Antiangiogenic Treatment with Three Thrombospondin-1 Type 1 Repeats versus Gemcitabine in an Orthotopic Human Pancreatic Cancer Model

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Abstract

**Purpose:** In this study, we investigated the antitumor efficacy of thrombospondin-1 three type 1 repeats (3TSR), the antiangiogenic domain of thrombospondin-1, in comparison and in combination with gemcitabine, in an orthotopic pancreatic cancer model.

**Experimental Design:** Human pancreatic cancer cells were injected into the pancreas of severe combined immunodeficient mice. The animals were treated with 3TSR, gemcitabine, 3TSR plus gemcitabine, or vehicle for 3 weeks. Subsequently, the effects of 3TSR and/or gemcitabine on tumor growth, tumor necrosis, microvessel density, cancer cell proliferation, apoptosis, and endothelial cell apoptosis were analyzed.

**Results:** After 3 weeks of treatment, 3TSR reduced tumor volume by 65%, and gemcitabine by 84%. Tumor volume was not statistically different between gemcitabine group and combinatorial treatment group. Extensive necrotic areas were observed in tumors from 3TSR-treated mice, whereas tumors from gemcitabine and combinatorially treated mice were less necrotic than control tumors. 3TSR reduced tumor microvessel density and increased tumor blood vessel endothelial cell apoptosis. In contrast, gemcitabine induced apoptosis and inhibited proliferation of cancer cells.

**Conclusion:** 3TSR, the antiangiogenic domain of thrombospondin-1, showed comparable antitumor efficacy to gemcitabine in a human pancreatic cancer orthotopic mouse model. No synergistic effect was found when the two drugs were combined and possible reasons are discussed in detail. A delicate balance between normalization and excessive regression of tumor vasculature is important when initiating alternative combinatorial regimens for treatment of patients with pancreatic cancer.

Pancreatic cancer is an unsolved health problem. The 5-year survival rate is only 3% and median overall survival is <6 months, a situation that has remained unchanged for the past three decades (1). Surgical resection is the only potentially curative therapy for pancreatic cancer, with a 5-year survival of 20% at best (2). The majority of patients are potential candidates for systemic treatment because the tumor is already unresectable at diagnosis due to metastasis or the presence of locally advanced disease (1). Since 1997, gemcitabine has been widely accepted as first line treatment for patients with advanced pancreatic cancer (3). In later phase III clinical studies with gemcitabine, the median overall survival is still merely 164 to 195 days, and patients mainly benefit in terms of disease stabilization and clinical benefit index (based on pain reduction, performance status improvement, and weight gain; refs. 4, 5). Although phase II studies suggest that the combinations of gemcitabine and other therapeutic agents may have greater activity against pancreatic cancer, randomized controlled phase III trials are currently in progress. Published phase III studies indicate that the combinations of gemcitabine with 5-fluorouracil, marimastat (matrix metalloproteinase inhibitor), or tipifarnib (farnesyltransferase inhibitor) did not prolong overall survival compared with gemcitabine alone in patients with pancreatic cancer (4–6).

