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A fusion protein containing murine vascular endothelial growth factor and tissue factor induces thrombogenesis and suppression of tumor growth in a colon carcinoma model*

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Abstract: Induction of tumor vasculature occlusion by targeting a thrombogen to newly formed blood vessels in tumor tissues represents an intriguing approach to the eradication of primary solid tumors. In the current study, we construct and express a fusion protein containing vascular endothelial growth factor (VEGF) and tissue factor (TF) to explore whether this fusion protein has the capability of inhibiting tumor growth in a colon carcinoma model. The murine cDNA of VEGF A and TF were amplified by reverse transcriptase polymerase chain reaction (RT-PCR), and then cloned into prokaryotic expression plasmid pQE30 with a linker. The expression product recombinant VEGF-TF (rVEGF-TF) was purified and proved to have comparable enzyme activity to a commercial TF and the capability of specific binding to tumor vessels. Significant decrease of tumor growth was found in the mice administered with rVEGF-TF on Day 6 after initiated rVEGF-TF treatment ($P<0.05$), and the tumor masses in 2 of 10 mice were almost disappeared on Day 14 after the first treatment. In addition, valid thrombogenesis and tumor necrosis were observed in the tumor tissues injected with rVEGF-TF. Our results demonstrate that occlusion of tumor vasculature with rVEGF-TF is potentially an effective approach for cancer therapy.

Key words: Thrombogenesis, Vascular endothelial growth factor (VEGF), Tissue factor (TF), Recombinant fusion protein
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INTRODUCTION

Vascular endothelial growth factor (VEGF) is a member of a family of structurally related proteins that act as ligands for the family of VEGF receptors. VEGF exerts its effects on the development of new blood vessels (angiogenesis) and survival of immature blood vessels (vascular maintenance) by binding to and activating two structurally related membrane receptor tyrosine kinases, VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2), which are mainly expressed by endothelial cells in the blood vessel wall (Ferrara, 2004; Robinson and Stringer, 2001; Tao *et al*., 2006; Zachary, 2001). The binding of VEGF to these receptors initiates a signaling cascade that ultimately stimulates vascular endothelial cell growth, survival and proliferation. Recent studies have demonstrated that VEGFR-2 [also called Flk-1 (fetal liver kinase-1) in mice or KDR (kinase insert domain receptor) in humans] is the main receptor responsible for the angiogenic activity of VEGF (Millauer *et al*., 1993; Gille *et al*., 2001). The extracellular region of KDR consists of 7 immunoglobulin-like domains. The third domain is critical for ligand binding, and the second and fourth ones are important for ligand association (Shinkai *et al*., 1998). Overexpression of KDR is found on activated endothelial cells of newly formed blood vessels

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and is strongly associated with invasion and metastasis in human malignant diseases (Brown *et al*., 1993). Thus, targeting occlusion of the VEGF-related newly formed blood vessels in the tumor tissues seems to be an attractive approach for cancer therapy.

Tissue factor (TF) is the major initiating receptor for the thrombogenic cascades (Davie *et al*., 1991). Assembly of cell surface TF with Factor VII/VIIa generates the functional TF:VIIa complex. This complex can rapidly activate the serine protease zymogens Factors IX and X by limited proteolysis, leading to the formation of thrombin and, ultimately, a blood clot (Huang *et al*., 1997; 2006). Therefore, in the current study, we explored the feasibility of targeting occlusion of the newly formed blood vessels in solid tumors by thrombogenesis (blood coagulation) using a VEGF and truncated TF recombinant fusion protein in a murine colon carcinoma model.

