A Novel Role for Tissue Factor Pathway Inhibitor-2 in the Therapy of Human Esophageal Carcinoma

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Abstract

Esophageal cancer is characterized by rapid clinical progression and poor prognosis, due to early-stage invasion of adjacent tissues and metastasis. Tissue factor pathway inhibitor-2 (TFPI-2) has been implicated as a metastasis-associated gene in many types of tumors. Here we describe the potential involvement of TFPI-2 in the development of esophageal carcinoma. Western blotting revealed that TFPI-2 was downregulated in 75% of esophageal carcinomas and in most esophageal carcinoma cell lines. Immunohistochemistry revealed that TFPI-2 was significantly downregulated in tumor tissues and in lymph node metastases. Experimental overexpression of TFPI-2 in KYSE450, KYSE510, YES2, and EC9706 cells significantly inhibited their invasive ability. Overexpression of TFPI-2 in EC9706 cells inhibited xenograft tumor growth and invasion into surrounding tissues, as well as reduced lung metastasis. Further studies demonstrated that recombinant TFPI-2 protein significantly inhibited the activity of matrix metalloproteinases and tumor-related angiogenesis. Parenteral treatment with recombinant TFPI-2 protein significantly suppressed xenograft growth and metastasis. Together, these data indicate that TFPI-2 inhibits tumor invasion and angiogenesis both in vitro and in vivo, and suggest a potentially important therapeutic role for recombinant TFPI-2 in the treatment of malignant esophageal carcinomas.

Introduction

Human esophageal carcinoma is one of the most common causes of cancer death worldwide, and is particularly prevalent in China (Parkin, 2001; Enzinger and Mayer, 2003). The prognosis for patients with esophageal cancer is poor, due in large part to early-stage invasion of adjacent tissue and metastasis (Furihata et al., 2001). Proteic degradation of the extracellular matrix (ECM) is an essential step in the invasion of foreign tissues by malignant cells (Blindt et al., 2002; Rollin et al., 2007). Proteinase inhibitors that are capable of blocking tumor-derived proteinases might therefore be effective therapeutic agents against tumor metastasis (Herman et al., 2001; Pawlak et al., 2004). Human tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type serine proteinase inhibitor that is synthesized and secreted into the ECM by endothelial cells, smooth muscle cells, fibroblasts, keratinocytes, and urothelial cells (Rao et al., 1995; Udagawa et al., 1998). TFPI-2 readily inhibits trypsin, plasmin, chymotrypsin, cathepsin G, plasma kallikrein, and the factor VIIa–tissue factor complex (Chard et al., 2004). TFPI-2 is thought to play a significant role in the regulation of plasmin-mediated ECM degradation during tumor cell invasion and metastasis, wound healing, and angiogenesis (Rao et al., 1998; Shinoda et al., 1999; Izumi et al., 2000; Konduri et al., 2001b; Golino et al., 2004; Yanamandra et al., 2005; Choi et al., 2007; George et al., 2007). In this study we show that TFPI-2 acts as a tumor suppressor in esophageal carcinoma. TFPI-2 was downregulated in primary esophageal carcinoma tissues and lymph node metastases. In addition, we describe the inhibitory effect of TFPI-2 on the growth and metastasis of esophageal carcinoma tumors.

Materials and Methods

Tissue samples

Paired samples of fresh normal esophageal tissues and esophageal carcinoma tissues from the same patient were collected by the Department of Pathology in the Chinese Academy of Medical Sciences Cancer Hospital (Beijing, China). Each patient had received a pathologically and clinically confirmed diagnosis of esophageal squamous cell carcinoma. Primary tumor regions and the corresponding histologically normal esophageal mucosa were separated by experienced pathologists and immediately stored at −70°C.

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until use. None of the patients received treatment before surgery, and all signed informed consent forms before sample collection.