Given the dearth of drugs available for the treatment of pancreatic cancer, antiangiogenesis strategy might represent a novel approach. Angiogenesis, the formation of new blood vessels, is crucial for tumor growth and metastasis, and the angiogenic phenotype of a tumor is determined by the net balance between positive and negative angiogenic regulators in the tumor microenvironment (7, 8). Human pancreatic cancer cells overexpress proangiogenic molecules vascular endothelial cell growth factor (VEGF), interleukin-8, basic fibroblast growth factor, and platelet-derived endothelial cell growth factor (9, 10), whereas angiogenesis inhibitors, such as angiostatin, antithrombin, and thrombospondin-1, are also produced by pancreatic cancer cells (11, 12). The predominance of proangiogenic factors in the tumor microenvironment...
Thrombospondin-1 is the first naturally occurring antiangiogenic factor described and is a potent tumor inhibitor (15, 16). The antitumor efficacy of thrombospondin-1 involves angiogenesis inhibition and transforming growth factor β (TGF-β) activation (16, 17). 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 (18). In tumor cells harboring ras mutation, such as most pancreatic cancer cells, thrombospondin-1 is one of the key factors in the angiogenic switch driven by ras (19). For therapeutic purpose, the antiangiogenesis and antitumor efficacy of thrombospondin-1 has been shown by systemic treatment, adenovirus-mediated gene therapy, or using tumor cells genetically modified to overexpress thrombospondin-1 in mouse models (including human prostate cancer, colon cancer, melanoma, and cutaneous squamous cell carcinoma; refs. 20–23). However, thrombospondin-1 is a complex molecule with multiple biological functions, so the clinical use of the intact molecule is limited. The antiangiogenic domain of thrombospondin-1, three thrombospondin-1 type 1 repeats (3TSR), might prove to be a promising alternative for clinical administration as an antiangiogenic agent. Previously, recombinant forms of human TSRs have been purified in our lab and showed strong antiangiogenesis and antitumor efficacy in human melanoma and lung cancer xenografts through TGF-β−independent and TGF-β−dependent mechanisms, based on the response of tumor cells to TGF-β (24). In a smaller study, we had shown the antitumor and antiangiogenesis efficacy of 3TSR in an orthotopic human pancreatic cancer model (25).

Antiangiogenic strategies will have to be studied carefully both in vitro and in vivo before testing in human. Comparisons of these novel strategies with current chemotherapy regimens are of utmost importance for developing therapeutic approaches. Moreover, testing combinatorial strategies in animal models will show synergies and perhaps also show any pitfalls that may present themselves when drugs with different mechanisms of action are combined. Our study compared gemicitabine with a novel antiangiogenic agent, 3TSR, the antiangiogenic component of human thrombospondin-1. In this study, the therapeutic efficacy of 3TSR is comparable to that of gemicitabine, despite its distinct mechanism of action. The combination of 3TSR and gemicitabine, however, did not result in an additive effect, which allows us to comment with some deeper insight into the combination of antiangiogenic treatment and chemotherapy in pancreatic cancer management.

**Cell culture.** Human pancreatic cancer cell line AsPC-1 (American Type Culture Collection, Rockville, MD) 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 three thrombospondin-1 type 1 repeats.** Recombinant human 3TSR containing all the three type 1 repeats of human thrombospondin-1 was cloned and purified as previously described (24). The amino acid sequence of 3TSR is shown in Fig. 1. 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 endotoxin units/μg as determined using the QCL-1000 assay kit (BioWhittaker, Walkersville, MD). Proteins were filter sterilized and the protein concentration was determined.

**Proliferation assay and flow cytometry.** AsPC-1 cells were plated on 96-well plates at a density of 5 × 10³ cells/well. Twenty-four hours after plating, cells were changed into media containing 2% serum for overnight incubation, then cells were treated with media with 0.5% serum containing 1 μmol/L 3TSR, 1 μmol/L gemicitabine, or the combination. 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 instructions of the manufacturer. For flow cytometry, AsPC-1 cells were seeded onto six-well plates at a density of 2 × 10⁵ cells/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 were added to each sample, the cells were analyzed by flow cytometry at 488 nm and data were analyzed with ModFit LT software (Verity Software House, Inc., Topsham, ME).

**Gemicitabine sensitivity under hypoxia.** AsPC-1 cells were seeded onto 96-well or six-well plates as described above. After 24 hours, cells were treated with gemicitabine, and control cells were cultured in full media. On the start of gemicitabine treatment, cells were cultured under normal condition or in a hypoxic incubator (Heraeus Instruments, Inc., Tewksbury, MA) with 3% oxygen. Cell proliferation assay was carried out as described above. Cells treated with gemicitabine under normoxia or hypoxia were also analyzed with flow cytometry after treatment for 72 hours.