MATERIALS AND METHODS

Materials

Plasmid pQE30, Qiaquick PCR Purification Kit, Qiaquick Gel Extraction Kit, a monoclonal antibody (mAb) against 6× His tag and nickel-nitrilotriacetic acid (Ni-NTA) agarose were purchased from Qiagen (USA). *Escherichia coli* JM109 was purchased from Pharmacia (USA). Murine colon carcinoma cell line (CT26) was purchased from American Type Culture Collection (ATCC, USA). TRIzol reagent was purchased from Invitrogen (USA). One step reverse transcriptase polymerase chain reaction (RT-PCR) Kit, endonuclease, RNase A, T4 DNA ligase, DNA marker and isopropy-β-D-thiogalactoside (IPTG) were purchased from TaKaRa (Japan) or MBI Fermentas (Lithuania). Urea, Tris, sodium dodecyl sulfate (SDS), glycocine, Coomassie blue and polyvinylidene difluoride (PVDF) membrane were purchased from Bio-Rad (USA). mAb against TF and labeled streptavidin biotin reagents were purchased from Dako (USA). VECTASTAIN *Elite* ABC Kit was purchased from Vector Laboratories (USA). RPMI-1640 culture media was purchased from Gibco (USA). Purified Factors X, Xa, VII and VIIa were from Enzyme Research Laboratories (USA). BALB/c mice were purchased from the Animal Center of Hainan Province, China.

Plasmid construction and identification

Murine total RNA was isolated from murine embryo livers using TRIzol reagent and subjected to RT-PCR (using one step RT-PCR Kit from TaKaRa) for the amplification of the murine VEGF A cDNA (1~415 nucleotide residues) and the murine TF cDNA (135~1020 nucleotide residues), respectively. The upstream primers for VEGF A and TF were 5′-ATAGCATGCATGAACTTCTGCTCTCTTGG-3′ and 5′-ATTGGTACCATGGCGATCCTCGTGCGC CCG-3′, respectively. The downstream primers for amplification of VEGF A and TF were 5′-ATA GTTACCGCCTTGGCTTGTCAC-3′ and 5′-TACA AGCTTCCTCTATGCCAAGCGCGACGG-3′, respectively. The amplified products of VEGF A and TF were cloned into pQE30 plasmids, and the two resulted recombinant plasmids were named as pQE-VEGF and pQE-TF, respectively. In addition, the TF cDNA was then amplified with another upstream primer 5′-CTTGGTACCGGTGGAGGCGG TTCAGGCGGAGGTGGCTCTGGCGGTGGCGGA TCGATGGCGATCCTCGTGCGCCCG-3′ [containing a $(Gly_4Ser)_3$ linker] and subcloned in the recombinant plasmid pQE-VEGF, and this recombinant plasmid vector was named as pQE-VEGF-TF. All the recombinant plasmids were conformed by endonuclease digestion analysis and sequencing to be identical to those in GenBank (GI: 37545171 for murine VEGF A and GI: 201924 for murine TF).

Expression and purification of recombinant proteins

E. coli JM109 containing plasmid pQE-VEGF-TF, or pQE-VEGF, or pQE-TF was grown overnight in LB media at 37 °C until the optical density (OD) was between 0.4 and 0.6. At this time point, IPTG (1 mmol/L) was added to the media and maintained at 37 °C for 3~5 h. Culture media were collected and expressed proteins were analyzed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The blank JM109 strain not containing any expression plasmids and the JM109 strain containing the blank vector pQE30 were used as controls. The purification of the corresponding recombinant proteins was done using Ni-NTA agarose affinity chromatography column following the manufacturer's instructions from Qiagen. Thereafter, the purified proteins were extensively dialyzed to

allow refolding. The refolded proteins were further purified by ion exchange and gel filtration chromatography, then lyophilized and stored at 4 °C. The purified recombinant proteins were also confirmed by SDS-PAGE and Western blot analysis, and the endotoxin units were less than 0.5 μg as determined by *limulus* amebocyte lysate assay.

