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A recombined fusion protein SP5.2/tTF induce thrombosis in tumor blood vessel

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Tumor vascular targeting is one of the most promising strategies in tumor therapy. Here we used *E.coli* to express a recombinant SP5.2/tTF fusion protein, which, as a tumor vascular targeting agent, consists of SP5.2 (a peptide selectively binding and targeting VEGFR-1 on tumor endothelial cells) and truncated tissue factor (tTF)and aimed to explore its anti-tumor activities.

The SP5.2/tTF expression construct was synthesized by polymerase chain reaction (PCR) and recombined into plasmid pET22b(+). The fusion gene was verified by restriction mapping and sequencing. SP5.2/tTF was expressed in *E. coli* and then purified on a nickel-affinity chromatography column. The purified product was detected by SDS-PAGE. The pro-coagulant activity and binding of SP5.2/tTF to human umbilical vein endothelial cells (HUVECs) were monitored by FX activation analysis and fluorescent scanning confocal microscopy, respectively. The effect of SP5.2/tTF on tumor growth was analyzed in BALB/c mice bearing sarcoma 180 (S180) tumor. The tissue localization of SP5.2/tTF and its effect on tumor vessel thrombosis were observed by in vivo fluorescence imaging and histological studies, respectively.

The fusion gene was successfully cloned into pET22b(+). SP5.2/tTF was abundantly expressed in bacterial cells and efficiently purified by nickel-affinity chromatography. Functional studies showed that the protein retained both the coagulation activity of tTF and the binding capacity of SP5.2 to HUVECs. In tumor xenograft studies, SP5.2/tTF selectively targeted the tumor, induced thrombosis, and led to retardation and even regression of tumor growth (growth inhibition ratio = 70%, $P < 0.05$).

The recombinant fusion protein SP5.2/tTF inhibited tumor growth by selectively inducing thrombosis in tumor blood vessels.

Key words: truncated tissue factor, SP5.2, fusion protein, tumor vascular targeting, thrombosis, anti-tumor

It is known that tumor vasculature can sufficiently supply diversified nutrients during the initiation and development of the malignant neoplasms [1-3]. As early as 1994, the American Society of Clinical Oncology (ASCO) recognized that destroying tumor blood vessels would be an effective means to inhibit tumor growth and metastasis [1]. Currently employed therapies such as surgery, chemotherapy and radiotherapy often result in only temporary remissions and subsequent relapse.

Vascular targeting therapy currently involves two different strategies: the inhibition of neovascularization and the destruction of existing tumor vasculature. The former strategy inhibits the formation of new vessels, and is the cause of anti-angiogenic drugs [4, 5]. The second strategy led to occlusion of pre-existing tumor blood vessels and extensive hemorrhagic necrosis of tumors. This strategy mainly used agents that can selectively bind to tumor vessels and were so-called tumor vascular targeting agents [6-8].

Inducing tumor thrombosis is one of the most promising vascular targeting therapies. The tTF-ligand is a ligand-based vascular targeting agent and can selectively induce tumor thrombosis. Tissue factor (TF), also called blood coagulation factor III or tissue thromboplastin, having procoagulant activity and proteolytic function [9-12]. The extracellular domain of TF is a crucial component in activating the coagulation cascade and is called truncated tissue factor (truncated TF, tTF) [13]. tTF crosslinks to the target ligand and is a coagulation factor that can strongly and specifically bind to the surface of tumor vascular cells. Interestingly, it does not act as a coagulant when

it is freely circulating in the blood [6, 14-16]. It is known that tTF can specifically target tumor vessels after binding ligands, such as antibodies targeting various markers (VCAM-1 [8], MHC class II [17], etc.), chTNT-3 targeting tumor necrotic regions [18], chTV-1 targeting a vessel antigen [19, 20], ED-B domain of fibronectin, GRGDSP (RGD) targeting integrins and GNGRAHA (NGR) targeting aminopeptidase N (CD13). These studies indicate that there are limits to these tTF-ligands because of selective targeting to different tumor vessel markers, and some have low affinities for tumors. Combining tTF with other molecules or finding novel markers might have increased therapeutic value.

Vascular endothelial growth factor (VEGF) plays a vital role in tumor neovascularization [21]. Vascular endothelial growth factor receptor-1 (VEGFR-1, Flt-1), the main tyrosine kinase receptor of VEGF, is expressed on endothelial cells [22]. Since its overexpression is correlated with tumor angiogenesis and progression, VEGFR-1 may be an effective anti-cancer therapeutic target. tTF-ligand targeting to VEGFR-1 has not been reported in the literature [23, 24]. SP5.2 (NGYEIEWYSWVTHGMY-NH2), identified by phage display library screening, is a VEGFR-1-specific antagonist protein [23, 25]. SP5.2 has a higher affinity for VEGFR-1 than VEGF [25]. It can effectively inhibit VEGF binding to its receptor and prevent proliferation of cultured primary HUVECs induced by recombinant human VEGF165 [7]. Compared to original SP5.2, conjugates of SP5.2 with proteins significantly rose in binding affinity to VEGFR-1 [25]. This reveals that the potentially therapeutic activity of SP5.2 could be developed by the formation of conjugate protein [7, 25]. SP5.2 can not only be used as a VEGF antagonist, but also can be used as a ligand, targeting it to tumor vessels. Thus, it may be a novel and effective ligand of tumor vascular targeting agents in tumor therapy.