**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 as previously described (25). Female severe combined immunodeficient mice (Taconic, Germantown, NY), 4 to 6 weeks of age, were used. A suspension of 1 × 10⁶ pancreatic cancer cells was surgically implanted into the body of pancreas. Treatment was initiated 1 week after tumor cell implantation. Forty mice were separated into four groups: 3TSR (n = 10), 3 mg
Differences were considered statistically significant when program (26). All power values of above statistics are larger than 0.90. The power of all the analysis were calculated with PS Power and Sample Size volumes, vessel density, proliferation, and apoptosis. Sample size and total number of endothelial cells expressed as an average of the ratio of apoptotic endothelial cells to the endothelial cells. Apoptotic endothelial cells were quantified and localization of 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA) to counterstain the nuclei.

**Immunohistochemistry and immunofluorescence double staining.** Immunohistochemical staining was done as described (25). Five-micrometer-thick sections were used for CD31 (BD Pharmingen, San Diego, CA), CD31/terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling (TUNEL) double fluorescence, proliferating cell nuclear antigen (PCNA; Zymed Laboratories Inc., South San Francisco, CA), or H&E staining. For CD31 and TUNEL double immunofluorescence, TUNEL (Promega Corp., Madison, WI) labeling was done after CD31 staining, and tissue sections were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA) to counterstain the nuclei.

**Quantification of microvessel density, proliferating cell nuclear antigen, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, tumor necrotic area, and apoptotic endothelial cells.** For the quantification of microvessel density, 80 to 100 “hotspot” fields of CD31 staining at $20 \times (0.584 \times 0.438 \text{ mm}^2)$ from four to five tumors each group were captured using a Spot digital camera mounted to a Nikon TE300 microscope. Fields at $20 \times$ were also captured from each group for PCNA or TUNEL analysis (40 fields each). 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. Images were analyzed with IP-Lab software (Scanalitics, Inc., Fairfax, VA) to quantify tumor microvessel number, average area, and microvessel density, as well as tumor cell proliferation, apoptosis, and tumor necrosis. For PCNA and TUNEL quantification, tumor cell proliferation/apoptotic index was expressed as the number of PCNA/TUNEL-positive cells divided by the total cell number per field. Percentage tumor necrosis was determined by dividing total area of a tumor section with necrotic area. To quantify apoptotic endothelial cells, endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nuclei of apoptotic cells. Total number of endothelial cells was determined by localization of 4',6-diamidino-2-phenylindole – stained nuclei within endothelial cells. Apoptotic endothelial cells were quantified and expressed as an average of the ratio of apoptotic endothelial cells to the total number of endothelial cells.

**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. Sample size and power of all the analysis were calculated with PS Power and Sample Size Program (26). All power values of above statistics are larger than 0.90. Differences were considered statistically significant when P ≤ 0.05.

**Results.** The in vitro effects of three thrombospondin-1 type 1 repeats and gemcitabine on AsPC-1 pancreatic cancer cells. To characterize the direct effects of 3TSR and gemcitabine on AsPC-1 pancreatic cancer cells, we treated cells in vitro and analyzed cell proliferation and apoptosis. Figure 2A shows that 3TSR did not affect AsPC-1 cell proliferation after 24, 48, or 72 hours of exposure compared with control. In contrast, gemcitabine inhibited AsPC-1 cell proliferation by 24% after 48 hours of treatment, and by 42% after 72 hours. Combination of 3TSR and gemcitabine had no additive effects compared with gemcitabine alone. Flow cytometry analysis showed same pattern as the proliferation assay (Fig. 2B). There was no significant difference between 3TSR-treated pancreatic cancer cells and control cells in cell cycle progression or apoptosis. Seventy-two hours of gemcitabine treatment significantly blocked cell cycle progression and induced AsPC-1 cell apoptosis. Treatment with a combination of 3TSR and gemcitabine resulted in the same level of apoptosis as gemcitabine alone. We also repeated the above experiments using another pancreatic cancer cell line, MiaPaCa-2. These cells were more sensitive to gemcitabine (27). 3TSR did not affect cell proliferation or apoptosis either, and there was no significant difference between cells treated with 3TSR plus...
gemcitabine and with gemcitabine alone (data not shown). Thus, 3TSR treatment showed no direct effects on proliferation or apoptosis of pancreatic cells in vitro and had no synergy when combined with gemcitabine.