Western blot analysis

Western blot analysis was performed as described previously (Tan *et al*., 2004; Jiao *et al*., 2006). Briefly, recombinant proteins or lysates of bacterial cells were separated by SDS-PAGE. Gels were transported onto a PVDF membrane by a mini trans-blot system (Bio-Rad, USA). The membrane blots were blocked at 4 °C in 5% (w/v) nonfat dry milk, then washed and probed with a rat mAb against 6× His tag at 1:500 (v/v). Blots were then washed and incubated with a biotinylated secondary antibody (biotinylated rabbit anti-rat IgG) followed by transfer to VECTASTAIN *Elite* ABC Kit.

Spectozyme Factor Xa assay

To verify the clotting abilities of the recombinant VEGF-TF (rVEGF-TF) and commercial recombinant TF (rTF), a Spectozyme Factor Xa assay (Factor X activation assay) was performed as described previously (Ruf *et al*., 1991). Briefly, various concentrations (0.1~100 nmol/L) of rTF or rVEGF-TF acquired in the current study were mixed with 100 nmol/L Factor VII in Tris-buffered saline buffer and incubated at 37 °C for 10 min, to which 5 nmol/L Factor X was added. The mixture was then incubated at room temperature for another 10 min, to which 100 mmol/L ethylene diamine tetraacetic acid (EDTA) were added to quench the reaction. Thereafter, 2 nmol/L chromogenic substrate Spectozyme Factor Xa was added, and the mixture was detected at 405 nm by enzyme-linked immunosorbent assay (ELISA) reader (ELX808, Bio-Tek, USA) in the first 5-min time period.

Determination of tumor specific binding and histological analysis

BALB/c mice at 6 to 8 weeks of age were firstly inoculated with 2×10^6 live CT26 tumor cells into the right flank until the tumors grew to about 6~8 mm in diameter. Then, the mice were randomly divided into 3 groups of 6 mice each. In rVEGF-TF group, each mouse was administered intravenously with 20 μg of the purified rVEGF-TF in 200 μl of normal saline (this dose was determined by an optimal-dose preliminary experiment) via tail vein. In rVEGF group, each mouse was administered intravenously with 20 μg of the purified rVEGF. In rTF group, each mouse was administered intravenously with 20 μg of the purified rTF. Three mice in each group were sacrificed 5 min after the recombinant proteins injections, and the other 3 mice were sacrificed 1 h after the recombinant proteins injections. The tumor tissues and major organs such as the heart, lung, kidney, liver and brain were collected and frozen in liquid nitrogen. Thereafter, to determine the specific binding to the newly formed blood vessels in tumor tissues by rVEGF-TF, frozen sections from these tumor tissues were fixed in acetone, washed with phosphate buffered solution (PBS), incubated with rat anti-TF antibody (IgG), and then further incubated with a second rabbit fluorescein isothiocyanate (FITC)-conjugated antibody against rat IgG. Slides were examined by fluorescence microscopy.

To assess the thrombogenesis in tumor tissues by histological analysis, tumor tissues and major organs from the above two time points were collected and fixed in 10% (w/v) buffered neutral formalin overnight, embedded in 2% (w/v) paraffin, sectioned, and stained with hematoxylin and eosin (H & E). Thrombosis of vessels was assessed as either total or incomplete depending on the extent of closely packed erythrocytes, blurring of the vessel outline, and the presence of aggregated platelets and fibrin deposition as described previously (Hu *et al*., 2003).

In vivo determination of anti-tumor effects

For the investigation of the therapeutic efficacy of the rVEGF-TF in anti-tumor activity in vivo, BALB/c mice at 6 to 8 weeks of age were inoculated with 2×10^6 live CT26 tumor cells into the right flank and remained untreated until palpable tumors of distinct size (about 6~8 mm in diameter) appeared in the mice. Then, the mice were randomly divided into 4 groups of 10 mice each. In Group 1 (rVEGF-TF group), each mouse was administered intravenously with 20 μg of the purified rVEGF-TF in 200 μl of normal saline via tail vein, and then another dose one week later. In Group 2 (rVEGF group), each mouse

was administered with 20 μg of the purified rVEGF as done in Group 1. In Group 3 (rTF group), each mouse was administered with 20 μg of the purified rTF as done in Group 1. In Group 4 (NS group), each mouse was injected with 200 μl of normal saline (NS) twice without any recombinant protein. The above procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. The tumor size and survival rate were monitored at a 3-d interval, and the tumor volume (*TV*) was calculated by the following formula: $TV=0.52ld^2$, where l is the tumor length and d is the tumor width.