On the basis of these studies, we generated a novel fusion protein, SP5.2/tTF, consisting of tTF and an SP5.2 peptide coupled to the N-terminal region. We explored the feasibility of selectively targeting VEGFR-1 to induce thrombogenesis, thus inhibiting tumor growth in solid tumors. The SP5.2/tTF fusion protein was expressed and purified to investigate its potential for peptide-directed targeting and blood coagulation.

Materials and methods

Materials. The tTF-pSK(+) vector was obtained from Dr. Epstein (South Western Medical Center, Dallas, TX). The plasmid pET22b(+) and *E. coli* BL21(DE3) were purchased from Novagen (San Diego, California, USA). Qiaquick PCR Purification Kit, Qiaquick Gel Extraction Kit, DNA and protein marker were purchased from Tiangen (Beijing, China). Nickel-nitrilotriacetic acid (Ni-NTA) agarose was obtained from Qiagen (Santa Clarita, California, USA). Restriction endonucleases, T4 DNA ligase and other molecular biology reagents were purchased from New England Biolabs (Beverly, MA, USA). GIBCO Fetal bovine serum (FBS) and Low glucose Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen-Life technologies (Carlsbad, California, USA). Penicillin-streptomycin solution, factors X and VII were obtained from Sigma Chemical Co (St. Louis, Missouri, USA). Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). BALB/c nude mice were obtained from the Animal Center of Xiamen University (Xiamen, Fujian, China). The animal study was approved by the Ethics Committee of Xiamen University. The Hoechst Staining Kit and Hematoxylin and Eosin (H&E) Staining Kit were purchased from the Beyotime Institute of Biotechnolgy (Beijing, China). Histostain-Plus IHC Kit was purchased from NeoBioScience (Beijing, China).

Construction of the fusion gene. Based on the motif of tTF and using two glycine amino acids as a linker to protect the configuration of tTF, the sequence encoding tTF was amplified as a 693 bp DNA fragment by polymerase chain reaction (PCR) using the tTF-pSK(+) plasmid as the template. Primers used were P1 (5'-T***CCATGG***GCTCTGGCACTACA-3' *Nco*I) and P2 (5'-GTG***CTCGAG***TTCTCTGAATTCC-3' *Xho*I). According to the literature, the amino acid sequence of the SP5.2 peptide is NGYEIEWYSWVTHGMY-NH2 [23, 25]. The tTF PCR product and an oligonucleotide P3 with sequence (5'-AA***CCATGG***TTAACGGCTATGAAATTGAATGGTATAGCTGGGTGACCCACGGCATGTATGGCGGCTCTGGCACTACAAATACTGTGG-3' *Nco*I) were annealed to construct the template for SP5.2/tTF. A fusion gene encoding SP5.2/tTF was amplified by PCR using the primer P4 (5'-AA***CCATGG***TTAACGGCTATGAA-3' *Nco*I) and P2. The amplified sequence was cloned into digested plasmid pET22b(+). The resulting product was identified by restriction endonuclease analysis and by agarose gel electrophoresis [26]. Integrity of the fusion was verified by sequencing.

Expression and purification of the fusion protein. The resulting plasmid encoding SP5.2/tTF was transformed into *E. coli*. The medium of germiculture was Luria broth (LB) medium with 1% ampicillin. The expression of fusion protein was stimulated with various concentrations of isopropyl-β-D-thiogalactoside (IPTG) or various incubation time until the germiculture reached ~0.5 at OD600nm. The bacterial cells were stimulated and expression results were analyzed by SDS-PAGE. After identifying optimum condition, the cells were centrifuged (12,000 × g; 4 °C; 15 min) and collected. Per gram collection (wet weight) added 5 ml lysis buffer (20 mM Tris-HCl, pH 8.0; 0.5 M NaCl; 1 mM EDTA; 10 % lysozyme). Cells were then incubated for 90 min and centrifuged (12,000 × g; 4 °C; 15 min). Resuspended and sonicated bacterium in washing buffer (20 mM Tris/HCl, pH 8.0; 0.5 M NaCl; 2 M urea; 2% Triton X-100) and per gram of the inclusion bodies (wet weight) was solubilized in 5 ml solubilization buffer (20 mM Tris/HCl, pH 8.0; 8 M urea; 1 mM β-mercaptoethanol; 2 % Triton X-100). The suspension was incubated (overnight; RT) and centrifuged (12,000 × g; 30 min; 4 °C). The supernatant purified by a Ni-NTA column., purification and refolding

were administered with the protocol of a His Bind Buffer kit. Purified protein in the IMAC eluate was dialyzed against PBS solution. The final products were denatured and analyzed by SDS-PAGE.

Factor X activation. As described previously, Factor X activation assay [27] was conducted to evaluate the tTF moieties' blood coagulation abilities of the proteins. Briefly, various concentrations of SP5.2/tTF or tTF (0.01, 0.1, 1 and 10 μmol/l) were mixed in Tris-buffered saline buffer with 100 nM Factor VII and incubated (37 °C; 10 min). Then mixed with 5 nM Factor X and incubated at RT (10min). Quenched the reaction with 100 mM EDTA. After that, the mixture with 2 nM Spectrozyme Factor Xa was read at $OD_{405\,nm}$ in a 3 min time period.

Cell culture. In low glucose DMEM medium (hEGF, 12% FBS and 1% penicillin–streptomycin), HUVECs were cultured at incubator (37 °C; 5% $CO_{2,}$ humidified atmosphere).

