

Antiangiogenic therapy for human pancreatic carcinoma xenografts in nude mice

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Abstract

AIM: To investigate the anti-tumor effects of antiangiogenic therapy (a combination of TNP-470, an antiangiogenic compound, with gemcitabine, an antimetabolite) on human pancreatic carcinoma xenografts and its mechanism.

METHODS: A surgical orthotopic implantation (SOI) model was established by suturing small pieces of SW1990 pancreatic carcinoma into the tail of pancreas in nude male mice. Mice then received either single therapy ($n = 24$) or combined therapy ($n = 32$). Mice receiving single therapy were randomly divided into control group, G100 group receiving 100 mg/kg gemcitabine IP on d 0, 3, 6 and 9 after transplantation, and T30 group receiving 30 mg/kg TNP-470 s.c on alternate days for 8 wk. Mice receiving combined therapy were randomly divided into control group, T15 group, G50 group and combination group (TNP-470 30 mg/kg and gemcitabine 50 mg/kg). Animals were killed 8 wk after transplantation. Transplanted tumors, liver, lymph node and peritoneum were removed. Weight of transplanted tumors, the T/C rate (the rate of mean treated tumor weight to mean control tumor weight), change of body weight, metastasis rate, and 9-wk survival rate were investigated. Tumor samples were taken from the control group, T30 group, G100 group and combination group. PCNA index (PI) and microvessel density (MVD) were investigated by immunohistochemical staining for PCNA and factor VIII, respectively.

RESULTS: There was a significant inhibitory effect on primary tumor growth of pancreatic carcinoma in G100 group, compared to T30 group, whereas tumor metastasis was significantly inhibited in T30 group compared to G100 group. There was no significant improvement in survival rate in these two groups. No significant inhibitory effect on tumor growth and metastasis in T15 group and G50 group. However, significant anti-tumor and anti-metastatic effects were observed in the combination group with a significant improvement in survival rate. The inhibitory effect on tumor growth in combination group enhanced 2 times in comparison with G50 group and 5 times in comparison with T15 group. Moreover, 25% of the animals bearing

tumors were cured by the combination therapy. The levels of MVD and PI were 14.50 ± 5.93 and 0.41 ± 0.02 , 12.38 ± 1.60 and 0.30 ± 0.07 , 7.13 ± 2.99 and 0.37 ± 0.03 , and 5.21 ± 1.23 and 0.23 ± 0.02 respectively in the control group, G100 group, T30 group and combination group. A significant inhibitory effect on PI level and MVD level was observed in G100 group and T30 group respectively whereas both MVD and PI levels were significantly inhibited in the combination group ($P < 0.05$).

CONCLUSION: Antiangiogenic therapy shows significant anti-tumor and anti-metastatic effects, and is helpful to reduce the dosage of cytotoxic drugs and the side effects. These effects are related to the antiangiogenic effect of TNP-470 and cytotoxic effect of gemcitabine.

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Key words: Pancreatic carcinoma; TNP-470; Angiogenesis Inhibitors; Xenografts

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INTRODUCTION

Exocrine pancreatic carcinoma is now the fifth leading cause of cancer in the United States, Japan and Europe, with an overall 5-year survival rate of less than 5%^[1]. One of the major causes of death is peritoneal dissemination and liver metastasis^[2]. As all solid tumors, pancreatic carcinoma depends on the development of an adequate blood supply through angiogenesis for growth at both primary and secondary sites. Inhibition of neoangiogenesis is a new and attractive target for tumor therapy, since it theoretically offers the hope of long-term control of tumor progression. TNP-470 is a potent angiogenesis inhibitor, which has been shown to have a marked inhibitory effect on tumor growth and metastasis both *in vivo* and *in vitro*^[3-5].

The anti-neoplastic actions of angiogenic inhibitors and cytotoxic agents are clearly different. Treatment with antiangiogenic agents could interact in a positive way with a variety of anti-cancer therapies, and the anti-metastatic and anti-tumor effects of combination therapy were stronger than those of angiogenic inhibitors alone and cytotoxic agents alone^[5-8]. Therefore, Satoh *et al*^[9] (1998) first proposed the concept of angiocytotoxic therapy. However, the effect of antiangiogenic therapy on pancreatic carcinoma and its precise mechanism have not been elucidated completely.

In this study, TNP-470 and gemcitabine served as representatives of angiogenic inhibitors and cytotoxic agents respectively. The aims of this study were to assess the anti-tumor and anti-metastasis effects of TNP-470 and gemcitabine alone or in combination using a patient-like model of pancreatic carcinoma by surgical orthotopic implantation (SOI) and to clarify the mechanism of angiocytotoxic therapy.

MATERIALS AND METHODS

Drugs and reagents

TNP-470 was a kind gift from Takeda Chemical Industries (Osaka, Japan). Its structure and metabolism were described previously. For *in vivo* use, TNP-470 was suspended in a vehicle of 3% ethanol and 5% gum arabic in saline. Gemcitabine (Eli Lilly Inc.) was dissolved in saline. RPMI 1640 and heat-inactivated fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY).