Statistical analysis

For comparison of individual time points, analysis of variance (ANOVA) was used. $P<0.05$ was considered statistically significant. Error bars represent the standard deviation (*SD*).

RESULTS

Gene cloning and plasmid construction

Total RNA of mouse liver tissue was extracted by TRIzol reagent and then subjected to RT-PCR with amplified DNA measured by 1% (w/v) agar gel electrophoresis. As can be seen from Fig.1, the resultant PCR cDNA products of TF (Lane 2) and VEGF A (Lane 3) were approximately 900 and 400 bp as predicted, respectively.

The amplified products of TF and VEGF A cDNA were cloned into pQE30 plasmids, which obtained the plasmid pQE-TF and pQE-VEGF. The cDNA of TF was then subcloned in the recombinant pQE-VEGF plasmid vector, which obtained recombinant plasmid pQE-VEGF-TF. These three recombinant plasmids were then conformed by endonuclease digestion analysis and sequencing (data not shown) to be identical to those in GenBank (GI: 37545171 for murine VEGF A and GI: 201924 for murine TF). In Fig.1, Lanes 4~7 show the fragments digested by the corresponding endonucleases, and the sites of the digested DNA fragments to the recombinant pQE30 empty plasmid (Lane 4), pQE-TF (Lane 5), pQE-VEGF (Lane 6) and pQE-VEGF-TF (Lane 7) were also as predicted, respectively.

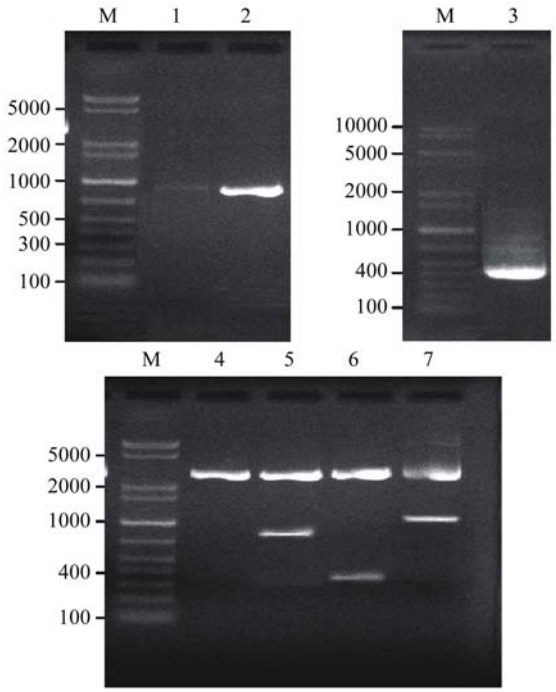

Fig.1 Amplification of TF and VEGF A cDNA, and analysis of the corresponding recombinant plasmids
Lanes 1 and 2: The cDNA of TF (about 900 bp) at different conditions of PCR; Lane 3: The cDNA of VEGF A (about 400 bp); Lane 4: The fragment of empty pQE30 plasmid digested by *Kpn*I (about 3.4 kb); Lane 5: The fragments of recombinant pQE-TF plasmid digested by *Kpn*I and *Hin*dIII [about (3.4+0.9) kb]; Lane 6: The fragments of recombinant pQE-VEGF plasmid digested by *Sph*I and *Kpn*I [about (3.4+0.4) kb]; Lane 7: The fragments of recombinant pQE-VEGF-TF plasmid digested by *Sph*I and *Hind*III [about (3.4+1.4) kb]. All the digested fragments were as predicted

Expression and purification of the recombinant proteins

The corresponding recombinant plasmids (pQE-VEGF-TF, pQE-VEGF and pQE-TF) were transfected into *E. coli* JM109, respectively. The expressions of corresponding rVEGF-TF, rVEGF and rTF were induced using 1 mmol/L IPTG, and the lysates were collected. The corresponding recombinant proteins were then purified using a Ni-NTA agarose affinity chromatography column and monitored at 280 nm (Fig.2a), and the purified proteins were further detected and certified by SDS-PAGE (Fig.2b) and Western blot analysis (Fig.2c).