Fluorescent labeling. SP5.2/tTF and tTF proteins were labeled with fluorescein. The concentration of Rhodamine B isothiocyanate (RBITC) used was 1 mg/ml in DMSO. According to the total amount of proteins (tTF or SP5.2/tTF), 0.01 mg fluorescein per mg protein were mixed at 4 °C overnight.

Confocal microscopy. HUVECs (1×10^5 cells/ml) were inoculated into a 6 well cell culture cluster with glass coverslips and cultured. Once cells reached 50-60% confluence, the supernatant was removed. The coverslips with cells were washed with PBS (pH 7.4) three times and fixed with 2 ml 4% paraformaldehyde for 30 min. After being washed (ditto), 2 ml of 0.4 mg/ml tTF-RBITC or SP5.2/tTF-RBITC were added, respectively. Afterward the coverslips with cells were incubated for 1h and washed (ditto) in the dark. The coverslips were then incubated with Hoechst 33258 and examined under the laser scanning confocal microscope (LEICA, Germany) and photographed.

Localization of the fusion protein in tumors. Groups of BALB/c mice (6 week-old; female) were chosen and 0.2 ml inoculum with 2×10^6 S180 cells/ml was subcutaneously (s.c.) injected into the right flank of the mice. The tumors grew for several days until they reached 250±50 mm^3 in volume. Six mice were then divided into two groups randomly. Mice in each group were administered intravenously via tail vein with 50 μl (2 mg/ml) of SP5.2/tTF-RBITC or tTF-RBITC, respectively. Mice treated with the proteins were given a general anesthesia at 2, 4, 8, 12, 24, 48 and 72 h. The RBITC fluorochrome was detected using the IVIS spectrum imaging platform (Caliper Life Sciences, MA, USA). To determine the distribution of the fluorescent proteins, the mice were sacrificed. Tumor tissues and some major organs (brain; kidney; lung; liver; heart) were collected, fixed with formaldehyde, embedded in OCT and frozen in liquid nitrogen. The frozen sections were cut into sections of 5 μm thickness, stained with Hoechst 33258, and then washed with PBS. Slides were observed by fluorescence scanning confocal microscopy.

Mouse tumor models. Mice were reared and treated according to the procedure above. The mice bearing S180 tumors that reached 250±50 mm^3 in volume were divided into three groups randomly. Two groups of mice were administered intravenously via tail vein with 50 μl (2 mg/ml) of the purified SP5.2/tTF or tTF daily for six days, respectively. The remaining group were injected with 50 μl PBS or saline solution as controls. The tumors were measured with calipers to determine the three-dimensional parameters and the tumor volume was calculated according to the formula (length × $width^2 \times \pi/6$).

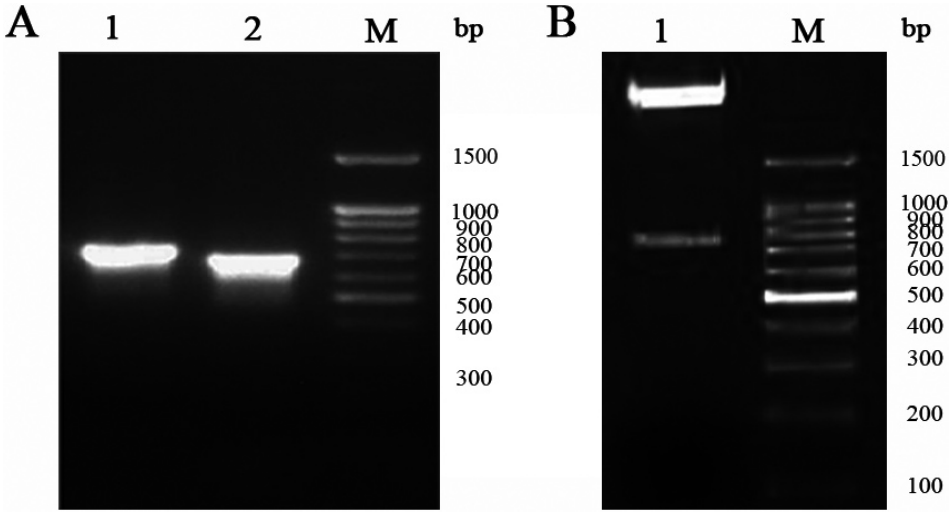

Figure 1. The gene tTF and SP5.2/tTF were determined by PCR and the recombined plasmid was identified by *Nco*I and *Xho*I Restrition endonuclease analysis. (A) DNA analysis of PCR products by agarose gel electrophoresis. Lane 1, PCR products of SP5.2/tTF (732 bp); lane 2, PCR products of tTF (693 bp); M, DNA ladder. (B) DNA analysis of *Nco*I and *Xho*I products by agarose gel electrophoresis. Lane 1, *Nco*I and *Xho*I Restrition endonuclease map of SP5.2/tTF/pET22b(+); M, DNA ladder.

Histological analyses. To estimate the expression of VEGFR and vascular distribution in normal and tumor vessels, tissues were cut into 5 μm-thickness frozen sections and stained immunohistochemically with a rabbit IgG specific for mouse VEGFR at 4°C overnight. Then tissues derived from the PBS, SP5.2/tTF and tTF treated mice were similarly cut, treated with H&E staining and stained immunohistochemically with a rabbit IgG specific for mouse fibrin to estimate the extent of thrombosis. Control slides were treated with the isotype MAb or omission of the primary antibody as a negative control in an identical manner. After incubation of goat anti-mouse IgG conjugated to HRP, sections were stained with diaminobenzidine (DAB) substrate solution and counterstained with hematoxylin. The positive cells showed brown. H&E-stained and immunohistochemical (IHC) sections were assessed signs of thrombosis and fibrin deposition under conventional light microscopy.