To partially elucidate why pancreatic cancer cells failed to respond to 3TSR, we measured levels of CD36, a 3TSR receptor, in six pancreatic cancer cell lines (AsPC-1, MiaPaCa-2, BxPC-3, HS766-T, PANC-1, and SU86). None of the cell lines expressed detectable CD36 on Western blot (25).

Three thrombospondin-1 type 1 repeats and gemcitabine showed no synergy in tumor growth inhibition in an orthotopic human pancreatic cancer mouse model. The therapeutic effects of 3TSR and gemcitabine were tested in the AsPC-1 orthotopic pancreatic cancer model. The AsPC-1 orthotopic model was chosen because of its reproducible tumor take, pattern of spread, and timing of tumor progression. One week after AsPC-1 cells were surgically implanted into the pancreas, mice were treated for 3 weeks with 3TSR (3 mg/kg/d), gemcitabine (150 mg/kg, twice a week), or the combination. Control mice had an average tumor volume of 1,065 mm$^3$, which is consistent and repeatable in this orthotopic model. Three weeks of 3TSR treatment reduced the tumor volume to 372 mm$^3$ (65.1% tumor volume reduction, $P < 0.001$; Fig. 3A). The smallest average tumor volume was observed in gemcitabine treatment group. Mice treated with 3TSR plus gemcitabine bore an average tumor volume of 219 mm$^3$ (79.4% reduction), which was significantly smaller than that in 3TSR-treated or control group ($P < 0.01$), and was not statistically different from that of gemcitabine-treated mice (167 mm$^3$, 84.3% reduction, $P = 0.18$). Liver metastasis was sporadic in this model and was not systematically analyzed.

To exclude the possibility that the lack of synergy between 3TSR and gemcitabine is an artificial or cell line–specific phenomenon in the AsPC-1 orthotopic model, we tested 3TSR and/or gemcitabine treatment in a MiaPaCa-2 orthotopic pancreatic cancer model. Similar to the results in the AsPC-1 model, both 3TSR and gemcitabine significantly inhibited tumor growth (3TSR, 83.7% reduction in tumor volume; gemcitabine, 87.3%), and 3TSR plus gemcitabine (87.4% reduction in tumor volume) resulted in a comparable tumor inhibition compared with 3TSR or gemcitabine alone.

Three thrombospondin-1 type 1 repeats increased whereas gemcitabine decreased the tumor necrotic areas. To evaluate the changes in tumor necrosis after 3TSR and/or gemcitabine treatment, we quantified tumor necrotic areas in sections across the center of the tumors. As a fast-growing tumor, orthotopically grown AsPC-1 tumors had an average of 34% central necrotic areas (Fig. 3B and C). 3TSR treatment significantly increased the tumor necrotic area to 54% ($P < 0.0001$ versus control). In contrast, average necrotic area in gemcitabine-treated tumor was 6% ($P < 0.0001$ versus control). Some gemcitabine-treated tumors were completely viable and showed no necrosis at all. Tumors from mice treated with 3TSR plus gemcitabine showed larger necrotic areas than tumors from gemcitabine-treated mice, but the difference was not statistically significant (10% versus 6%, $P = 0.18$). In this case, the combination of 3TSR and gemcitabine worked more like gemcitabine alone by decreasing tumor necrosis compared with control.

