Antiangiogenic Treatment with the Three Thrombospondin-1 Type 1 Repeats Recombinant Protein in an Orthotopic Human Pancreatic Cancer Model

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ABSTRACT

Purpose: This study investigates the antiangiogenesis and antitumor efficacy of a recombinant protein composed of the three type 1 repeats (3TSR) of thrombospondin-1 in an orthotopic human pancreatic cancer model and provides useful preclinical data for pancreatic cancer treatment.

Experimental Design: Human pancreatic cancer cells (AsPC-1) were injected into the pancreas of severe combined immunodeficient mice. The animals were treated with 3TSR (3 mg per kg per day) or PBS for 3 weeks. Subsequently, the effects of 3TSR on tumor growth, microvessel density, cancer cell proliferation, apoptosis, and endothelial cell apoptosis were analyzed. The in vitro effects of 3TSR on human pancreatic cancer cells were also studied.

Results: 3TSR treatment significantly reduced angiogenesis and tumor growth of orthotopic pancreatic cancer. 3TSR-treated mice had a 69% reduction in tumor volume (316.6 ± 79.3 versus 1,012.2 ± 364.5 mm³; \( P < 0.0001 \)), and a significant increase in tumor necrotic area. After 3TSR treatment, both the vessel number and average microvessel size were significantly decreased, and microvessel density was decreased from 8.0% to 3.7% (\( P < 0.0001 \)). The apoptotic rate of tumoral endothelial cells in 3TSR-treated tumors increased to 14.7% comparing to 4.2% in control tumors (\( P < 0.0001 \)). 3TSR showed no direct effects on pancreatic cancer cell proliferation or apoptosis either in vivo or in vitro.

Conclusion: 3TSR, a domain of a natural occurring angiogenesis inhibitor, showed potent therapeutic effect in pancreatic cancer by inhibiting tumor angiogenesis and may prove to be a promising agent for clinical pancreatic cancer treatment.

INTRODUCTION

Pancreatic cancer remains the fourth leading cause of cancer death in the United States. Patients with pancreatic cancer have few viable therapeutic options. Five-year survival rates remain around 5% and a median survival of <6 months has remained unchanged for the last three decades. Surgical resection is still the only curative therapy, although in 80% of patients the tumor is already unresectable at diagnosis due to metastasis or local extension (1, 2). Even in the 15% to 20% of patients undergoing potentially curative resection, the 5-year survival is only 20% (3). So novel therapeutics are urgently needed, and new treatment development mainly depends on the better understanding of biological behavior and genetic mechanisms of this aggressive cancer.

The process of tumor angiogenesis first proposed by Folkman (4) is becoming more thoroughly understood. Angiogenesis is a highly complex process, involving signals from the matrix, stroma, epithelial tissue, and the endothelial cell itself. The balance between stimulators and inhibitors of angiogenesis is tightly regulated and normally allows for restricted angiogenesis in adult tissues. Either an increase in proangiogenic (stimulatory) signals or a decrease in antiangiogenic (inhibitory) signals may disturb this balance and initiates angiogenesis. To stimulate angiogenesis, human pancreatic cancer cells overexpress proangiogenic molecules vascular endothelial growth factor (VEGF), interleukin 8, basic fibroblast growth factor, and platelet-derived endothelial cell growth factor (5, 6). However, pancreatic cancer cells also produce angiogenesis inhibitors, such as angiostatin, antithrombin, and thrombospondin-1 (7, 8), and thrombospondin-1 expression was increased in human pancreatic cancer extracellular stroma, especially at the invasion front of the tumor (8, 9). In the tumor microenvironment, proangiogenic factors outbalance antiangiogenic ones generating a proangiogenic response, which results in an increased blood vessel density (10, 11). In patients with pancreatic cancer, intratumoral microvessel density was an independent prognostic factor for survival on multivariate analysis (12, 13). To restore the tightly regulated angiogenesis balance in the tumor microenvironment, therapeutic approaches can be aimed at up-regulation of antiangiogenic factors or down-regulation of proangiogenic factors or both.