Reagents and antibodies

Monoclonal antibodies recognizing TFPI-2 were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibody against TFPI-2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO). Matrigel was obtained from BD Biosciences (Palo Alto, CA). A Cell Counting Kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). Three effective small interfering RNA (siRNA) duplex sequences targeting TFPI-2, and a corresponding negative control siRNA, were designed and synthesized by Ribobio (Guangzhou, China). The target sequences were as follows:

siRNA 1: 5'-CCAACAATTTCACACCTG-3'
siRNA 2: 5'-CAGATGAAGCTACTTGTAT-3'
siRNA 3: 5'-CAATGTGACTCGCTATTAT-3'

Molecular cloning of human TFPI-2

TFPI-2 mRNA was amplified by the polymerase chain reaction (PCR), using the forward primer 5'-CGCCGGATCC TTCTCGGAC-3' and the reverse primer 5'-GAATGTCCTC GATTGCTTTCCGA-3'. The amplification product was subcloned between the BamH I and XhoI sites of the mammalian vector pcDNA4.0 (Invitrogen, Carlsbad, CA), which encodes c-Myc and poly(His) epitopes. The sequence of the recombinant vector was confirmed by automated nucleotide sequencing.

Reverse transcription-polymerase chain reaction

Cells were harvested in TRIzol reagent (Invitrogen), and total RNA was isolated according to the manufacturer’s instructions. Single-stranded cDNA was synthesized from 4 μg of total RNA, using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen), with an oligo(dT)18-mer as the primer, in a final reaction volume of 25 μl. In TFPI-2 sense, 5'-CAG GAG CCA ACA GGA AAT AAC-3', antisense, 5'-GAA TAC GAC CCC AAG AAA TGA-3', and the reverse primer, in a final reaction volume of 25 μl, using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen), with an oligo(dT)18-mer as the primer, in a final reaction volume of 25 μl. The amplification product was subcloned between the BamH I and XhoI sites of the mammalian vector pcDNA4.0 (Invitrogen, Carlsbad, CA), which encodes c-Myc and poly(His) epitopes. The sequence of the recombinant vector was confirmed by automated nucleotide sequencing.

Western blotting

For Western blot analysis, tissue sample and cellular protein bands were visualized after exposure of the membrane to Kodak X-ray film (Carestream Health, Rochester, NY).

Cell culture conditions and transfection

The esophageal carcinoma cell (ESCC) line EC9706 (a gift from M. Wang, Chinese Academy of Medical Sciences Cancer Institute Hospital, Peking University Medical School, Beijing, China) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Other ESCC lines (KYSE30, KYSE150, KYSE180, KYSE410, KYSE450, KYSE510, and Yes2) were generously provided by Y. Shimada (Kyoto University Graduate School of Medicine, Kyoto, Japan).

The TFPI-2 expression vector was transfected into EC9706 and KYSE150 cells with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s recommendations. Cells carrying the recombinant or empty (control) TFPI-2 vector were selected by culturing in the presence of Zeocin (200 ng/ml; Invitrogen) for more than 4 weeks. Two stably TFPI-2-transfected EC9706 clones (C9 and C10) and one TFPI-2-negative clone (C7) were chosen for subsequent experiments. Transfection of duplex siRNAs was performed in the same manner. The cells were used in assays of cell migration and invasion 72 hr after transfection.

Cell proliferation assay

Cells were trypsinized and then resuspended in complete medium and seeded onto 96-well plates at 2×10³ cells per well. Cells were counted with the Cell Counting Kit-8 on the indicated days according to the manufacturer’s instructions, with the optical density (OD) measured at 450 nm. All experiments were performed in triplicate and repeated twice.

Invasion assay

Invasion assays were carried out in a 24-well Transwell unit on a polycarbonate filter with 8-μm pores coated with Matrigel. After a 24-hr incubation period, the cells that had passed through the filter into the lower wells were stained, counted, and photographed. All experiments were performed in triplicate and repeated twice.

Immunohistochemistry

Paraffin-embedded xenograft tumor or human tissue sample sections were assayed. For immunohistochemical staining, sections were dewaxed and rehydrated and then incubated with 3% H₂O₂ to quench endogenous peroxidase activity. Sections were then blocked, incubated overnight with primary antibody (15 μg/ml in phosphate-buffered saline [PBS]), incubated with biotinylated secondary antibody, developed, and counterstained with hematoxylin. At least five areas of each section were examined at a magnification of x200. The expression score was determined on the basis of staining intensity and the percentage of immunoreactive cells. Tissues were rated on a numeric scale: 0, no staining; 1, faint staining, or moderate to strong staining in 25% of cells; 2, moderate or strong staining in 25–50% of cells; or 3, strong staining in 50% or more of cells.