Tumor microvessel density and in vivo endothelial cell apoptosis after three thrombospondin-1 type 1 repeats and/or gemcitabine treatment. CD31-stained tumor sections were analyzed to quantify tumor blood vessel density. Table 1 and Fig. 4A show that 3TSR treatment significantly decreased both the number and average size of microvessel. Microvessel density, defined as the percentage of microvessel area in a given tumor field, was...
the microvessel density of 3TSR-treated tumors (3.1%, gemcitabine-treated tumors, and also statistically higher than 3.8%, which was significantly lower than that in control or with 3TSR plus gemcitabine, the microvessel density was (19.2%, did not significantly change tumor cell proliferation index of 7.1% (Fig. 4B) and cell nuclei were counterstained with 4',6-diamidino-2-phenylindole, so that the percentage of apoptotic endothelial cells was calculated in areas of necrotic tumor. A basal microvessel endothelial cell apoptotic index of 3.7% was observed in pancreatic tumors from control mice. 3TSR or 3TSR combined with gemcitabine significantly increased the endothelial cell apoptotic index (3TSR, 13.4%; 3TSR plus gemcitabine, 12.3%; P < 0.0001 versus control). Compared with 3TSR, gemcitabine alone had less proapoptotic efficacy in tumor microvessel endothelial cells and resulted in an endothelial cell apoptotic index of 7.1% (Fig. 4B).

Pancreatic cancer cell proliferation and apoptosis after three thrombospondin-1 type 1 repeats and/or gemcitabine treatment. Pancreatic cancer cell proliferation was quantified as the number of PCNA-positive cells versus total cells (per microscopic field). In tumors from control mice, tumor cell proliferation index was 19.3% (Fig. 4A and C). 3TSR treatment did not significantly change tumor cell proliferation index (19.2%, P > 0.9), whereas gemcitabine significantly reduced tumor cell proliferation index to 9.1% (P < 0.0001). 3TSR plus gemcitabine was less efficacious than gemcitabine alone, resulting in a pancreatic cancer proliferation index of 14.2% (P < 0.0001 versus gemcitabine). The tumor cell apoptotic index was measured as the percentage of TUNEL-positive cells versus total number of tumor cells (per microscopic field). 3TSR treatment did not induce tumor cell apoptosis compared with the control, whereas gemcitabine or 3TSR plus gemcitabine treatment significantly increased pancreatic cancer cell apoptosis, and the apoptotic index was comparable between gemcitabine and combinatorial groups (Fig. 4A and D).

Hypoxia decreased sensitivity of AsPC-1 human pancreatic cancer cells to gemcitabine-induced apoptosis and proliferation inhibition in vitro. Because 3TSR did not affect gemcitabine-induced apoptosis or inhibition of proliferation in pancreatic cancer cells in vitro, we tried to explain why the combination of 3TSR and gemcitabine was less effective than gemcitabine alone in inhibiting tumor cell proliferation in vivo. One possibility was that 3TSR treatment increased tumor hypoxia as a result of antiangiogenesis, and in hypoxic environments, tumor cells might become resistant to the antiproliferative effect of gemcitabine. To test this hypothesis in vitro, AsPC-1 cells were treated with gemcitabine under normoxic or hypoxic condition, and cell proliferation inhibition and apoptosis were compared. As described above, gemcitabine significantly inhibited AsPC-1 cell proliferation in a dose-dependent manner. Hypoxia did not significantly affect cell proliferation or cell cycle progress, whereas AsPC-1 cells were less sensitive to gemcitabine when cultured under hypoxia (Fig. 5). After 48 and 72 hours of gemcitabine treatment (from 0.1 to 10 μmol/L), AsPC-1 cell proliferation indices were about 20% higher when cells were cultured under hypoxia compared with cells treated under normal condition (Fig. 5A). Figure 5B also shows that under hypoxic conditions, AsPC-1 cells were less sensitive to gemcitabine-induced apoptosis.