Thrombospondin-1 is the first naturally occurring antiangiogenic factor described and a potent tumor inhibitor (14, 15). The antitumor efficacy of thrombospondin-1 involves angiogenesis inhibition and transforming growth factor-\( \beta \) (TGF-\( \beta \)) activation (15, 16). By binding to transmembrane receptor CD36, thrombospondin-1 inhibits migration and induces apoptosis in microvessel endothelial cells and consequently inhibits angiogenesis (15–19). In thrombospondin-1 null mice, melanoma cells grew approximately twice as fast as those in wild-type mice, indicating the inhibitory effect of stromal-derived thrombospondin-1 on tumor growth (20).
When transfected to overexpress thrombospondin-1, human cancer cells (prostate cancer and cutaneous squamous cell carcinomas) grew at lower rates in nude mice and showed decreased microvessel density (21, 22). Systemic treatment with thrombospondin-1 significantly reduced tumor volume and microvessel density in human melanoma and colon cancer xenografts (23, 24). Besides its antiangiogenesis efficacy, thrombospondin-1 activates latent TGF-β and suppresses tumor cells that respond to TGF-β inhibition (16). However, thrombospondin-1 is a complex macromolecule (450 kDa) with numerous other receptors, and its use may be clinically limited because of its size, difficulty in large-scale production, and more importantly, concerns about side effects that might result from its multiple other biological functions, such as the induction of vascular smooth muscle cell chemotaxis by the type 3 repeats and COOH-terminal domains (25). The antiangiogenic domain of thrombospondin-1, 3TSR, provides a promising alternative for clinical application, because both antiangiogenic and TGF-β activation (KRFK) sequences are located within 3TSR (16). Previously, recombinant forms of human TSRs have been purified in our lab using a eukaryotic expression system and showed strong antiangiogenesis and antitumor efficacy in human melanoma and lung cancer xenografts through TGF-β-independent and -dependent mechanisms (26). To date, the effect of thrombospondin-1 and 3TSR on pancreatic cancer progression has not been reported. Interestingly, thrombospondin-1 has been shown to be the key factor in the angiogenic switch driven by Ras (27), which is mutated in 75% to 100% of pancreatic cancers (2).

In this study, we used an orthotopic pancreatic cancer model to determine the therapeutic effect of 3TSR on pancreatic cancer. An orthotopic model recapitulates many clinical features of human pancreatic cancer, and the pancreas microenvironment may be important for appropriate tumor growth factor expression and the progression of pancreatic cancer (28). We have tested the orthotopic growth of three pancreatic cancer cell lines (AsPC-1, MiaPaCa-2, and CFPAC). All of the three cell lines grow more rapidly in the pancreas than their s.c. counterparts. AsPC-1 orthotopic model was chosen for future studies because of its extremely reproducible tumor take, pattern of spread, and timing of tumor progression. Our data shows that 3TSR, the antiangiogenic domain of thrombospondin-1, reduces tumor volume and microvessel density in an orthotopic pancreatic cancer model. In vitro analysis of the effects of 3TSR on human pancreatic cancer cells was also done.

MATERIALS AND METHODS

Cell Culture. Human pancreatic cancers cell AsPC-1, BxPC-3, Su86 (American Type Culture Collection, Rockville, MD), HTB66-T, PANC-1, and MiaPaCa-2 (kindly provided by Dr. Ronald DePinho, Dana-Farber Cancer Institute, Boston, MA) were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin. Cells were grown in 5% CO2/95% air at 37°C in a humidified incubator.

Generation of Recombinant Human 3TSR. Recombinant human 3TSR was cloned and purified as previously described (26). The peptide was mixed with Polymyxin B-Agarose (Sigma Chemical Co., St Louis, MO) for 30 minutes at room temperature to remove endotoxin. The endotoxin levels were <0.05 EU/μg as determined using the QCL-1000 assay kit (Bio Whittaker, Walkersville, MD). Proteins were filter sterilized, and the protein concentration was determined.