Detection of the TF enzyme activity

The enzyme activities of rVEGF-TF and rTF acquired in the current study were analyzed by

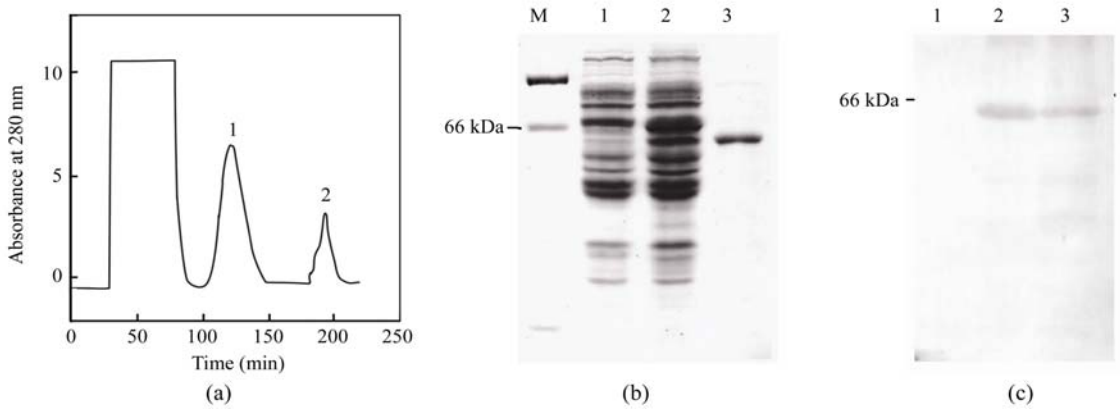

Fig.2 Expression and purification of the recombinant proteins. (a) The rVEGF-TF was purified by Ni-NTA agarose affinity chromatography. Peak 1 shows the nonspecific binding protein and peak 2 shows the recombinant protein rVEGF-TF; (b) Pro-expression (Lane 1), expression (Lane 2) and purification (Lane 3) of rVEGF-TF by SDS-PAGF; (c) Pro-expression (Lane 1), expression (Lane 2) and purification (Lane 3) of rVEGF-TF by Western blotting. The data for rVEGF and rTF were not shown

Spectozyme Factor Xa assay, compared with a commercial TF (cTF). As shown in Fig.3, the enzyme activities of rVEGF-TF and rTF at different time points were similar (P>0.05), almost comparable with the activity of cTF. In addition, the half-maximal activities of the three fusion proteins were observed at a concentration of ~100 nmol/L, which were also comparable with the activity of cTF (Fig.3), indicating the rVEGF-TF is active enough for subsequent experiments.

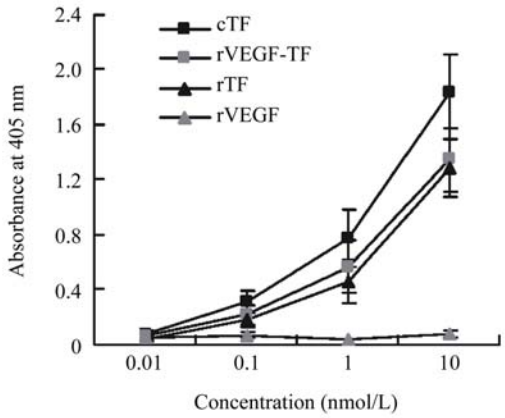

Fig.3 The enzyme activities of rVEGF-TF and rTF were comparable with the commercial TF (cTF) determined by the Spectozyme Factor Xa assay