Tumor growth and metastasis

Female nu/nu mice, obtained from the Jackson Laboratory (Vital River Lab Animal, Beijing, China), were kept in a specific pathogen-free facility at the Experimental Center of the Chinese Academy of Medical Sciences (Beijing, China).
Female mice, aged 4–6 weeks, were used in these experiments. Parent EC9706, TFPI-2-transfected EC9706 clones C9 and C10, or vector-transfected cells were subcutaneously injected into 16 nude mice. Thirty days, and again at 90 days, after injection, eight mice were killed and examined for subcutaneous tumor growth or metastasis development. To allow metastatic nodules to be detected in lungs, the lungs were fixed in Bouin’s solution, after which tissues were embedded in paraffin and sections stained with hematoxylin and eosin. Microvessels were detected with anti-CD31 antibody (ab28364; Abcam, Cambridge, UK), with each section examined in five areas at a magnification of ×200.

Expression and purification of TFPI-2 protein

Full-length human TFPI-2 was overexpressed as a C-terminal His-tagged fusion protein in *Escherichia coli* BL21 (DE3). A recombinant plasmid derived from pET30b, bearing a histidine tag and cDNA encoding full-length TFPI-2, was prepared according to standard procedures. Wild-type His-tagged TFPI-2 preparations were expressed in *E. coli* grown in rich medium containing kanamycin (100 mg/liter) and induced at 37°C with 1 mM isopropyl thiolgalactopyranoside at mid-log phase (A<sub>600</sub> = 0.6–0.8). Overexpressed proteins were recovered from cell lysates in the form of inclusion bodies, after sonication in 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl, 5 mM 2-mercaptoethanol, and 10 mM imidazole (buffer A). Inclusion bodies were recovered by high-speed centrifugation (20,000 × g for 60 min) and thoroughly washed overnight at room temperature before solubilization in buffer A containing 6 M guanidine hydrochloride. The solubilized inclusion bodies were recovered by high-speed centrifugation and were filtered through 0.22-μm Nalgene filters, and then applied to a His-Trap column as directed by the manufacturer’s protocol (Chand et al., 2004; Kong et al., 2004).

Refolding of purified recombinant TFPI-2

Recombinant TFPI-2 (rTFPI-2) was first diluted to an absorbance of 0.08–0.10 at 280 nm, using a buffer consisting of 20 mM Tris-HCl (pH 8.0), 6 M urea, 0.01% Brij 35, and 0.3 M NaCl, after which 1-cysteine powder was added to a final concentration of 2 mM. The solution was incubated at room temperature for 24 hr and then diluted 1:1 with water and further incubated, first at room temperature for 24 hr and then at 4°C for up to 2 weeks. Before use, the refolded rTFPI-2 was concentrated against a solution containing 15 mM Tris-HCl (pH 7.4) and 0.15 M NaCl (Rao et al., 2000).

Suppression of tumor growth by rTFPI-2

Tumor-bearing mice were matched by size and distributed into groups. Mouse weights varied less than 5% within each treatment cohort. Treatment began 3 days after injection of tumor cells, when mice received either 100 μg of rTFPI-2 (about 5 mg/kg; high dose), 10 μg of rTFPI-2 (about 0.5 mg/kg; low dose), or dialysis buffer alone. Therapeutic and control doses were systemically administered in 200 μl of PBS once daily for 5 days, followed by twice weekly administration until treatment was completed. Tumor volumes were calculated according to the following formula: volume = length × width<sup>2</sup>/2. After the last treatment, mice were killed to measure tumor weight.

Statistical analysis

All data are presented as means ± SD. Statistical analysis was performed with SPSS statistical software (SPSS, Chicago, IL). The Student two-tailed t test was used to compare groups. p < 0.05 was considered significant.

Results

**TFPI-2 is downregulated in human esophageal carcinoma and in ESCC lines**

Gene expression analysis revealed that TFPI-2 was weakly expressed in ESCC lines (Fig. 1A). To assess TFPI-2 protein expression in esophageal carcinoma tissues, 12 sample pairs, each consisting of esophageal carcinoma tissue and normal esophageal tissue from the same patient, were subjected to Western blotting. In eight cases (75%) TFPI-2 expression was lower in tumor tissue than in the companion normal tissue (Fig. 1B). We then extended this analysis to 92 pairs of esophageal carcinoma and normal tissues. In 63 cases (68%) TFPI-2 expression appeared to be downregulated in tumors compared with the companion normal tissue. Lymph node metastatic foci also showed lower TFPI-2 expression than normal tissues, with the following immunostaining scores: normal, 1.98 ± 0.71; tumor, 1.06 ± 0.89; lymph node metastasis, 0.84 ± 0.98 (Fig. 1C).