Proliferation Assay and Flow Cytometry. AsPC-1 cells were plated into 96-well plate at the density of 5,000 cells per well. Twenty-four hours after plating, cells were changed into media containing 2% serum. After overnight incubation, cells were treated with 1 μmol/L 3TSR in media with 0.5% serum. Cells cultured in media containing 0.5% serum served as control. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay (R&D systems, Minneapolis, MN) at 24, 48, and 72 hours after treatment respectively, according to the manufacturer’s instructions. For flow cytometry, AsPC-1 cells were seeded into 6-well plate at the density of 2 × 105 cells per well and underwent treatment described above. Cells were harvested at 72 hours after treatment, washed with PBS (pH 7.4), and fixed in 70% ethanol overnight. Cells were resuspended in 0.5 mg/mL RNase in PBS, and incubated at 37°C for an hour. After 20 μL of 1 mg/mL propidium iodide was added to each sample, the cells were analyzed by flow cytometer at 488 nm.

CD36 Western Blot. Subconfluent cells were harvested using 2× cell lysis buffer [0.1 mol/L Tris, 2% SDS, 0.5 mol/L sucrose, 40 mmol/L DTT, 2 mmol/L EDTA (pH 7.5)], and protein concentration was determined with Bio-Rad detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA). Samples were separated in 8% SDS gel, and transferred onto nitrocellulose membrane; human platelet lysate served as the positive control. After blocking in 5% dry milk, the membrane was incubated in monoclonal FA6-152 mouse anti-human CD36 antibody (Cell Sciences, Inc., Canton, MA) for 2 hours at ambient temperature, followed by 2 hours incubation in sheep anti-mouse antibody. Signal detection was done using SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology, Inc., Rockford, IL).

Tumor Models. All animal work was done in the animal facility at Beth Israel Deaconess Medical Center, Boston, MA, in accordance with federal, local, and institutional guidelines. Female immunocompromised (severe combined immunodeficient) mice (Taconic, Germantown, NY) 4 to 6 weeks of age were used. The mice were acclimated, caged in groups of four or less in a barrier care facility. Pancreatic cancer cell AsPC-1 was grown in cell culture as described above. Subconfluent cells were harvested and the cell concentration was adjusted to 5 × 106 cells/mL. Mice were shaved and the abdominal skin was cleaned with betadine and ethanol. Animals were anesthetized with 2.5% Avertin (250 mg/kg). A 1-cm incision was made in the left subcostal region, and the pancreas was exposed. A suspension of 1 × 106 tumor cells was injected into the body of pancreas. Peritoneum and skin were closed with a 4.0 surgical suture, and antibiotic ointment was applied to the wound. Treatment was initiated 1 week after tumor cell implantation. Mice were randomized into 3TSR group or control group, with eight mice in each group. Mice in the 3TSR group were treated with daily i.p. injection of 3TSR at 3 mg (162 nmol) per kg per injection, whereas control mice received daily injection of an equivalent volume of PBS (pH 6.0). Treated mice were closely monitored for any signs of...
progressive disease and sacrificed if they became moribund. Mice in all groups were sacrificed and underwent necropsy 28 days after tumor cell implantation. Tumor volume was calculated as π/6 × length × width × height.