Specific binding to the tumor blood vessels

Immunofluorescence was used to determine the specific binding to the newly formed blood vessels in tumor tissues. Frozen sections from tumor tissues and major organs were incubated with rat anti-TF antibody (IgG) and a second rabbit FITC-conjugated antibody against rat IgG. Slides were then examined by fluorescent microscopy. Five minutes after injection of the recombinant proteins, slides of tumor tissues from the mice injected with rVEGF-TF (Fig.4a) or rTF (Fig.4b) were found fluorescent signals, which showed the characteristic morphous of blood vessels. In contrast, the slides of tumor tissues from the mice injected with rVEGF were not found to have any fluorescence signals (Fig.4c). However, 1 h after injection of the recombinant proteins, only the slides of tumor tissues from the mice injected with rVEGF-TF had fluorescent signals (Fig.4d), and the slides from the mice injected with rTF or rVEGF had no florescent signals (Figs.4e and 4f). In addition, we also found that, 1 h after injection of the three recombinant proteins, all slides of major organs such as the heart, lung, kidney, liver and brain from the mice had no fluorescent signals (data not shown). These results demonstrate that rVEGF-TF has capability of specific targeting to the tumor vessels.

Suppression of tumor growth and thrombogenesis in tumor tissues

CT26 colon carcinoma was established in BALB/c mice, and the tumor volume was monitored at 3-d intervals. Our results (Fig.5) show that tumors in the mice injected with rVEGF, rTF and NS grew progressively. At every time point, there was no any significant difference in the three group mice treated

with rVEGF, rTF or NS. In contrast, a significant inhibition of tumor growth was observed in the mice injected with rVEGF-TF. Compared with the control groups, tumor volume in the mice injected with rVEGF-TF significantly decreased (P<0.05) from Day 6 after the first treatment (Fig.5). The tumor masses in 2 mice injected with rVEGF-TF were almost disappeared at Day 14 after the first treatment.

In addition, a histological study was performed to determine whether intravenous administration of the rVEGF-TF induced selective thrombosis in the tumor tissues of mice bearing CT26 colon carcinoma. Within 1 h, almost all vessels throughout the tumor were thrombosed, containing occlusive platelet aggregates, packed erythrocytes and fibrin, and necrosis was shown in the tumor cells (Fig.6a). In contrast, blood vessels in mice treated with rTF (Fig.6b) and rVEGF (Fig.6c) were intact and no thrombogenesis was found.

