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 isopropyl-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′-ATTGGTACCATGGCGATCCCTCGTGCGC CCG-3′,

respectively. The downstream primers for amplification of VEGF A and TF were

5′-ATAGCATGCATGAACTTCTGCTCTCTTGG-3′ and

5′-ATTGGTACCATGGCGATCCCTCGTGCGC CCG-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′-CTTGATGCTTCTTGGCTTGTCAC-3′ [containing a (Gly4Ser)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 confirmed 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