**Immunohistochemistry.** Tumor tissue was harvested, fixed in 4% paraformaldehyde at 4°C for 2 to 4 hours, and incubated in 30% sucrose at 4°C overnight. Tumor tissues were embedded in tissue freezing medium snap frozen in liquid nitrogen, and stored at −80°C. Five-micrometer-thick sections were mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX). Adjacent sections were used for CD31, CD31/terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) double fluorescence, proliferating cell nuclear antigen (PCNA), or H&E staining. For CD31 and PCNA staining, samples were washed in PBS (pH 7.4), and incubated with 3% hydrogen peroxide in methanol (v/v) for 20 minutes to block endogenous peroxidase, washed with PBS and incubated in corresponding protein blocking solution for 1 hour. For CD31 staining, the sections were incubated with the rat anti-CD31 (BD PharMingen, San Diego, CA) antibody [1:200] in a humidified chamber for 15 to 18 hours at 4°C, rinsed with PBS, followed by 60 minutes incubation with biotinylated rabbit anti-rat secondary antibody [1:200] at ambient temperature. Sections analyzed for PCNA were incubated in biotinylated anti-PCNA antibody (1:200; Zymed Laboratories, Inc., South San Francisco, CA) for 1 hour at ambient temperature. Positive reaction was visualized using VECTASTAIN Elite avidin-biotin complex peroxidase reagent and 3,3’-diaminobenzidine substrate (Vector Laboratories, Inc., Burlingame, CA). Control samples exposed to secondary antibody alone showed no specific staining.

**Immunofluorescence Double Staining for CD31 (Endothelial Cells) and Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling (Apoptotic Cells).** Frozen tissue sections were incubated with rat monoclonal anti-CD31 antibody as described above, rinsed with PBS, and incubated with goat anti-rat IgG conjugated to Texas Red (1:500; Vector Laboratories) for 60 minutes at ambient temperature in the dark. After sections were washed with PBS containing 0.1% Brij 99 (v/v), TUNEL labeling was done using a commercial kit (Promega Co., Madison, WI) according to the manufacturer’s instructions. Background reactivity was determined by processing slides in the absence of terminal deoxynucleotidyl transferase (negative control); maximum reactivity was observed by preincubating the tissue sections with DNase I to confirm the quality of the specimen.

Tissue sections were mounted with Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (Vector Laboratories) to counterstain the nuclei. Representative sections from three tumors per group were analyzed with a Nikon TE300 microscope. Images were captured with a Spot digital camera mounted to a Nikon TE300 microscope. Fields at ×20 were also captured from each group for PCNA or TUNEL analysis. All images for analysis were captured from viable tumor areas. Images of H&E-stained sections from four tumors per group (three sections per tumor) were captured using a dissection microscope for tumor necrosis analysis. IP-Lab software was used to quantify tumor microvessel number, average area, microvessel density, and tumor cell proliferation rate, apoptotic rate, and necrotic area.

**Statistics.** All tumor volumes and quantified variables were expressed as the mean ± SD. Student’s t test was used to compare tumor volumes, vessel density, proliferation, and apoptosis of treated tumors versus untreated control. Sample size and power of all the analysis were calculated with PS Power and Sample Size Program (29). All power value of above statistics are larger than 0.90. Differences were considered statistically significant when P ≤ 0.05.

**RESULTS**

**3TSR Showed No Effect on AsPC-1 Pancreatic Cancer Cell Proliferation or Apoptosis In vitro.** First, we examined the direct effect of 3TSR on AsPC-1 pancreatic cancer cells. Cells were treated for 72 hours in vitro and proliferation and apoptosis were measured. All the data were generated from two or more independent experiments. Figure 1A shows that AsPC-1 pancreatic cancer cells showed no change in the proliferation rates after 24, 48, and 72 hours of 3TSR treatment in vitro [3-(4,5-dimethylylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay]. There was no significant change in cell cycle progression or cell apoptosis after 72 hours of 3TSR exposure (Fig. 1B). The expression of CD36, a 3TSR receptor, was examined using Western blotting. A high level of CD36 expression was detected in human dermal microvessel endothelial cells, whereas none of the six pancreatic cancer cell lines expressed detectable CD36 (Fig. 2).