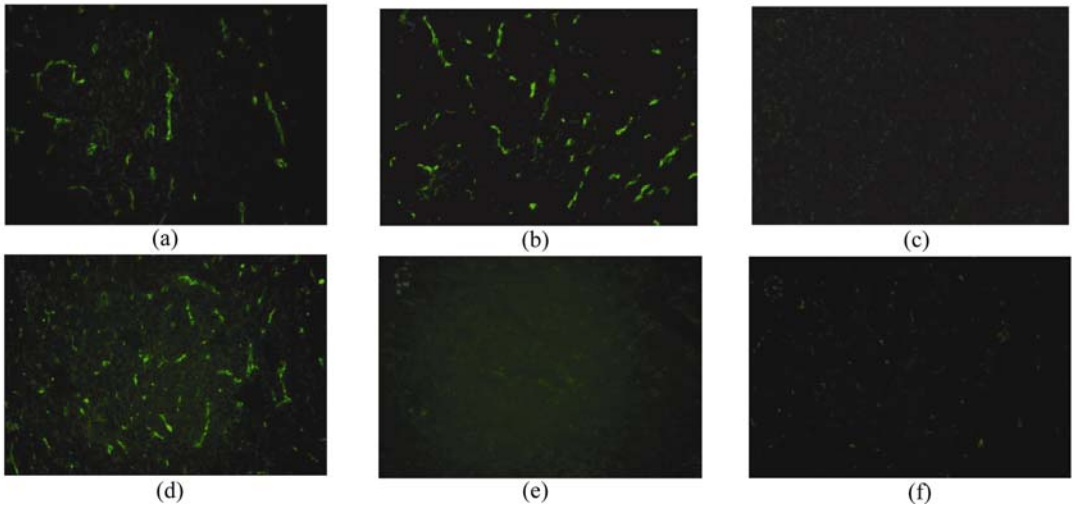

Fig.4 Specific targeting to the tumor vessels by rVEGF-TF. The tumor tissues from mice injected with rVEGF-TF (a) or rTF (b) showed florescent signals 5 min after the injection of the correspondent recombinant protein. The tumor tissues from mice injected with rVEGF were not found to have any fluorescence signals 5 min (c) and 1 h (f) after injection. However, 1 h after the injection, only tumor tissues from the mice injected with rVEGF-TF had fluorescent signals (d), and the florescent signals from the mice injected with rTF disappeared (e), suggesting that specific binding to the tumor vessels was highly related to the specific binding between VEGF and VEGFR

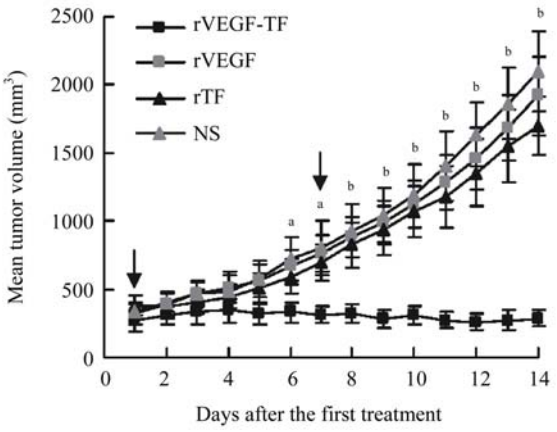

Fig.5 Tumor volumes at different time points after the first treatment. rVEGF-TF: Mice injected with the recombinant fusion protein rVEGF-TF; rVEGF: Mice injected with rVEGF; rTF: Mice injected with rTF; NS: Mice injected with NS. Arrows represent the time points of treatment. ^{a}P<0.05, ^{b}P<0.01. Data are shown as mean±*SD* (n=10 in each group)

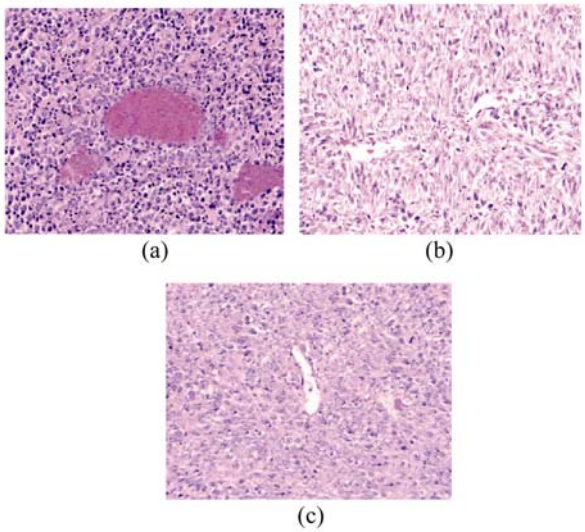

Fig.6 Histology of the tumor tissues. Significantly thrombosis and tumor necrosis are found in the tumor tissues from the mice injected with rVEGF-TF (a), but not in the mice injected with rVEGF (b) or rTF(c)