Table 1. The effects of 3TSR and/or gemcitabine on tumor vasculature in the AsPC-1 orthotopic human pancreatic cancer model

<table>
<thead>
<tr>
<th>Count/area</th>
<th>Average area (μm²)</th>
<th>Microvessel density (% of ROI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.1 ± 32.8</td>
<td>483.7 ± 248.8</td>
</tr>
<tr>
<td>3TSR</td>
<td>28.8 ± 11.1*</td>
<td>298.3 ± 130.3</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>44.8 ± 21.1</td>
<td>378.7 ± 190.9</td>
</tr>
<tr>
<td>3TSR + gemcitabine</td>
<td>38.9 ± 11.81</td>
<td>278.7 ± 145.01</td>
</tr>
</tbody>
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NOTE: Eighty to one-hundred hotspot fields of CD31 staining at ×20 (0.584 × 0.438 mm²) from four to five tumors each group were captured, and microvessel number, average size, and microvessel density were quantified. Microvessel density was expressed as the percentage of total microvascular area in a 0.584 × 0.438 mm² field (ROI, region of interest).

*P < 0.01 compared with all other groups.
*P < 0.05 compared with control group.
*P < 0.01 compared with gemcitabine group.

*P < 0.0001 compared with control or 3TSR. In tumors treated with 3TSR plus gemcitabine, the microvessel density was 3.8%, which was significantly lower than that in control or gemcitabine-treated tumors, and also statistically higher than the microvessel density of 3TSR-treated tumors (3.1%, P < 0.01).

As shown in Fig. 4A, apoptotic endothelial cells were identified via CD31/TUNEL double labeling, and cell nuclei were counterstained with 4',6-diamidino-2-phenylindole, so that the percentage of apoptotic endothelial cells was calculated in areas of necrotic tumor. A basal microvessel endothelial cell apoptotic index of 3.7% was observed in pancreatic tumors from control mice. 3TSR or 3TSR combined with gemcitabine significantly increased the endothelial cell apoptotic index (3TSR, 13.4%; 3TSR plus gemcitabine, 12.3%; P < 0.0001 versus control). Compared with 3TSR, gemcitabine alone had less proapoptotic efficacy in tumor microvessel endothelial cells and resulted in an endothelial cell apoptotic index of 7.1% (Fig. 4B).

Discussion

We compared 3TSR, an antiangiogenic domain of thrombospondin-1, with gemcitabine in treatment of human pancreatic cancer using an orthotopic mouse model. 3TSR treatment reduced tumor volume by 65.1% compared with control, whereas gemcitabine caused an 84.3% tumor reduction. Although the average tumor volume in gemcitabine-treated mice was statistically smaller than that in 3TSR-treated group, the viable tumor burden was comparable between the two groups because tumors from 3TSR-treated mice were characterized by extensive areas of necrosis, which has also been observed in other tumor models treated with thrombospondin-1 (20, 21), and might be a consequence of tumor ischemia due to inhibition of tumor angiogenesis. In contrast, gemcitabine treatment significantly inhibited tumor cell proliferation but had little effect on tumor blood supply. As a result of
relatively decreased demand for blood supply, tumors from gemcitabine-treated mice had significantly less necrotic areas than those of control.

This study confirms the different mechanistic actions of 3TSR and gemcitabine. In this pancreatic cancer model, 3TSR mainly worked as an antiangiogenic agent and showed no direct effects on pancreatic cancer cell proliferation or apoptosis either in vitro or in vivo. 3TSR inhibited tumor growth by decreasing tumor microvessel density and induced tumor-associated endothelial cell apoptosis. In contrast, gemcitabine mainly targeted proliferating tumor cells, resulting in decreased proliferation and increased apoptosis of the tumor cells in vivo. Although a moderate decrease in microvessel density and a moderate increase in endothelial cell apoptosis were observed in tumors from gemcitabine-treated mice, the anti-tumor efficacy of gemcitabine mainly resulted from inhibition of proliferation and induction of apoptosis of pancreatic cancer cells.