Results

Construction of the fusion gene. The tTF and SP5.2/tTF expression constructs were amplified by PCR and subcloned into pSK(+) vector. Analysis of the PCR products by agarose gel electrophoresis showed that the tTF (693 bp) and SP5.2/tTF (732 bp) products were the predicted size (Fig. 1A). *Nco*I and *Xho*I digestion of the expression vector indicated that the

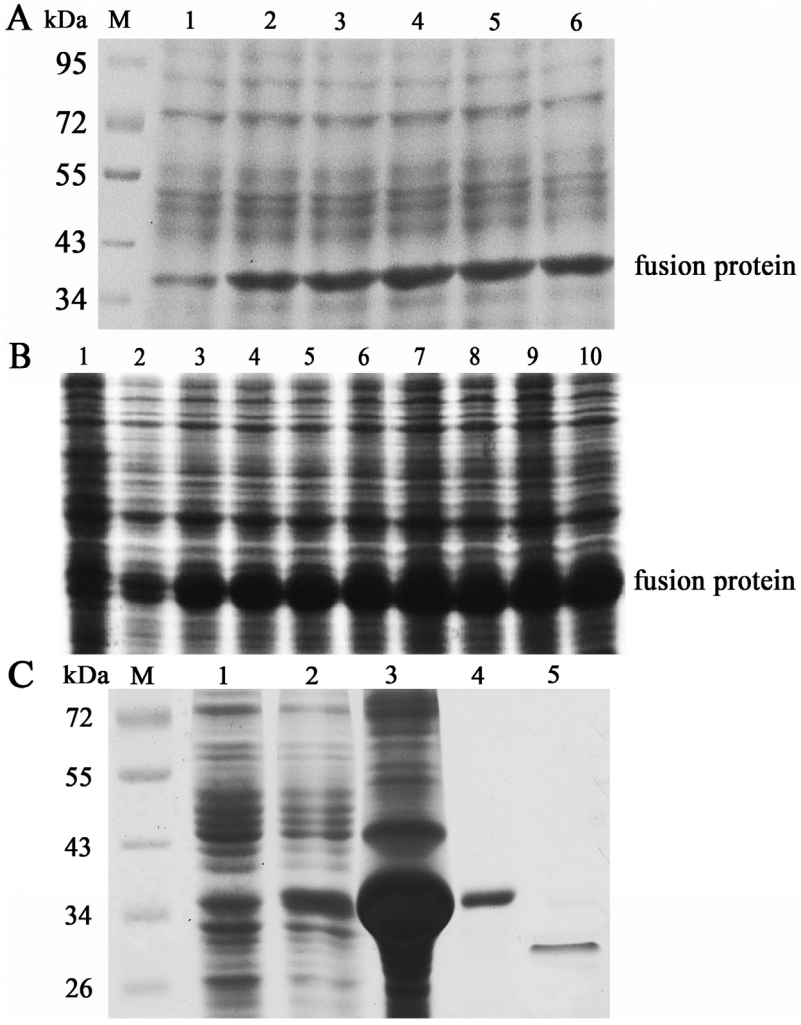

Figure 2. (A) SDS-PAGE analysis for the expression of the fusion protein SP5.2/tTF induced by various concentrations IPTG with 6h in *E. coli*. M, protein marker; lane 1, total protein from *E. coli* without induction. lanes 2-6, total protein from *E. coli* with 0.2, 0.4, 0.6, 0.8 and 1 mmol/l IPTG induction, respectively; (B) SDS-PAGE analysis for the expression of the fusion protein SP5.2/tTF induced by 0.6mmol/l IPTG with various times in *E. coli*. lane 1, total protein from *E. coli* without induction. lanes 2-10, total protein from *E. coli* by 0.6 mmol/l IPTG induction at 1, 2, 3, 4, 5, 6, 7, 8 and 9 h, respectively; (C) SDS-PAGE analysis of SP5.2/tTF expression and purification. Lane M, molecular weight markers; lane 1, total acterial proteins induced without IPTG; lane 2, total acterial proteins contained of SP5.2/tTF protein that induced with IPTG; lane 3, inclusion body of total acterial proteins contained of SP5.2/tTF protein that induced with IPTG; lane 4, Ni-NTA affinity column final purifed SP5.2/tTF fusion protein (35 kDa); lane 5, purified tTF.

plasmid was correctly recombined (Fig. 1B). Subsequent sequencing indicated that the SP5.2/tTF fusion was constructed successfully.

Expression and purification of the fusion protein. SDS-PAGE analysis was used to examine the expression of SP5.2/tTF induced by various concentrations IPTG. Expressed recombinant protein reached maximum yield at 0.6 mmol/l of IPTG (Fig. 2A). A time series of IPTG incubation indicated that recombinant proteins reached their maximum yield after a 6 h incubation with 0.6 mmol/l of IPTG (Fig. 2B). The fusion protein was mostly deposited into inclusion bodies. However, after solubilization and purification with NTI-agarose beads, a highly purified product of the predicted size (35 kDa) was obtained (Fig. 2C).

tTF activity of the fusion protein. To assess the clotting activity of the SP5.2/tTF fusion protein, we performed *in vitro* Factor X activation assays. SP5.2/tTF and tTF exhibited similar ability to activate Factor X. When the concentration of either protein exceeded 1 μmol/l, they activated Factor X in a dose-dependent manner (Fig. 3).

