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Continuous administration of the three thrombospondin-1 type 1 repeats recombinant protein improves the potency of therapy in an orthotopic human pancreatic cancer model

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Abstract

Thrombospondin-1 is one of most important natural angiogenic inhibitors. The three thrombospondin-1 type 1 repeats (3TSR), an anti-angiogenic domain of thrombospondin-1, is a promising novel agent for anti-angiogenic treatment. In the present study, we showed 3TSR was biologically stable at least for 7 days in mini-osmotic pumps in vivo, and continuous administration of 3TSR decreased the dosage and improved the potency of therapy in an orthotopic pancreatic cancer model. By using different dosage and delivery routes, we proved that the anti-tumor efficacy of 3TSR was correlated with its anti-angiogenic efficacy. 3TSR treatment also decreased tumor vessel patency and blood flow. The results indicate the advantage of continuous administration of angiogenic inhibitors and provide rationale for using such delivery methods for cancer treatment.

Keywords

Pancreatic cancer; Thrombospondin-1; Angiogenesis; Anti-angiogenic therapy; Continuous administration; Orthotopic model

1. Introduction

Pancreatic cancer is the most deadly of all malignancies. To date, pancreatic cancer still represents a challenging therapeutic problem because it is widely recognized to be resistant to surgery, radiotherapy, and chemotherapy; furthermore, no major advances in the treatment of this disease were achieved during recent years [1]. The pharmacological control of angiogenesis might provide a novel regimen to the management of pancreatic cancer, because the growth and persistence of solid tumors depends on angiogenesis. In patients with pancreatic cancer, intratumoral microvessel density has been identified as an independent prognostic factor for survival on multivariate analysis [2,3]. In preclinical studies, natural angiogenic inhibitors, such as endostatin, angiostatin, and thrombospondin-1, have shown promising anti-tumor effects in various pancreatic cancer models [4–7].

Thrombospondin-1 is the first naturally occurring anti-angiogenic factor described and a potent tumor inhibitor [8,9]. The anti-angiogenic domain of thrombospondin-1 has been mapped to the type 1 repeats of thrombospondin-1. 3TSR, a recombinant protein of 21 kDa that contains all three type 1 repeats, designated 3TSR, provides a promising alternative for clinical administration. Using an orthotopic human pancreatic cancer model, we have shown 3TSR exerts strong anti-angiogenesis and anti-tumor efficacy [7].

Evidence suggests that a consistent level of angiogenic inhibitors might improve the anti-angiogenic and anti-tumor efficacy [4,10–12]. In the present study, we showed that continuous administration of 3TSR reduced the effective dosage of 3TSR by fourfold, and improved the therapeutic potency in mice with orthotopic pancreatic tumors.

2. Materials and methods

2.1. Cell culture

Human pancreatic cancer cells, AsPC-1 (American Type Culture Collection, Rockville, MD), were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin–streptomycin. Human dermal microvessel endothelial cells (HDMEC) were kindly provided by the Cell Biology Core of the Cancer Biology and Angiogenesis Division at Beth Israel Deaconess Medical Center. The cells were cultured in vitrogen precoated dishes and maintained in EBM-2 (Clonetics Corp., San Diego, CA) containing 20% fetal bovine serum, 1 µg/ml hydrocortisone acetate, 50 mol/L dibutyryl-cAMP, 200 units/ml penicillin, 100 units/ml streptomycin, and 250 µg/ml amphotericin.

2.2. In vivo stability and biological activity of 3TSR

Recombinant human 3TSR was cloned and purified as previously described [13]. To determine the stability of 3TSR in mini-osmotic pumps in vivo, we implanted pumps (ALZET Osmotic Pumps, Cupertino, CA) loaded with 3TSR subcutaneously into SCID mice (Taconic, Germantown, NY). Pumps were removed after 4 or 7 days incubation, and the remaining 3TSR solution inside the pumps was aspirated under sterile conditions. The samples were analyzed for Western blotting with a customized chicken anti-TSR antibody (Aves Labs, Inc., Tigard, Oregon). Identical gels were stained with Coomassie blue.