Inhibition of angiogenesis by thrombospondin-1 is reportedly mediated by the interaction of TSRs with CD36 receptor
on the endothelial cell membrane (28). Thrombospondin-1 binds CD36 receptor and sequentially activates p59 fyn, caspase-3, and p38 mitogen-activated protein kinase, then causing microvessel endothelial cell apoptosis (29). Thrombospondin-1 also induces endothelial cell apoptosis via the CD95 death receptor pathway (30). Previously, we showed that 3TSR inhibited endothelial cell migration at similar levels as thrombospondin-1 based on the same molar concentrations (24). 3TSR also induced microvessel endothelial cell apoptosis in vitro in a CD36-dependent manner, and both the intrinsic and extrinsic apoptotic pathways 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. Besides the intact TSR proteins, several TSR-derived peptides (with or without mutation, as shown in Fig. 1) have been reported to have antiangiogenic functions. One TSR mimetic peptide, ABT-510 (derived from GVITRIR with two mutations; see Fig. 1 legend), has entered clinical trials for solid tumors (31). It is possible that multiple sites that inhibit angiogenesis through distinct mechanisms are clustered in 3TSR (28). Actually, the three-dimensional crystal structure showed that the TSR polypeptide folds so that two of the active sequences, WSHWSWP and GVITRIR, contribute side chains to a single positively charged groove (32), which indicates that different reportedly active sequences of 3TSR may constitute the same receptor binding site, and the three-dimensional structure of the 3TSR may be crucial for optimal biological activity (32). One example may be the lower dose requirement of 3TSR (3 mg/kg/d) compared with ABT-510 (50 mg/kg, twice a day) to achieve similar therapeutic efficacy in mouse models (31), despite the higher molecular weight of 3TSR. Like ABT-510, 3TSR is also biologically active at least for 1 week in vivo (in mini-osmotic pumps; data not shown), however, the pharmacokinetic data of 3TSR are not yet available.

We hypothesized that the combination of gemcitabine and 3TSR would result in a synergistic or additive tumor inhibitory effect in this pancreatic cancer model because the two agents target two different tumor compartments (tumor cells and tumor blood vessels, respectively). Gemcitabine has been previously shown to enhance the therapeutic efficacy of anti-VEGF treatment in both orthotopic and s.c. human pancreatic cancer models (33, 34). In addition, the combination of thrombospondin-1 and irinotecan showed stronger antitumor efficacy in a s.c. colon cancer model (23). In our study, however, the average tumor volume showed no statistical difference between gemcitabine-treated mice and those treated with 3TSR plus gemcitabine. This is not an artificial or cell line–specific phenomenon because the same results were also obtained when we tested the combination of 3TSR and gemcitabine using another pancreatic cancer cell line, MiaPaCa-2, which is more sensitive to gemcitabine in vitro. Our in vitro data indicated that 3TSR did not affect the antiproliferative or proapoptotic effect of gemcitabine on pancreatic cancer cells, so the potential antagonism between 3TSR and gemcitabine in vivo might result from the effects of 3TSR on the tumor vasculature or stromal reactions activated by 3TSR.