**Transfection of TFPI-2 inhibits invasion of esophageal carcinoma cells**

To determine the role of TFPI-2 in tumor progression, the pcDNA4.0-TFPI-2-c-Myc vector was transiently transfected into ESCC lines EC9706, KYSE450, KYSE510, and YES2 (Fig. 2A and C). In a Transwell assay, cells from the EC9706 line (Fig. 2B) as well as other cell lines (Fig. 2D) showed a reduced ability to invade the matrix after they had been transiently transfected with TFPI-2. Similar results were seen with cells stably transfected with TFPI-2 (Fig. 2E and F). In accordance with these gain-of-function results, experimentally induced downregulation of TFPI-2 by RNA interference (RNAi) promoted tumor cell invasion (Fig. 2G). TFPI-2 did not influence the proliferation and migration of cells (Fig. 2H and I).

**TFPI-2 inhibits growth and spontaneous metastasis of esophageal tumors in vivo**

To define the function of TFPI-2 in vivo, we subcutaneously injected TFPI-2-overexpressing EC9706 clones (clones C9 and C10), EC9706-vector control cells, and parental EC9706 cells into athymic mice; after 30 days these mice were killed. TFPI-2 inhibited tumor growth and reduced tumor burden significantly (Fig. 3A). In the EC9706-TFPI-2 group, the xenograft tumor showed a low invasive pattern in hematoxylin and eosin (H&E)-stained sections (Fig. 3B). TFPI-2 also significantly inhibited spontaneous metastasis of EC9706 cells to lung. At 120 days after subcutaneous inoculation, the lungs of mice in the control cohort had clearly evident metastatic nodules, whereas these were barely visible on the lung surface of the TFPI-2-transfected cohort (Fig. 3C). H&E staining further confirmed the presence of lung metastases (Fig. 3C). TFPI-2 upregulation also inhibited tumor-related angiogenesis: EC9706-TFPI-2 clones C9 and C10 had significantly fewer microvessels than their corresponding controls (Fig. 3D).
Recombinant TFPI-2 inhibits invasion and proliferation of esophageal tumor cells

Electrophoresis demonstrated that purified TFPI-2-His-tagged fusion protein was refolded in the refolding buffer (Fig. 4A). The biological properties of refolded TFPI-2 (rTFPI-2) were tested for their ability to inhibit matrix degradation by EC9706 cells. As shown in Fig. 4B, 2 μM rTFPI-2 significantly inhibited the activity of cell matrix metalloproteinase (MMP)-2 and MMP-9. The invasive ability of EC9706 cells was also inhibited, in a dose-dependent manner, by rTFPI-2 (Fig. 4C). We studied the effect of TFPI-2 on capillary formation by coculturing human vascular endothelial cells with refolded TFPI-2. After 6 hr, cells were assessed for the formation of capillary networks. As seen in Fig. 4D, rTFPI-2 inhibited the formation of capillary-like structures by endothelial cells.

Engrafted tumors were treated with rTFPI-2. Mice were killed 30 days postinjection. The growth of tumors was significantly suppressed by rTFPI-2, compared with those treated with dialysis buffer. The average volume in the rTFPI-2 high-dose group was significantly lower than that in the control group (Fig. 4E). The tumor burden of the high-dose rTFPI-2 group (low dose [10 μg], 36 ± 15; high dose [100 μg], 11 ± 7) was also less than that of the group treated with dialysis buffer (45 ± 13) (p < 0.01; Fig. 4F). There was no obvious difference in the body weight of mice in the treated and control groups (data not shown).

