targeted photodynamic therapy for breast cancer. Breast Cancer Res Treat 126(3):589–600. doi:10.1007/s10549-010-0957-1
17. Hu Z, Li J (2010) Natural killer cells are crucial for the efficacy of Icon (factor VII/human IgG1 Fc) immunotherapy in human tongue cancer. BMC Immunol 11:49. doi:10.1186/1471-2407-11-49
18. Bora PS, Hu Z, Tezel TH, Sohn JH, Kang SG, Cruz JM, Bora NS, Garen A, Kaplan HJ (2003) Immunotherapy for choroidal neovascularization in a laser-induced mouse model simulating exudative (wet) macular degeneration. Proc Natl Acad Sci USA 100(5):2679–2684
19. Tezel TH, Bodek E, Sommez K, Kaliappan S, Kaplan HJ, Hu Z, Garen A (2007) Targeting tissue factor for immunotherapy of choroidal neovascularization by intravitreal delivery of factor VII-Fc chimeric antibody. Ocul Immunol Inflamm 15(1):1–10
20. Lu F, Hu Z, Sinard J, Garen A, Adelman RA (2009) Factor VII-verteporfin for targeted photodynamic therapy in a rat model of choroidal neovascularization. Invest Ophthalmol Vis Sci 50(8):3890–3896. doi:10.1167/iovs.08-2833
21. Krikun G, Hu Z, Osteen K, Bruner-Tran KL, Schatz F, Taylor HS, Toti P, Arcuri F, Konigsberg W, Garen A, Booth CJ, Lockwood CJ (2010) The immunoconjugate “icon” targets aberrantly expressed endothelial tissue factor causing regression of endometriosis. Am J Pathol 176(2):1050–1056. doi:10.2353/apath.2010.090757
22. Courty J, Loret C, Moenner M, Chevallier B, Lagente O, Courtois Y, Barritault D (1985) Bovine retina contains three growth factor activities with different affinity to heparin: eye derived growth factor I, II, III. Biochimie 67(2):265–269
23. Tang Y, Borgstrom P, Maynard J, Kozioz J, Hu Z, Garen A, Deisseroth A (2007) Mapping of angiogenic markers for targeting of vectors to tumor vascular endothelial cells. Cancer Gene Ther 14(4):346–353
24. Ferrara N (2002) VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2(10):795–803. doi:10.1038/nrc909
25. Klagsbrun M, Sullivan R, Smith S, Rybka R, Shing YE (1987) Purification of endothelial cell growth factors by heparin affinity chromatography. Methods Enzymol 147:95–105
26. Afuwape AO, Kiriakidis S, Paleolog EM (2002) The role of the angiogenic molecule VEGF in the pathogenesis of rheumatoid arthritis. Histol Histopathol 17(3):961–972
27. Fujimoto J, Sakaguchi H, Hirose R, Wen H, Tamaya T (1999) Angiogenesis in endometriosis and angiogenic factors. Gynecol Obstet Investig 48(Suppl 1):14–20. doi:10.1159/000052864
28. Zucker S, Mirza H, Conner CE, Lorenz AF, Drews MH, Bahou WF, Jesty J (1998) Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase activation and cell proliferation. Int J Cancer 75(5):780–786
29. Carson SD, Ross SE, Bach R, Guha A (1987) An inhibitory monoclonal antibody against human tissue factor. Blood 70(2):490–493
30. Ye X, Fukudome K, Tsuneyoshi N, Satoh T, Tokunaga O, Sugawara K, Mizokami H, Kimoto M (1999) The endothelial cell protein C receptor (EPCR) functions as a primary receptor for protein C activation on endothelial cells in arteries, veins, and capillaries. Biochem Biophys Res Commun 259(3):671–677. doi:10.1006/bbrc.1999.0846
31. Yoneda Y, Arioka T, Imamoto-Sonobe N, Sugawa H, Shimonishi Y, Uchida T (1987) Synthetic peptides containing a region of SV 40 large T-antigen involved in nuclear localization direct the transport of proteins into the nucleus. Exp Cell Res 170(2):439–452
32. Cheng J, Xu J, Duanmu J, Zhou H, Booth CJ, Hu Z (2011) Effective treatment of human lung cancer by targeting tissue factor with a factor VII-targeted photodynamic therapy. Curr Cancer Drug Targets 11:1069–1081. doi:10.2174/156800911798070320
33. Watanabe T, Yasuda M, Yamamoto T (1999) Angiogenesis induced by tissue factor in vitro and in vivo. Thromb Res 96(3):183–189
34. Ghosh S, Pendurthi UR, Steine A, Esmon CT, Rao LV (2007) Endothelial cell protein C receptor acts as a cellular receptor for factor VIIa on endothelium. J Biol Chem 282(16):11849–11857. doi:10.1074/jbc.M609283200
35. Disse J, Petersen HH, Larsen KS, Persson E, Esmon N, Esmon CT, Teyton L, Petersen LC, Ruf W (2011) The endothelial protein C receptor supports tissue factor ternary coagulation initiation complex signaling through protease-activated receptors. J Biol Chem 286(7):5756–5767. doi:10.1074/jbc.M110.201228
36. Smith NR, Baker D, James NH, Ratcliffe K, Jenkins M, Ashton SE, Sproat G, Swann R, Gray N, Ryan A, Jurgensmeier JM, Womack C (2010) Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. Clin Cancer Res 16(14):3548–3561. doi:10.1158/1078-0432.CCR-09-2797
37. Christofori G, Naik P, Hanahan D (1995) Vascular endothelial growth factor and its receptors, flt-1 and flk-1, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. Mol Endocrinol 9(12):1760–1770
38. Witmer AN, Dai J, Weich HA, Vrensen GF, Schlingemann RO (2002) Expression of vascular endothelial growth factor receptors 1, 2, and 3 in quiescent endothelia. J Histochem Cytochem 50(6):767–777
39. Hu Z (2011) Factor VII-targeted photodynamic therapy for breast cancer and its therapeutic potential for other solid cancers and leukemia. In: Gunduz E, Gunduz M (ed) Breast cancer—current and alternative therapeutic modalities. InTech, pp 175–196. doi:10.5772/20398
40. Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. New Engl J Med 349(5):427–434. doi:10.1056/NEJMoa021491
41. Alidzanovic L, Starlinger P, Schauer D, Maier T, Feldman A, Buchberger E, Stift J, Koeck U, Pop L, Gruenberger B, Gruenberger T, Brostjan C (2016) The VEGF rise in blood of bevacizumab patients is not based on tumor escape but a host-blockade of VEGF clearance. Oncotarget. doi:10.18632/oncotarget.11084
42. Tong JP, Chan WM, Liu DT, Lai TY, Choy KW, Pang CP, Lam DS (2006) Aqueous humor levels of vascular endothelial growth factor and pigment epithelium-derived factor in polypoidal choroidal vasculopathy and choroidal neovascularization. Am J Ophthalmol 141(3):456–462. doi:10.1016/j.ajo.2005.10.012