In two areas, 3TSR and gemcitabine seemed to function in a somewhat antagonistic fashion: tumor necrosis and tumor cell proliferation. This lack of synergy between gemcitabine and 3TSR is an important finding in our study and is clinically relevant because lack of synergy was also observed in recent phase III clinical trials combining gemcitabine and novel molecularly targeted agents in multiple human malignancies (4, 5, 35). A number of possible explanations for this lack of synergy can be proposed. When the combination of 3TSR and gemcitabine was used in this model, (a) 3TSR might have reduced tumor blood supply to a critical level thus hindering the delivery of gemcitabine to the tumor cells. Reduced delivery of gemcitabine then would have resulted in a higher proliferative index in the combination treatment group than in the gemcitabine group. A tumor vascular normalization phase has been observed during anti-VEGF treatment (36),
however, with our experimental design it is difficult to ascertain whether a tumor vascular normalization phase occurred during 3TSR treatment. (b) Gemcitabine treatment may have inhibited tumor cell proliferation to a point that demand for increased blood supply was no longer relevant, thus decreasing the therapeutic efficacy of 3TSR. This notion would be supported by the presence of decreased necrosis in tumors from gemcitabine and combination treatment groups compared with those from the control group. (c) 3TSR induced ischemia and a hypoxic tumor microenvironment, thus causing tumor cells to become resistant to gemcitabine. Our in vitro data suggested that hypoxia reduced both the proliferation inhibition and apoptosis induction effects of gemcitabine. Hypoxia also increased the resistance to gemcitabine-induced apoptosis in another pancreatic cancer cell line through phosphatidylinositol 3-kinase/Akt/nuclear factor κB pathways (37). (d) 3TSR may activate stromal/epithelial interactions that protect tumor cells from chemotherapy. For example, 3TSR activates TGF-β in the tumor microenvironment (24, 38). Although AsPC-1 cells and MiaPaCa-2 cells do not directly respond to TGF-β because of mutated SMAD4 (MiaPaCa-2) or TGF-β receptors (AsPC-1; refs. 39, 40), TGF-β increased desmoplasia and epithelial-mesenchymal transdifferentiation in pancreatic tumors (41, 42). It has been shown that pancreatic cancer cells acquired the more resistance when cocultured with fibroblasts or grown on collagen-coated plates in vitro (43, 44). However, the in vivo effect of such tumor-stromal interactions on chemotherapy is unknown. In fact, in ongoing studies, we are using purified TSR-derived peptides, such as ABT-510, which does not have TGF-β receptors which are present on many pancreatic cancer cell lines (47, 48).

Thus, it is hard to extrapolate the results of current study to other TSR-derived peptides, such as ABT-510, which does not have TGF-β activation sequence. It is also not clear whether the lack of synergy between 3TSR and gemcitabine resulted from some specific features of gemcitabine. To address this question, we are testing the combination of 3TSR and docetaxel, which acts faster and has a broader cell cycle spectrum, in this pancreatic cancer model.

However, our data on the lack of synergism between 3TSR and gemcitabine make it more interesting that such synergy had been seen between gemcitabine and anti-VEGF treatments in studies using other pancreatic cancer models (33, 34). In the I.3.6pl orthotopic pancreatic cancer model, gemcitabine did not decrease pancreatic cancer cell proliferation, and yet increased tumor hypoxic areas (33). Because in that model tumor cell proliferation was not decreased, the demand of tumor for blood supply was still high and may have resulted in increased sensitivity of tumor cells to the ischemia induced by anti-VEGF treatment. Furthermore, the combination of gemcitabine and anti-VEGF treatment resulted in significantly higher endothelial cell apoptotic indices than either agent alone both in vitro and in vivo (33, 34), and was more potent in decreasing tumor blood vessel density (33). Because VEGF is crucial to endothelial cell survival, blockage of VEGF signaling pathway may not only induce endothelial cell apoptosis but may also sensitize endothelial cells to the cytotoxicity of gemcitabine (45).

In conclusion, 3TSR, the antiangiogenic domain of thrombospondin-1, showed comparable antitumor efficacy to gemcitabine in a human pancreatic cancer orthotopic mouse model. No synergistic effects of these two drugs were found when combinatorial treatment was used. Further investigations in both alternative timing and combination of antiangiogenic and cytotoxic regimens will be pursued to achieve better therapeutic efficacy against pancreatic cancer. The need for a delicate balance between normalization of tumor vasculature and excessive regression of tumor vessels is becoming more obvious to those investigators at the forefront of bringing this bench research to the bedside (36). Selection of the dose and administration schedule will have to be carefully studied when combinations of chemotherapy and antiangiogenic treatment are contemplated. One successful example is the promising results of combining antiangiogenic drugs with metronomic chemotherapy (49). To maximally reduce the viable tumor burden, optimal regimens need to create a maximal time window allowing delivery of chemotherapeutic agents and oxygen and at the same time rendering the tumor incapable of vascular expansion. Although clearly more cumbersome and time consuming, it is our firm belief that logical combinations directed at different tumor compartments need to be proposed and carefully tested in preclinical animal models to allow both efficacious transfer to human trials and further molecular analysis of the different interactions at the tumor microenvironment level.

References


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