Animals

Four-week-old male BALB/c nu/nu mice, weighing 18-20 g, were obtained from the Experimental Animal Center, Sun Yat-Sen University. The animals were housed in microisolator cages with autoclaved bedding, food, and water. The mice were maintained on a 12-h light/12-h dark cycle.

Cell line

Human pancreatic carcinoma cell line SW1990 was kindly provided by the Animal Center, Sun Yat-Sen University. Cells were cultured in RPMI 1640 supplemented with 10% FCS, and maintained at 37 °C in 50 mL/L CO₂. A single cell suspension of approximately 5×10⁶ cells in 0.5 mL of culture medium was inoculated subcutaneously into the left axial region of the cervix of two BALB/c nu/nu mice to make source tumors.

Establishment of SOI model

Pancreatic tumor tissues were transplanted orthotopically in nude mice using the method of Furukawa *et al*^[10] with some modifications. These source tumors were excised when they grew to approximately 1 cm³, and minced into approximately 1 mm³ pieces in Hanks' balanced salt solution (HBSS) containing 100 units/mL penicillin and 100 µg/mL streptomycin.

Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (0.45 mg/g body weight). An incision was then made through the upper left abdominal pararectal line and peritoneum. The pancreas was carefully exposed and a tumor piece was transplanted on the tail of pancreas close to the portal area of the spleen with a 6-0 surgical suture. The pancreas was relocated into the abdominal cavity, which was then closed in two layers with 6-0 surgical sutures. Animals were kept in a sterile environment.

Effects of TNP-470 and gemcitabine alone on SOI model

Twenty-four male mice were randomly divided into 3 groups: control group was given saline solution, T30 group, which received 0.2 mL of TNP-470 solution (30 mg/kg, sc. on alternate days for 8 wk) from d 7 after tumor transplantation, G100 group, which received 0.2 mL of gemcitabine (100 mg/kg ip.) on d 0, 3, 6 and 9 after transplantation. Treatment was continued until animals were killed 8 wk after tumor transplantation.

The transplanted tumors, liver, lymph node and peritoneum were harvested, fixed in paraformaldehyde, and embedded in paraffin. Five-micron thin tissue sections were obtained and stained with hematoxylin and eosin for microscopic examination. Metastases were evaluated macroscopically and confirmed histologically. Besides, the weight of transplanted tumors, T/C rate (the rate of the mean tumor weight in treated animals to the mean tumor weight in control animals), change of body weight of mice and 9-wk survival rate were also investigated.

Effects of combination therapy of TNP-470 with gemcitabine on SOI model

Thirty-two male mice were randomly divided into 4 groups: control group, T15 group receiving TNP-470 (15 mg/kg), G50

group receiving gemcitabine (50 mg/kg) and combination group (TNP-470 30 mg/kg+gemcitabine 50 mg/kg). The experiment steps were the same as that mentioned above.

Immunohistochemistry

Expression of proliferating cell nuclear antigen (PCNA) and microvessel density (MVD) in tumors were examined by immunohistochemical method for PCNA and factor VIII respectively. The samples were from the control group, T30 group, G100 group and combination group. Primary antibody used was polyclonal antibody of von Willebrand factor (factor VIII-related antigen) at a dilution of 1:100, monoclonal antibodies to PCNA at a dilution of 1:100. Negative controls were generated by substituting the primary antibody with distilled water. Two independent experienced pathologists counted separately, and consensus counting was done for dispute.

Microvessel density Criteria for positive staining and microvessel counting were those established by Weidner *et al*^[11] with a minor modification^[2-3]. The counting was first proceeded at 100× magnification for "hot spot" representing the area of the highest microvessel density (MVD), then switched to 200× magnification for clear depiction and better counting. For each slide, five "hot spots" were counted, and the mean count represented the final MVD. MVD was counted according to the standards that any stained endothelial cell or cells were identified as an independent vessel. A brown stain structure clearly separated from the adjacent microvessels was regarded as a single countable microvessel. However, apparent vasa or vasa with red blood cells could be regarded as vessels^[11].

PCNA index (PI) PCNA staining was confined to the nuclei. Although the nuclear staining varied in intensity, all identifiable staining was considered positive. Sections were counted at high power (×400), and five fields were assessed randomly for tumor or non-tumor pancreatic cells. Five hundred cell nuclei were found respectively, and the rate of positive cells over total cells counted was defined as the PCNA index^[12].

Statistical analysis

Statistical analysis was carried out by SPSS packed program. Variance test, chi-square test and Mann-Whitney test were used. *P*<0.05 based on a two-tailed test was considered statistically significant.