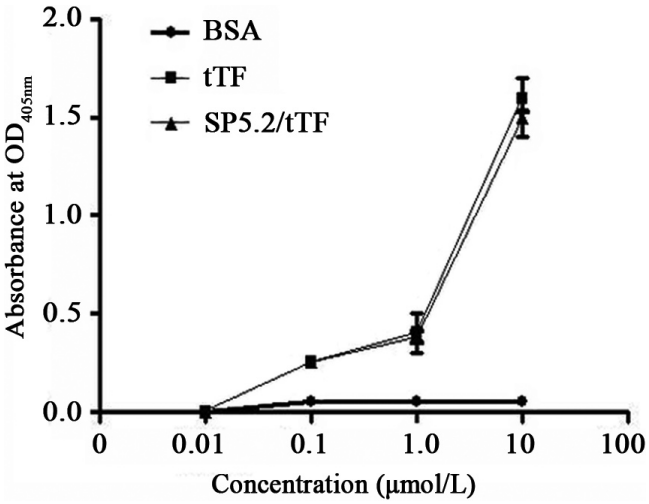

Figure 3. The detection of tTF-coagulation activity of SP5.2/tTF by Factor X coagulation assay, tTF, SP5.2/tTF (positive control) and BSA (negative control). The SP5.2/tTF obviously retained the tTF-clotting activity, compare with the tTF.

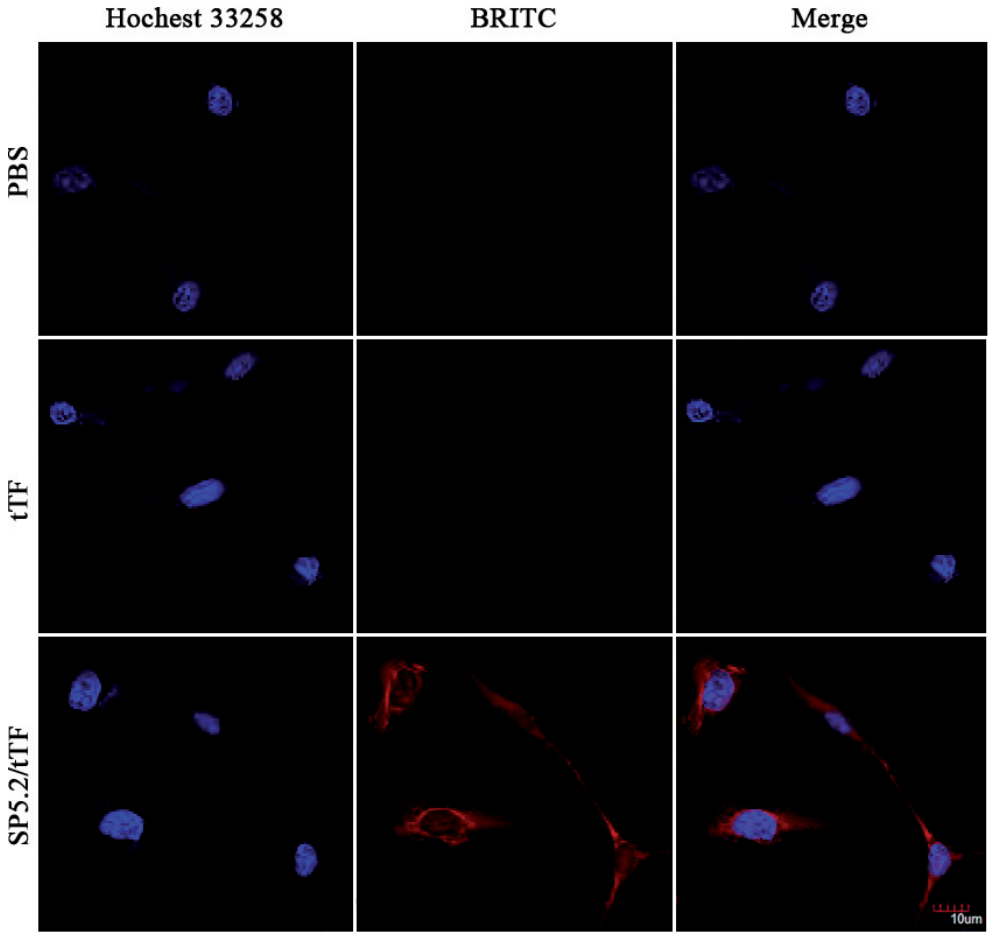

Figure 4. Binding and internalization of SP5.2/tTF by laser scanning confocal microscope analysis. The HUVECs were cultured with PBS and tTF-RBITC (negative controls), SP5.2/tTF-RBITC (positive controls) for 2 h, respectively. As shown, it could be observed the SP5.2/tTF binding to the cells surface, while the protein tTF did not see. The fusion protein SP5.2/tTF still possess potent binding affinity to VEGFR-1 over-expressing HUVECs.