FIG. 1. Tissue factor pathway inhibitor-2 (TFPI-2) is downregulated in human esophageal carcinomas. (A) RT-PCR and Western blot analysis of TFPI-2 expression in six esophageal carcinoma cell (ESCC) lines and the normal cell line HEK-293. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) Western blot analysis of TFPI-2 expression in 12 paired samples, each pair comprising esophageal carcinoma tissue (T) and normal esophageal tissue (N) from the same patient. (C) Top and bottom: Immunohistochemical analysis of TFPI-2 expression in a total of 92 paired esophageal tumor and normal tissue samples. Red lines mean the average. + + +, strong staining in 50% or more of cells; −, no staining. **p < 0.01. (See supplemental data at http://www.liebertonline.com/doi/pdfplus/10.1089/hum.2008.129)
The TFPI-2 transcript is abundant in several adult human tissues. Conversely, TFPI-2 mRNA levels fall with increasing malignancy in tumors of the breast, stomach, colon, pancreas, larynx, kidney, endometrium, glial cells, and several other human tissues (Konduri et al., 2003; Sato et al., 2005; Steiner et al., 2005; Nobeyama et al., 2007; Wong et al., 2007). Our data showed that TFPI-2 was significantly downregulated in esophageal carcinomas, as well as in lymph node metastases. TFPI-2 expression has been reported to be regulated by the Ras/Raf/MEK/ERK signaling pathway (Kast et al., 2003). Each component of this pathway, when constitutively active, is able to induce high transcriptional activity of the hTFPI-2 gene. In esophageal tumor cell lines, TFPI-2 expression has been shown to be only marginally increased by treatment.
FIG. 3. TFPI-2 inhibits tumor growth and spontaneous metastasis of esophageal tumor cells in vivo. (A) Right: Tumor growth curve of the indicated groups. Left: Average tumor weight of the indicated groups 30 days after inoculation. *$p < 0.05$. (B) Top: H&E staining of EC9706 parental group, vector group, and C9 and C10 groups. The xenograft tumor showed a low-invasive pattern similar to that of the EC9706 parental and vector groups. Bottom: Immunohistochemical detection of TFPI-2 in exogenous tumor. (C) Left: Lung metastasis of the indicated groups 120 days after subcutaneous inoculation. Right: Plot indicating the metastatic loci visible on the lung surface. (D) Left: Immunohistochemical detection of blood microvessels in exogenous tumor, using anti-CD31. Right: Plot shows the average density of blood microvessels in the indicated groups. *$p < 0.05$. 
with phorbol myristate acetate (PMA), the activator of the Ras/Raf/MEK/ERK signaling pathway. However, sequential 5-Aza (5-aza-2-deoxycytidine)/PMA treatment was able to induce TFPI-2 expression in all cancer cells (see supplementary Fig. S1 at http://www.liebertonline.com/hum). TFPI-2 expression in ESCC thus appears to be governed by epigenetic mechanisms. As reported in other cancer cell lines, hypermethylation of the TFPI-2 promoter appears to be the principal means by which the TFPI-2 gene is silenced (Hube et al., 2003; Konduri et al., 2003; Sato et al., 2005; Steiner et al., 2005; Jiang et al., 2006; Guo et al., 2007).

Our results demonstrated that TFPI-2 was able to inhibit tumor cell invasion in vitro, and to suppress tumor growth and metastasis in vivo, by regulating ECM remodeling and tumor angiogenesis. These observations are in agreement with the results of several other reports (Konduri et al., 2001a; Rollin et al., 2005; Wong et al., 2007), which have suggested that TFPI-2 might act as a tumor suppressor. In addition, virally mediated gene transfer of TFPI-2 resulted in strong inhibition of tumor growth and angiogenesis (Sun et al., 2006; Ivanciu et al., 2007). These data suggest a potential role for TFPI-2 in the gene therapy of esophageal carcinoma.
TFPI-2 is a secreted protein that carries out its functions extracellularly. This led us to wonder whether TFPI-2 protein might be used directly as an anticancer drug. We therefore tested the antimalignancy effects of TFPI-2 in vitro and in vivo. Our results showed that rTFPI-2 directly inhibited both the activity of MMPs and the formation of tubes by endothelial cells. One report revealed that TFPI-2 significantly suppressed both vascular endothelial growth factor (VEGF)-stimulated and fibroblast growth factor (FGF)-stimulated endothelial cell proliferation (Ivanciu et al., 2007). This might explain the mechanism of TFPI-2-inhibited in vitro angiogenesis. Treatment with rTFPI-2 significantly suppressed tumor invasion and tumor-related angiogenesis, thereby reducing tumor growth and metastasis. In future work we will try to make the refolding of TFPI-2 protein a more straightforward process, because the refolded and functional protein is at present difficult to obtain.

It is well known that tumor growth and metastasis depend on invasion and angiogenesis. Our findings suggest that TFPI-2 inhibits tumor invasion and angiogenesis in vitro and in vivo. These results raise the intriguing possibility that TFPI-2 may have therapeutic value in the treatment of malignant esophageal carcinomas.

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Author Disclosure Statement

The authors have nothing to disclose.

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