**3TSR Inhibited the Growth and Increased Necrosis of Human Pancreatic Cancer in the Orthotopic Mouse Model.** One week after surgical tumor cell implantation into the pancreas, mice were randomized as outlined and treatment initiated for 3 weeks with 3TSR at 3 mg per kg per day or PBS as control. By the end of treatment, all mice were sacrificed and the tumor volume was measured. All mice survived treatment. No weight loss was seen in the treated animals, whereas control mice seemed sicker with bloody ascites and piloerection. As shown in Fig. 3, control animals had an average tumor volume of 1,012.2 ± 364.5 mm³, whereas the tumor volume was significantly reduced to 316.6 ± 79.3 mm³ after 3TSR treatment (a 69% reduction, P = 0.0001). We also tested the antitumor effect of 3TSR in a MiaPaCa-2 orthotopic model with a smaller sample size (n = 4 each group) and observed an ~80% reduction in tumor volume.

Central necrosis is a common phenomenon in fast-growing tumors. In AsPC-1 orthotopic model, necrosis presented in all analyzed tumors. In the sections across the center of the control
tumors, an average of 25.9% of the tumor was occupied by necrotic area. After 3TSR treatment, the average tumor necrotic area was significantly increased to 51.0% (P < 0.0001, Fig. 4).

3TSR Decreased Tumor Microvessel Density and Induced Blood Vessel Endothelial Cell Apoptosis In vivo.
We further analyzed the change in tumor microvessel density after 3TSR treatment. 3TSR significantly decreased both the number and the average size of tumor blood vessel. The average microvessel number in each 0.584 mm² field was 29 in 3TSR-treated tumors, compared with 49 per field in control tumors (P < 0.001). In addition, the average blood vessel size was significantly reduced from 537.1 to 378.3 mm² after 3TSR treatment (P < 0.001). Thus, microvessel density, defined as the percentage of total microvessel area in a given tumor field, was significantly decreased after 3TSR treatment (8.0% versus 3.7%, P < 0.0001; Figs. 5 and 6).

CD31/TUNEL immunofluorescence double staining revealed that 3TSR induced apoptosis of tumoral endothelial cell in vivo (Fig. 5). The basal level of apoptotic endothelial cell in control tumors was 4.2%, whereas 14.7% of tumoral endothelial cells were undergoing apoptosis after 3TSR treatment (P < 0.0001; Fig. 7).

VEGF in situ hybridization was done as described (30) to evaluate any gross change in VEGF expression in the pancreatic tumors after 3TSR treatment. Visual analysis of VEGF mRNA levels by an experienced pathologist (Dr. Lawrence F. Brown, Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA) indicated that there was no significant difference between tumors from control and 3TSR-treated mice.

3TSR Did Not Affect Pancreatic Cancer Cell Proliferation and Apoptosis In vivo. The direct effects of 3TSR on pancreatic cancer cell proliferation and apoptosis in vivo were evaluated via quantification of PCNA and TUNEL staining. Unlike the effect of 3TSR on microvessel endothelial cells, the proliferation and apoptosis levels of pancreatic cancer cells were not affected by 3TSR treatment. As shown in Fig. 8, the proliferation rate was 20.9% in control group and 20.5% in 3TSR-treated group (P = 0.8). The in vivo apoptotic rate of pancreatic cancer cells was 4.4% in control tumors, and 4.8% after 3 weeks of 3TSR treatment (P = 0.7).

DISCUSSION
Restoring the balance of angiogenesis stimulators and inhibitors in the tumor microenvironment is one strategy for the treatment of human malignancy. In an AsPC-1 orthotopic mouse model of human pancreatic cancer, we have shown that 3TSR, the antiangiogenic domain of the endogenous angiogenesis inhibitor thrombospondin-1, significantly reduced tumor volume. Three weeks of systemic 3TSR treatment caused a 69% reduction in tumor volume compared with PBS-treated control, and the tumor inhibitory effect of 3TSR was confirmed using another pancreatic cancer cell line (MiaPaCa-2) in this orthotopic model.
We further quantified the necrotic areas in AsPC-1 tumors, and large areas of necrosis were seen in 3TSR-treated tumors compared with control tumors. Extensive areas of necrosis were also reported in thrombospondin-1 overexpressing human cutaneous squamous cell carcinomas and prostate cancer xenografts (21, 22). In 3TSR-treated tumors, the increased tumor necrosis probably resulted from tumor ischemia, a consequence of decreased microvessel density after 3TSR treatment.