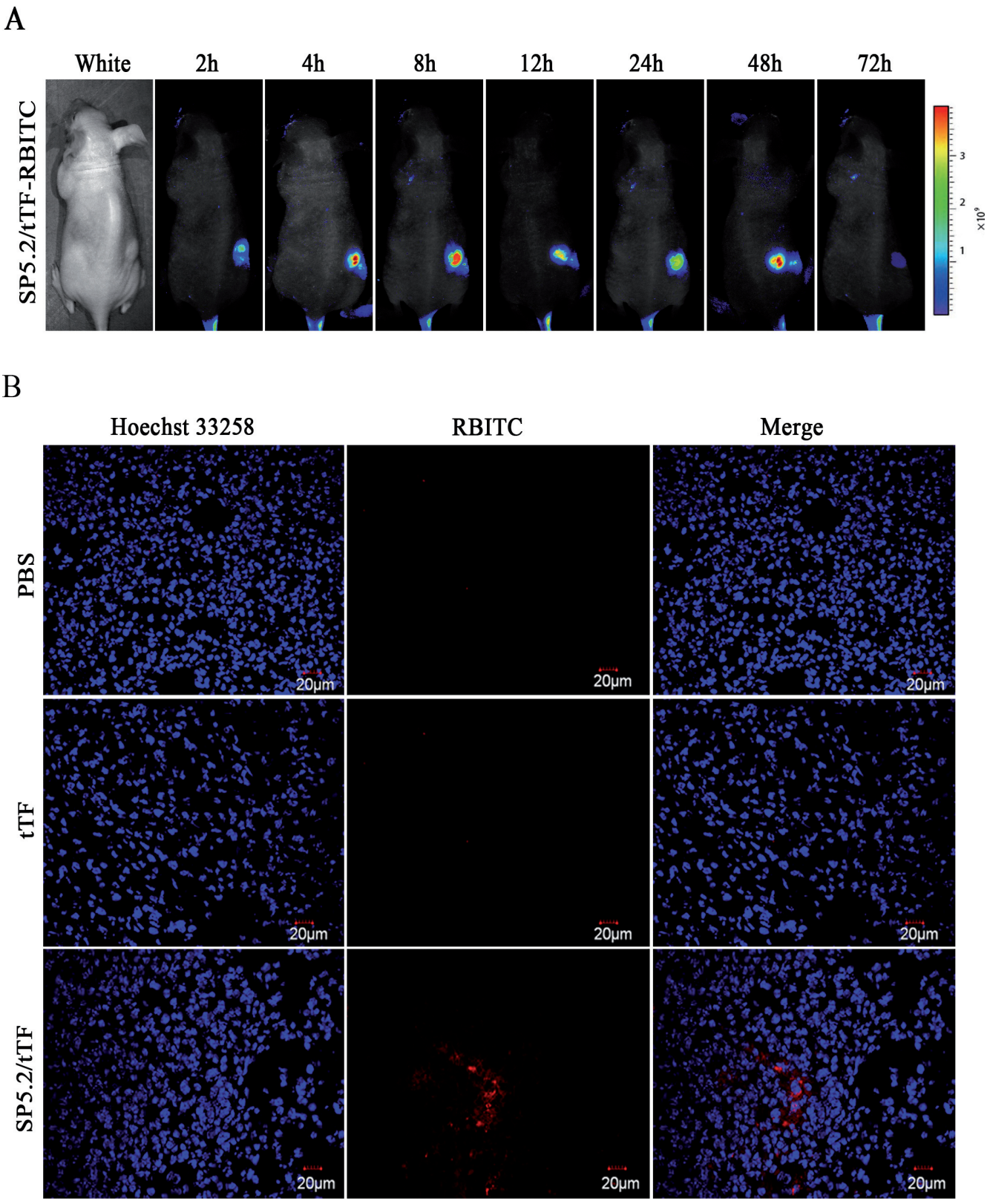

Figure 5. Application of RBITC fluorochrome-labelled protein. (A) The figures show pictures in vivo fluorescence imaging of S180 in BALB/c mice after injecting with proteins (tTF-RBITC or SP5.2/tTF-RBITC) at 2, 4, 8, 12 , 24, 48 and 72 h. (B) The tumor tissure of SP5.2 /tTF-RBITC and tTF-RBITC treated groups under fluorescent scanning confocal microscopy.

SP5.2 activity of the fusion protein. Laser scanning confocal microscopy was used to observe the binding of SP5.2/tTF to HUVEC cells. The SP5.2/tTF treated cells had a strong RBITC fluorescence signal and mainly colocalized with the cell membrane, but tTF treated cells had no fluorescence signal (Fig. 4).

Localization of the fusion protein in tumors. Fluorescence labeling was used to determine the localization of the SP5.2/tTF-RBITC fusion protein in tumor tissues. *In vivo* fluorescence imaging showed that the RBITC florescence signal was located within the mouse tumor and fluorescence intensity reached its peak 8 h after injection in the SP5.2/tTF-RBITC treated group. There was no RBITC fluorescence signal in other tissues of tumor-bearing mice or in any of the other treatment groups (Fig. 5A). Fluorescence scanning confocal microscopy indicated that RBITC signals were present in the tumor tissue sections derived from the mice injected with SP5.2/tTF-RBITC, while no RBITC signals were present in the tumor tissue of the other treatment groups (Fig. 5B). We also found that all major organs such as the heart, lung, kidney, spleen, liver and brain had no fluorescent signals (data not shown).