DISCUSSION

More and more studies have demonstrated that the growth and persistence of solid tumors and their metastases are angiogenesis-dependent (Folkman, 1990; Risau, 1997; Wickham *et al*., 1997). Among various molecules that stimulate angiogenesis, VEGF are shown to be the most important one (Paleolog, 1996). VEGF plays its function through interaction with VEGFRs, which are mainly expressed on the newly formed blood vessels in tumor tissues. VEGF is a family mainly including VEGF A, VEGF B, VEGF C, VEGF D and PlGF (placental growth factor). VEGF A is the most specific molecule binding to VEGFR-1 and VEGFR-2 which are mainly present at sufficient density on the surface of tumor vascular endothelium but absent in normal vascular endothelium (Folkman, 1992). Thus, VEGF A is potentially a promising candidate molecule targeting the newly formed blood vessels in tumor tissues. In the current study, we constructed a fusion recombinant protein rVEGF-TF containing VEGF A and TF, and found that rVEGF-TF has the capability of specifically targeting tumor vessels in a CT26 colon carcinoma model by immunofluorescence.

TF is the major initiating receptor for the thrombogenic cascades, leading to the formation of thrombin and, ultimately, a blood clot. Therefore, as a strategy for cancer therapy, the use of double-function recombinant fusion-protein that selectively occludes the newly formed vasculature of solid tumors offers several theoretical advantages over conventional tumor cell targeting in the therapy of solid tumors: (1) This approach may have the potential to circumvent the problem of acquired drug resistance (Kerbel, 1991; Boehm *et al*., 1997). The reasonable explanation is that drug resistant mutants are easily generated from tumor cells due to the genetic instability of tumor cells. On the contrary, genetically stable normal cells, such as vascular endothelial cells, would be far less adept at generating such mutants; (2) Occlusion of the newly formed blood vessels in tumor tissues may have the capability of overcoming the problem of tumor heterogeneity (Matsuno *et al*., 1999). At present, tumor heterogeneity has become a major problem with the tumor cell targeting therapy; (3) Physiological barriers for the high-molecular weight drugs (such as antibodies and immunoconjugates) to penetrate into solid tumors will be also circumvented by targeting a tumor's vasculature rather than tumor cells themselves (Jain, 1994; Dvorak *et al*., 1991). The possible reason is that, unlike tumor cells in the solid tumors, the vascular endothelial cells are directly accessible to circulating high-molecular weight drugs; (4) Many thousands of dependent tumor cells will die of nutrient and/or oxygen deprivation if a capillary or a sector of the capillary bed fails (Denekamp, 1993; Thorpe and Burrows, 1995). Therefore, killing of only a minority of vascular endothelial cells of tumors may be sufficient to eradicate most malignant cells in tumor tissues; (5) At present, nearly all solid tumors develop newly formed blood vessels, so therapeutic agents targeting occlusion of therapy such as rVEGF-TF in the current study could be applied to various types of solid tumors and other angiogenesis-associated diseases.

In the current study, we have correctly constructed and purified the VEGF and TF fusion protein rVEGF-TF. Except for being specific to tumor vessels, rVEGF-TF also has the TF enzyme activities that are almost comparable to the cTF. In addition, we also found that administration of 20 μg per mouse twice at a week interval in a mouse CT26 colon carcinoma model could significantly suppress tumor growth. Selective thrombosis and tumor cell necrosis were found in the tumor tissues from the mice injected with rVEGF-TF. The thrombosed vessels contained occlusive platelet aggregates, packed erythrocytes and fibrin. Based on these findings, we may thus conclude that the suppression of tumor growth in the current study is highly related to the induction of tumor occlusion by the recombinant thrombogen rVEGF-TF to the newly formed blood vessels in tumor tissues.

In the current study, our results illustrate the therapeutic potential of selective initiation of the blood coagulation cascade in tumor vasculature. At present, several molecules (Vlodavsky and Friedmann, 2001), such as endoglin (including endoglin-like molecule), endosialin, fibronectin isoform, osteosarcoma-related antigen, CD34, collagen type VIII, VEGFR, have been identified to be present at sufficient density on the surface of tumor vascular endothelium, but absent in normal vascular endothelium. Therefore, the induction of tumor occlusion by targeting a thrombogen to these tumor endothelial cell markers represents an intriguing approach to the eradication of primary solid tumors and vascularized metastases.

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