The biological activity of 3TSR was tested with an endothelial cell migration assay. HDMECs were harvested and 3TSR was added to the cell suspension to a final concentration of 10 ng/ml. Fresh 3TSR kept at 4 °C was used as control. HDMECs (1×10^5) were seeded into 6.5-mm vitrogen-coated tissue culture inserts (Costar, 8 µm pore). Medium with 2% BSA was added to the bottom wells with or without 10 ng/ml of VEGF (R&D Systems, Minneapolis, MN), and cells were incubated for 4 h at 37 °C. Cells that had not migrated were removed from the top membrane. The membrane was stained with 0.2% crystal violet in 2% ethanol, and cell number was counted under a microscope at $\times 20$ fields.

2.3. Tumor models

All animal work was performed in the animal facility at Beth Israel Deaconess Medical Center, Boston, MA, in accordance with federal, local, and institutional guidelines as previously described [7]. Female SCID mice, 4–6 weeks of age, were used. Animals were anesthetized and a 1 cm incision was made in the left subcostal region and the pancreas was exposed. A suspension of 1×10^6 pancreatic cancer cells in 100 µl of RPMI 1640 medium was injected into the body of pancreas. Peritoneum and skin were closed with a 4.0 surgical suture. Treatment was initiated 1 week after tumor cell implantation. Mice were randomized into five groups: (a) control ($n=8$); (b) 3TSR daily i.p. injection, 3 mg/kg per

day ($n=8$); (c) 3TSR daily i.p. injection, 0.75 mg/kg per day ($n=4$); (d) 3TSR continuous administration via mini-osmotic pumps, 1.5 mg/kg per day ($n=8$); (e) 3TSR continuous administration, 0.75 mg/kg per day ($n=4$). These pumps (internal volume, 100 μ l) continuously deliver test agents for 7 days. Pumps were implanted subcutaneously and replaced every 7 days. The control group received comparable i.p. injections of vehicle (phosphate buffer, 14.76 mM NaH₂PO₄, 5.24 mM Na₂HPO₄, 500 mM NaCl, pH 6.0, $n=4$) or s.c. implanted, vehicle-loaded pumps ($n=4$). Mice in all groups were sacrificed and underwent necropsy at 28 days after tumor cell implantation. Data were generated from three independent experiments with control and same stringent experimental settings. To evaluate the perfusion of tumor blood vessels, 1 mg fluorescein labeled dextran (10 mg/ml, 2,000,000 MW, Molecular Probes, Inc., Eugene, OR) was injected into the tail vein 5 min before the mice were sacrificed. Tumor volume was calculated as $\pi/6 \times \text{length} \times \text{width} \times \text{height}$.

2.4. Immunohistochemistry and immunofluorescence

Tumor tissue was harvested, fixed in 4% paraformaldehyde at 4 °C for 2–4 h, and incubated in 30% sucrose at 4 °C overnight. Tumor tissues were embedded in OCT compound, snap frozen in liquid nitrogen, and stored at –80 °C. Normal pancreatic tissue around the tumor was also harvested and processed together with tumor tissue. CD31 staining for tumor microvessel quantification was done on 5- μ m-thick sections as previously described [7]. To study the patency and perfusion of tumor blood vessels, dextran labeled samples were cut to 60 μ m-thick sections. CD31 immunofluorescence was done as described by Inai et al. [14].

2.5. Quantification of microvessel density (MVD) and tumor blood flow

For the quantification of microvessel density, 40 ‘hot spot’ fields of CD31 staining at $\times 20$ (0.584 $\times$ 0.438 mm²) were captured from four tumors each treatment group using a Spot digital camera mounted to a Nikon TE300 microscope. IP-Lab software (Scanalytics, Inc., Fairfax VA) was used to quantify tumor microvessel number, average area, and microvessel density. Fluorescence confocal microscopic digital projection images were also processed with IP-lab software to quantify tumor blood flow (dextran labeling of perfused blood vessels) and tumor vasculature (endothelial cells, CD31 immunoreactivity).