The antiangiogenesis efficacy of 3TSR was further shown via quantifying tumor microvessel in viable tumor areas. 3TSR significantly reduced both the tumor microvessel number and average size. Thus, the microvessel density, defined as the percentage of total microvessel area in a given tumor field, was significantly decreased after 3TSR treatment. Thrombospondin-1 also decreased the number and average size of blood vessel in human cutaneous squamous cell carcinomas and prostate cancer xenografts (21, 22), whereas neutralizing anti-VEGF antibody treatment in an orthotopic pancreatic model decreased vascular density as well as tumor volume, but the tumor blood vessel size remained unchanged (31).

Inhibition of angiogenesis by thrombospondin-1 involves the inhibition of endothelial cell migration and induction of apoptosis, and these effects are reportedly mediated by interaction of the TSRs with CD36 receptor on endothelial cell membrane (16). Thrombospondin-1 binds CD36 receptor and sequentially...

Fig. 4 3TSR increased the tumor necrotic area after 3 weeks of treatment. A, H&E staining of a cross-section through the center of representative tumors. Black line, tumor necrotic area. B, quantification of tumor necrotic area as the percentage of whole area of the tumor section.

Fig. 5 Immunohistochemical analyses. Tumors were harvested from control or 3TSR-treated mice after a 3-week treatment. The sections were immunostained for CD31 expression (top) to localize the blood vessels. Tumors from mice treated with 3TSR had a decrease in microvessel count, microvessel area, and microvessel density (MVD). Immunofluorescent double-labeling with CD31 (endothelial cells, Texas red) and TUNEL (apoptosis, FITC-green) was performed to identify tumor associated apoptotic endothelial cells. Apoptotic endothelial cells were identified as overlap of fluorescent red and fluorescent green signals, as indicated with the white arrows (middle). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue) to quantify % apoptotic endothelial cells (bottom).
activates p59 fyn, caspase-3, and p38 mitogen-activated protein kinase, then causing microvessel endothelial cell apoptosis (19). Several TSR-derived peptides inhibit endothelial cell migration in vitro and angiogenesis in vivo (cornea pocket assay and chorioallantoic membrane assay; refs. 16, 18, 32). However, TSR-derived peptides without CD36 binding sequence also have antiangiogenic function suggesting that multiple sites that inhibit angiogenesis through distinct mechanisms are clustered in 3TSR (16), and the three-dimensional crystal structure of the 3TSR may be crucial for optimal biological activity (33).

Previously, we showed that 3TSR inhibits endothelial cell migration at similar levels as thrombospondin-1 based on the same molar concentrations (26). 3TSR also induced microvessel endothelial cell apoptosis in vitro in a CD36-dependent manner, and both the intrinsic and extrinsic apoptotic pathways were involved to activate executioner caspase 3 in 3TSR-induced apoptosis. Moreover, 3TSR decreased endogenous and VEGF-induced c-Akt activity and induced endothelial cell apoptosis in the presence of VEGF. In our study, the apoptotic rate of tumoral vascular endothelial cells was increased by 3.5-fold after 3TSR treatment. The increase in intratumoral endothelial cell apoptosis and decrease in tumor microvessel density after 3TSR treatment suggest that exogenous 3TSR attenuated the proangiogenic microenvironment of the pancreatic tumor and induced apoptosis of vascular endothelial cells, thereby significantly inhibiting tumor angiogenesis. Theoretically, the inhibition of endothelial cell proliferation and migration may also be involved in 3TSR-induced antiangiogenesis, but they were not studied here.