Effect of the fusion protein on the tumor growth. The anti-tumor activity of SP5.2/tTF was examined in BALB/c mice bearing S180 carcinoma xenotransplants. The fusion protein significantly reduced tumor growth and, in some cases, resulted in tumor regression. These effects were not observed in the group treated with tTF protein (Fig. 6).

Effect of the fusion protein on the tumor thrombosis. In the tumors of SP5.2/tTF treated mice, thrombus and necrotic tumor tissue were observed by H&E staining (Fig. 7A; arrows indicate blood vessels or necrotic tumor tissue). In contrast, little intravascular embolization was observed in the tTF treated group, and non-tumor bearing tissues of mice treated with the fusion protein also appeared normal (Fig. 7B). Immunohistochemical staining of tumor vessels in the SP5.2/tTF group revealed that vessels exhibiting thrombosis were strongly stained with DAB. This stained pattern was greatly reduced in the control and tTF treatment groups (Fig. 7C). Immunohistochemical staining also revealed that the vascular staining was much stronger and vascular density was much greater in tumor tissue than that in normal vessels (Fig. 7D).

Discussion and conclusion. The ligand-directed approach was based on the selectivity of ligands to deliver drugs to targeted tumor cells or tissue. Many reports showed that tTF-ligand targeting tumor vascular markers can significantly inhibit tumor growth by selectively inducing intratumoral thrombosis. After intravenous administrating of tTF-RGD, the tumor growth of mice bearing fibrosarcoma (HT1080), melanoma (M21) and human adenocarcinomas (CCL185) retarded or regressed by inducing tumor vascular thrombotic occlusion [28]. Schwoppe et al. recently reported that tumor vascular thrombosis was clearly induced after subcutaneous or intravenous administration of tTF-RGD or tTF-NGR

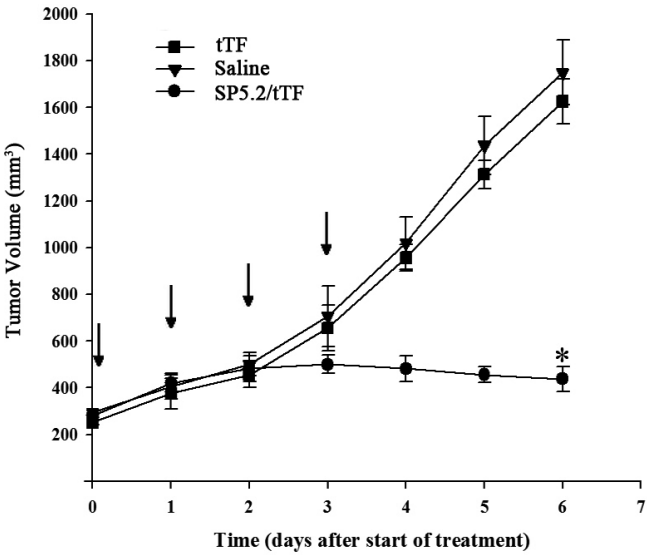

Figure 6. Effect of SP5.2/tTF fusion proteins on growth of S180 transplants in BALB/c mice. When the tumor reach 250±50 mm3, calculated growth inhibition of S180 tumor in saline (solid squares, n = 5), tTF (solid triangles, n = 5) and SP5.2/tTF (solid circles, n = 5) -treat groups. Arrow, time point of injection. Data are presented as means +/−SE; asterisk denotes statistical significance (Day 6: SP5.2/tTF vs tTF or saline, respectively; P < 0.05).

compared to tTF protein only, and the former significantly retarded or regressed growth of lung adenocarcinoma (A549) or malignant human mammary carcinoma (SKBR3) in nude mice [7].

To overcome the low affinities of known tTF-ligands for many tumors, it is important to develop new ligands. VEGFR-1 is abundantly expressed in endothelial cells in many types of tumors, and it plays a positive role in tumor angiogenesis and progression [22]. The SP5.2 peptide, which binds to VEGFR-1, has been confirmed as a promising agent to target the tumor vessel [25].

Based on the known crystal structure of the tTF-VIIa complex, we designed the SP5.2/tTF fusion protein using the SP5.2 peptide as a binding motif coupled to the N-terminus of tTF. A linker (G2) was used as a spacer to couple tTF to SP5.2 in order to separate these two moieties [11, 12, 29-32].

In this study we designed, generated, and purified the SP5.2/tTF fusion protein in order to assess the antitumor activity and its mechanism. Previous in vitro studies found that the fusion protein retained both the coagulation properties of tTF (Fig. 3) as well as the capacity to bind to HUVECs, which was mediated by SP5.2 (Fig. 4). In order to further verify its activity in vivo, we established a mouse xenograft model (S180) and injected the fluorescently-labeled protein through the tail vein. We found that the SP5.2/tTF protein was enriched in tumor tissue, while none was observed in normal tissues (Fig. 5). In addition, SP5.2/tTF showed favorable antitumor activity during the course of treatment (Fig. 6).