2.6. Statistics

All tumor volumes and quantified parameters were expressed as the mean $\pm$ SD. Unpaired Student’s *t*-test was used to compare tumor volumes, vessel density, and tumor blood vessel perfusion between treated tumors and un-treated control. Differences were considered statistically significant when $P<0.05$.

3. Results

3.1. 3TSR is stable and biologically active at least for 7 days in mini-osmotic pumps in vivo

First, we determined that 3TSR is stable in mini-osmotic pumps incubated in mice, and is therefore suitable for continuous delivery via these pumps. After 4 or 7 days of in vivo incubation, no degradation of 3TSR was apparent in Western blot or Coomassie blue staining (Fig. 1A). Furthermore, we tested biological activity of 3TSR after in vivo incubation with an endothelial migration assay. Fresh 3TSR samples kept at 4 °C significantly inhibits human microvessel endothelial cell migration by 80% compared with VEGF control (Fig. 1B). 3TSR samples that had been incubated in mini-osmotic pumps in vivo for 4 or 7 days showed similar inhibitory effects on human microvessel endothelial cell migration compared with 3TSR samples kept at 4 °C.

3.2. Continuous administration of 3TSR reduced dosage and improved therapeutic potency

Previously, we have determined that the optimal dosage of 3TSR is around 3 mg/kg per day [13]. In this study, we included an arm of 3TSR 3 mg/kg per day bolus injection as a standard of treatment, compared with 1.5 mg/kg per day continuous administration. The control animals developed large invasive tumors ($664.0 \pm 81.0 \text{ mm}^3$) with peritoneal seeding and lymph node metastasis. However, liver metastasis is sporadic in this model. The average tumor volume was comparable between mice received vehicle i.p. injection ($n=4$) and mice received implantation of vehicle-loaded pumps ($n=4$). After 3 weeks of treatment, daily injection of 3TSR at 3 mg/kg per day resulted in a significant tumor volume reduction in this AsPC-1 orthotopic model ($387.5 \pm 149.0 \text{ mm}^3$, $n=8$, $P=0.0004$ versus control, Fig. 2). Continuously administered 3TSR at 1.5 mg/kg per day also significantly inhibited the growth of orthotopic pancreatic tumors ($377.5 \pm 139.7 \text{ mm}^3$, $n=8$, $P=0.0002$ versus control), and the tumor volumes were comparable between mice treated with 3 mg/kg per day daily injection and 1.5 mg/kg per day via mini-osmotic pumps ($P=0.8$).

We further did a head-to-head comparison of 3TSR daily injection with continuous administration both at the dosage of 0.75 mg/kg per day. When 3TSR was given as daily injection at 0.75 mg/kg per day, the therapeutic effect was only marginal, and the tumor volumes were not significantly different compared with those of control ($573.6 \pm 176.1 \text{ mm}^3$, $n=4$, $P=0.2$ versus control). However, continuous delivery of 3TSR at 0.75 mg/kg per day significantly inhibited tumor growth compared with control ($392.7 \pm 154.0 \text{ mm}^3$, $n=4$, $P=0.002$, Fig. 2), and the average tumor volume was comparable with that of mice treated with 3 mg/kg per day daily injection.

Sample size and power of above analysis were calculated with PS power and Sample Size Program [15]. The power value was larger than 0.90 in all significant statistics. And according the experimental data, the theoretical sample size was ranged between 2 and 3 for each group with $\alpha=0.05$ and $\beta=0.80$.

3.3. The anti-tumor efficacy of 3TSR is correlated with its anti-angiogenic efficacy

As shown in Fig. 3, the microvessel density was 5.9% (of a $\times 20$ visual field, $0.584 \times 0.438 \text{ mm}^2$) in control tumors. Daily injection of 3TSR at 3 mg/kg per day significantly decreased both the number and average size of tumor vessels, and consequently, the microvessel density was reduced to 2.9% after treatment ($P<0.0001$). As described above, daily injection of 3TSR at 0.75 mg/kg per day did not result in a significant tumor volume reduction. Similarly, 3TSR 0.75 mg/kg per day bolus injection showed little effect on the vasculature of pancreatic tumors. However, when administered continuously via mini-osmotic pumps, 3TSR at both 1.5 and 0.75 mg/kg per day significantly decreased the number and average size of vessels as well as tumor microvessel density ($P<0.0001$ versus control, Fig. 3). The analyzed parameters in tumors from the two continuous administration groups were comparable with those in tumors from mice treated with 3 mg/kg per day bolus injection.