In our study, 3TSR showed no direct effects on pancreatic cells both in vitro and in vivo. Although thrombospondin-1-induced apoptosis in leukemia cells via a thrombospondin-1-CD36 interaction (34), this well-established endothelial cell apoptotic pathway does not exist in most solid tumors. In our study, none of the six pancreatic cancer cell lines expressed any detectable CD36. TGF-β is another downstream factor mediating the function of TSR/thrombospondin-1. In human pancreatic cancer, TGF-β, TGF-β receptors, and its intracellular effectors, the SMAD proteins, often mutate or change expression levels (35), and AsPC-1 cells do not express either TGF-β type II receptor or SMAD4 (36). Thus, it is conceivable that 3TSR cannot exert any direct effect on pancreatic cancer cells due to a lack of effector molecules.

Among all the antiangiogenic treatments for pancreatic cancer, anti-VEGF strategy is one of the best studied (31, 37–39), and resulted in an average 50% to 70% tumor volume reduction in orthotopic models (31, 37, 38). A recombinant anti-VEGF antibody, bevacizumab, and VEGF receptor tyrosine kinase inhibitor SU5416 have entered phase II clinical trials for colorectal cancer and renal cancer (40–42). Thrombosis, hypertension, and proteinuria were potential safety concerns of anti-VEGF antibody, especially at higher dosage (40, 41), although better therapeutic
feasible agent for clinical treatment of pancreatic cancer. A thrombospondin-1 orthotopic mouse model of human pancreatic cancer by angiogenesis inhibitor, showed potent therapeutic effect in an further investigated.

pathways (19), therapeutic contributions of these agents may be thrombospondin-1 exert antiangiogenic activity via different pancreatic cancer models (45, 46). Because endostatin and angiogenesis inhibitor, which showed therapeutic effects in s.c. fibrosarcoma model (44). Endostatin is another endogenous VEGF small interfering RNA had been reported using a mouse therapy. Actually, a synergy between thrombospondin-1 and anti-thrombospondin-1/3TSR for pancreatic cancer antiangiogenic combine low dose anti-VEGF antibody with another agent like vascular endothelial cell homeostasis, it may be practical to Because physiologic levels of VEGF are crucial for maintaining endothelial cell detachment and hypertrophy and proteinuria (43). 

antibody and soluble VEGF receptor caused rapid glomerular efficacy was observed when higher dose of anti-VEGF antibody was used (41). Further animal studies showed that anti-VEGF antibody and soluble VEGF receptor caused rapid glomerular endothelial cell detachment and hypertrophy and proteinuria (43). Because physiologic levels of VEGF are crucial for maintaining vascular endothelial cell homeostasis, it may be practical to combine low dose anti-VEGF antibody with another agent like thrombospondin-1/3TSR for pancreatic cancer antiangiogenic therapy. Actually, a synergy between thrombospondin-1 and anti-VEGF small interfering RNA had been reported using a mouse fibrosarcoma model (44). Endostatin is another endogenous angiogenesis inhibitor, which showed therapeutic effects in s.c. pancreatic cancer models (45, 46). Because endostatin and thrombospondin-1 exert antiangiogenic activity via different pathways (19), therapeutic contributions of these agents may be further investigated.

In summary, 3TSR, a domain of a natural occurring angiogenesis inhibitor, showed potent therapeutic effect in an orthotopic mouse model of human pancreatic cancer by inhibiting tumor angiogenesis, and may prove to be an effective, feasible agent for clinical treatment of pancreatic cancer. However, genetic changes in tumor cells may enable them to overcome the effect of antiangiogenic reagents like thrombospondin-1 (47). This turns out to be a clinical concern especially when long-term antiangiogenic treatment is involved. Thus, more effective therapeutic efficacy may be achieved when 3TSR is combined with other therapeutic approaches targeting pancreatic cancer cells, such as gemcitabine or radiotherapy.

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