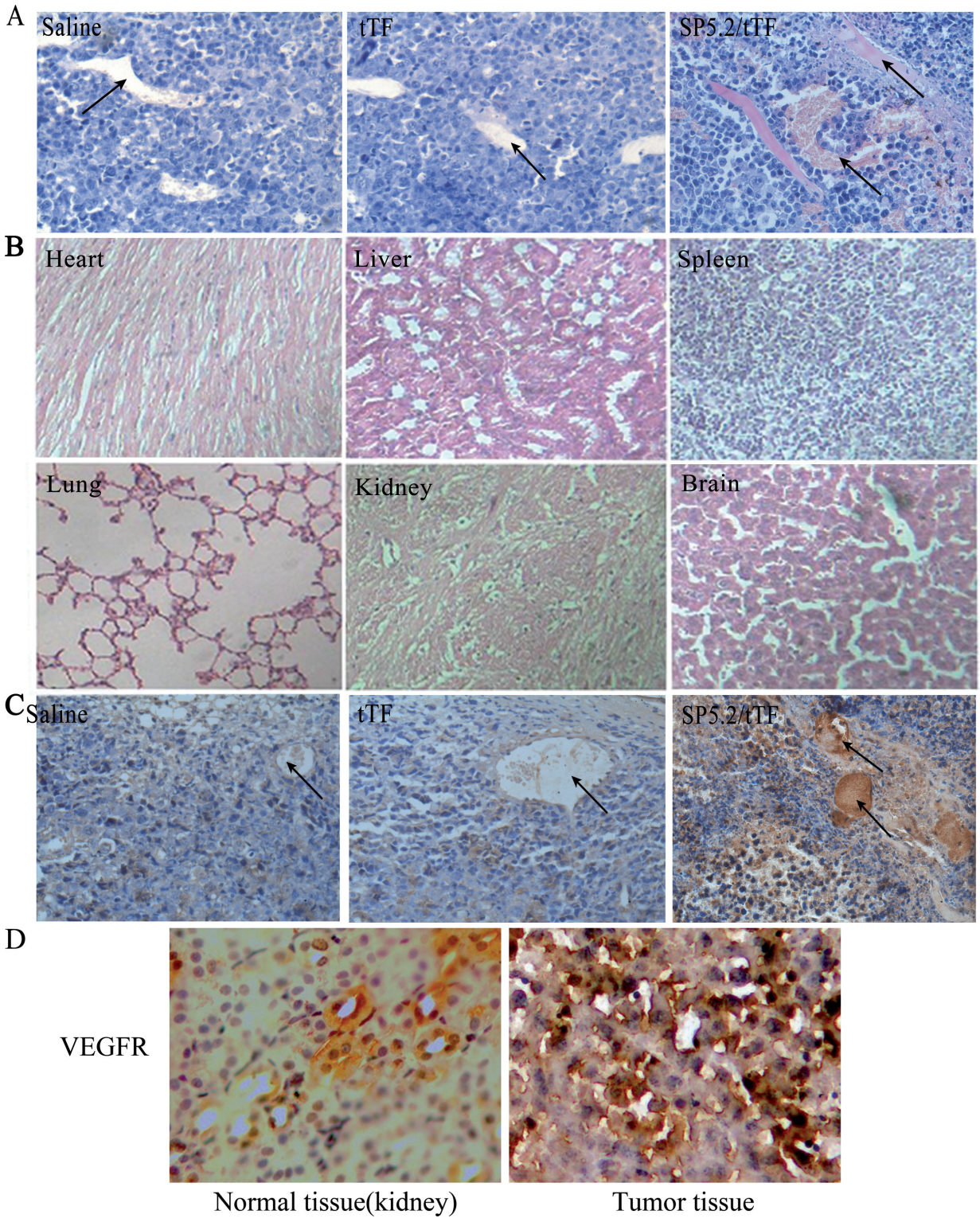

Figure 7. Histological analyses histological comparison of thrombosed vessels (A; B) and specific localization of anti-fibrin antibody (C) in BALB/c mice bearing S180 tumors 48 h after treatment with PBS (negative control), tTF and SP5.2/tTF (positive control). (A) A complete tumor blood vessels thrombosis and necrotic tumor tissue were observed in the SP5.2/tTF-treated group compared to tTF or Saline-treat group. (B) Normal organs of SP5.2/tTF-treated group. Note that blood vessels thrombosis and necrotic was not shown in all the areas here. (C) The thrombosis vessels of tumor were stained darkly with DAB and obvious fibrin deposition were observed. (D) The vessels of the tumor and normal kidney tissues stained darkly with DAB. Arrows indicate blood vessels or necrotic tumor tissue. Scale bar, 50 mm.

We also found that SP5.2/tTF clearly induced thrombosis in tumor vessels and the expression of VEGFR in the tumor tissue was greater than that in normal blood vessels (Fig. 7). It is known that the expression level of the target molecule VEGFR is lower than the corresponding reaction limit in normal tissue, and we found that SP5.2/tTF can specifically bind to VEGFR expressed on tumor vasculature. Once bound, it selectively induces vascular thrombosis in the tumor and effectively inhibits tumor growth.

As a target factor, the SP5.2 component of this fusion protein specifically bound VEGFR, while the effector tTF could selectively induce thrombosis in tumor blood vessels in the presence of phosphatidylserine. Due to the selective up-regulation of VEGFR in tumor vasculature as well as the presence of phosphatidylserine, SP5.2/tTF selectively induces embolization in tumor vessels and does not influence normal tissue. These findings suggest that SP5.2/tTF is safe and effective for tumor therapy.

Basing on these results, we conclude that SP5.2/tTF, targeting VEGFR-1 on tumor endothelia, can selectively induce extensive embolization in tumor blood vessels, thus causing effective anti-tumor therapy of solid tumor models in mice. Additional studies are warranted to optimize the effects of the thrombogenic agent and integrate this promising approach into current cancer treatment strategies.

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