3.4. 3TSR treatment decreased tumor blood vessel perfusion

We further analyzed the functional change in tumor microvasculature after 3TSR treatment at different dosage and delivery routes. We measured both the area and intensity of fluorescein dextran-labeled vessels as well as Texas Red-labeled CD31 signals, and tumor blood flow was quantified as the percentage of area or intensity of dextran-labeled vessels to that of CD31 staining. As shown in Fig. 4, either method showed the same pattern as that of tumor volume and microvessel density. Daily injection of 3TSR at 0.75 mg/kg per day showed little effect on pancreatic tumor blood flow compared with control, whereas 3TSR 3 mg/kg per day bolus injection significantly reduced tumor blood supply. When delivered

continuously via mini-osmotic pumps, 3TSR at both 1.5 and 0.75 mg/kg per day showed comparable efficacy to 3 mg/kg per day daily injection in decreasing tumor blood vessel perfusion. Fig. 4B also showed that the blood flow of normal pancreas from 3TSR treated mice was little affected after 3TSR treatment.

4. Discussion

Sustained systemic levels of angiogenic inhibitors appear more effective in inhibiting angiogenesis within the vascular bed of a tumor. We showed in the present study that continuous administration of 3TSR significantly reduced the effective dosage and improved therapeutic potency in an orthotopic human pancreatic cancer model. When given continuously via a mini-osmotic pump, 3TSR at a fourfold lower dose (0.75 mg/kg per day) accomplished the same tumor suppression as daily bolus injection (3 mg/kg per day). In contrast, the same lower dose was not sufficient in inhibiting tumor growth when given as daily bolus injection. These results are consistent with our previous data determining the dose–response curve of 3TSR [13]. The optimal dosage of 3TSR was around 2.5 mg/kg per day, and the anti-tumor efficacy of 2.5 mg/kg per day 3TSR daily injection was comparable with that of 10 mg/kg per day, whereas daily injection of 3TSR at 1 mg/kg per day did not significantly decrease tumor volume compared with control [13]. Our results indicated that continuous administration of 3TSR shifted dose–response curve to the left, and therefore improved the therapeutic potency. However, continuous administration of 3TSR did not improve the maximal therapeutic efficacy. According to the 3TSR dose–response data, the maximal therapeutic efficacy of 3TSR might be determined by other factors such as its receptor, CD36, levels on endothelial cells. Actually, the therapeutic efficacy of thrombospondin-1 was increased by enhancement of CD36 expression on endothelial cells [16].

Our data showed that 3TSR retained biological activity, and did not undergo any obvious proteolytic degradation in osmotic pumps in vivo for at least 7 days. However, to date, the half life and other pharmacological features of 3TSR are still unknown, because the blood concentration of 3TSR has been difficult to be measured due to the specificity of TSR antibodies. The results of this study indicated that continuous administration of 3TSR via an osmotic pump might achieve a more sustained circulating concentration, which resulted in more effective anti-angiogenic and anti-tumor effects.

Indeed, the anti-tumor efficacy of 3TSR is correlated with its anti-angiogenic efficacy. By measuring tumor volume and quantifying tumor microvessel density of pancreatic tumors from different treatment groups, we observed the similar pattern in tumor growth inhibition and tumor microvessel density decrease. This observed correlation between the anti-tumor and anti-angiogenic efficacy of 3TSR was also consistent with our previous mechanism studies. 3TSR does not inhibit proliferation or induce apoptosis of pancreatic cancer cells either in vitro or in this orthotopic model, but exerts its anti-tumor effects via inhibiting tumor angiogenesis and inducing apoptosis of tumor microvessel endothelial cells [7].

In this study, we also reported the functional impact of 3TSR on tumor blood vessels. There are compelling data showing the impact of thrombospondin-1 and TSRs on the structure of tumor blood vessel [7,17–19]. We showed here that 3TSR treatment also decreased vessel patency and blood flow of pancreatic tumors. The effective doses were consistent with those required for tumor growth inhibition. Loss of tumor vessel patency and blood flow preceding endothelial cell regression was also reported as an early phenomenon after VEGF signaling inhibition [14]. We previously reported that 3TSR induced apoptosis of tumor blood vessel endothelial cell, and these cells kept CD31 immunoreactivity when they underwent apoptosis [7]. These apoptotic endothelial cells might already lose their function,

thus resulted in the loss of patency and blood flow of tumor vessels. However, the exact mechanisms by which 3TSR reduced tumor vessel patency are still unknown. Our present finds warrant further investigation on the detailed functional analysis of tumor vasculature after 3TSR treatment, such as whether an early normalization phase exists before vessel regression, morphological basis of decreased vessel patency, the effects of 3TSR on tumor vessel permeability and interstitial fluid pressure, and platelets response to 3TSR treatment.

In summary, continuous administration of 3TSR, an anti-angiogenic domain of thrombospondin-1, decreased the effective dosage and improved the therapeutic potency in an orthotopic pancreatic cancer model. The therapeutic benefit of sustained levels of angiogenic inhibitor could be translated into clinical trials using intravenous infusion pumps [20]. Moreover, a long-lasting circulating level of 3TSR can also be achieved via gene therapy using integrated vectors such as adeno-associated virus, and such studies are ongoing in our laboratory.

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Abbreviations

3TSR	three thrombospondin-1 type 1 repeats
BSA	bovine serum albumin
HDMEC	human dermal microvessel endothelial cell
MVD	microvessel density
SCID	severe combined immunodeficient
VEGF	vascular endothelial cell growth factor

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Fig. 1.

3TSR is stable and biologically active at least for 7 days in mini-osmotic pumps *in vivo*. Pumps loaded with 3TSR were incubated in mice for 4 or 7 days, and the remained 3TSR was retrieved for analysis. A. No proteolytic degradation of 3TSR was apparent in Western blot or Coomassie blue staining. B. The biological activity of 3TSR was tested with the endothelial migration assay. Compared with 3TSR samples kept at 4 °C, 3TSR samples that had been incubated in mini-osmotic pumps *in vivo* showed similar inhibitory effects on human microvessel endothelial cell migration.

Fig. 2. Continuous administration of 3TSR reduced dosage and improved therapeutic potency. Treatment started 1 week after tumor cell implanted into the pancreas of mice, and lasted for 3 weeks. *, $P < 0.01$ versus control. The average tumor volume of mice treated with 3TSR 3 mg/kg per day bolus injection was not statistically different compared with that of mice received continuous delivery of 3TSR at 1.5 or 0.75 mg/kg per day.

Fig. 3.

The anti-tumor efficacy of 3TSR was correlated with its antiangiogenic efficacy. A. Representative CD31 staining of tumors from each treatment group. Bar, 100 μm . B. Quantification of tumor microvessel number, microvessel size, and tumor microvessel density. Tumor sections from four tumors of each group were analyzed. The microvessel density is defined as the percentage of total microvessel area in a $\times 20$ visual field ($0.584 \times 0.438 \text{ mm}^2$). *, $P < 0.05$ versus control.

Fig. 4.

The change of tumor vessel perfusion after 3TSR treatment at different dosage and delivery routes. A. Fluorescence confocal microscopic digital projection images of CD31-labeled tumor vasculature (Texas Red, top panel), dextran-labeled perfused blood vessels (FITC, middle panel) and merged images (bottom panel). White arrows indicate tumor vessels without perfusion. B. Vasculature of normal pancreas from tumor-bearing mice treated with 3TSR 3 mg/kg per day i.p. injection. Fluorescence confocal microscopic digital images in panels A and B were captured with a $\times 20$ objective, and the image size is $0.485 \times 0.485 \text{ mm}^2$. C and D. Quantification of tumor vessel patency. The percentage area (intensity) was calculated as the ratio of area (intensity) of dextran to that of CD31. The data represent quantification of two representative tumors from each group. *, $P < 0.001$.