RESULTS

Effects of monotherapy and its side effects

Anti-metastasis effect. We found that metastasis developed in 7 of 8 mice (87.5%) in the control group, in 5 of 8 mice (62.5%) receiving 100 mg/kg gemcitabine, in 2 of 8 mice (25%) receiving 30 mg/kg TNP-470. TNP-470 displayed a significant inhibitory effect on the metastasis of pancreatic carcinoma compared to the control and gemcitabine (*P*<0.05).

Anti-tumor effect Although TNP-470 (30 mg/kg) alone had no significant effect on tumor growth *in vivo*, gemcitabine (100 mg/kg) significantly inhibited primary tumor growth (*P*<0.05).

Survival rate Neither Gemcitabine (100 mg/kg) nor TNP-470 (30 mg/kg) showed a significant improvement in the survival rate of SOI models (*P*>0.05).

Side effects gemcitabine (100 mg/kg) led to severe diarrhea and weight loss during its administration, while 30 mg/kg of TNP-470 induced mild weight loss in the mice with pancreatic carcinoma (as shown in Table 1).

Effects of combination therapy and its side effects

Table 2 shows that low doses of TNP-470 alone and gemcitabine alone had no significant inhibitory effect on primary tumor

growth and metastasis of PCA *in vivo*. On the other hand, a significant anti-tumor and anti-metastatic effect was observed in the combination group. TNP-470 also enhanced the inhibitory effect of gemcitabine (50 mg/kg) from 23.64% to 67.27% (combination therapy), and 25% of the animals bearing tumors were cured by the combination therapy. Besides, only the combination group showed significant improvement on the survival rate of mice. All the therapies had no obvious side effects such as diarrhea and weight loss.

Mechanism of combination therapy

The levels of microvessel density in T30 group and combination group were significantly lower than those in G100 group and control group ($P < 0.05$), while there was no significant difference in MVD levels between T30 group and combination group, and between G100 group and control group ($P > 0.05$). In contrast, the level of PI in G100 group and combination group was significantly lower than that in T30 group and control group ($P < 0.05$), while there was no significant difference in PI levels between G100 group and combination group, and between T30 group and control group ($P > 0.05$).

Table 1 Tumor weight, survival rate, metastasis rate and BW in three groups (mean±SD)

Group	Tumor weight		Survival rate (%)	Metastasis rate (%)	Change of body weight of mice (g)
	(g)	(1-T/C,%)			
Control	0.64±0.04		50.0 (4/8)	87.5 (7/8)	-0.34±0.12
G100	0.19±0.01 ^a	70.31	50.0 (4/8)	62.5 (5/8)	-1.04±0.20
T30	0.38±0.03	40.62	62.5 (5/8)	25.0 (2/8) ^a	-0.99±0.14

^a $P < 0.05$ vs control.

Table 2 Tumor weight, survival rate, metastasis rate and BW in four groups (mean±SD)

Group	Tumor weight		Survival rate (%)	Metastasis rate (%)	Change of body weight of mice (g)
	(g)	(1-T/C,%)			
Control	0.55±0.03		37.5 (3/8)	100 (8/8)	-0.44±0.09
G50	0.42±0.02	23.6	62.5 (5/8)	75.0 (6/8)	-0.90±0.13
T15	0.48±0.02	12.7	62.5 (5/8)	62.5 (5/8)	-0.79±0.12
Combination	0.18±0.02 ^a	67.3	100 (8/8) ^a	12.5 (1/8) ^a	0.13±0.07 ^a

^a $P < 0.05$ vs control.

Table 3 MVD and Proliferation index (PI) in four groups (mean±SD)

Group	MVD	PI
Control	14.50±5.93	0.41±0.02
G100	12.38±1.60	0.30±0.07 ^a
T30	7.13±2.99	0.37±0.03
Combination	5.21±1.23 ^a	0.23±0.02

^a $P < 0.05$ vs other groups.

DISCUSSION

The pivotal role of angiogenesis in primary tumor growth and metastasis has been recognized many years before. It has been found that angiogenesis is essential for the growth of solid tumors at primary and at secondary sites^[3,13]. It is thought that new blood vessels in tumor are highly permeable and provide a route for cancer cells to enter the circulation^[25]. Therefore, anti-angiogenic agents might be clinically useful for the prevention of cancer

progression^[13-16]. Since the first successful efforts to inhibit endothelial cell growth were reported, many new drugs have been developed for the inhibition of tumor angiogenesis. TNP-470, a semisynthetic analogue of fumagillin, is one of the promising antiangiogenic drugs in clinical trials based on its efficacy and the lack of major adverse effects, and which has been reported to be highly effective against a wide variety of tumors and metastases mainly by preventing tumor neovascularization^[17-20].

Antiangiogenic therapy offers a number of potential benefits including lack of resistance to some agents, synergistic interaction to other modalities, lack of significant toxicity compared with conventional agents, and a potent antitumor effect. However, the anti-neoplastic actions and side effects of angiogenic inhibitors and cytotoxic agents were clearly different^[6,7,14]. Administration of angiogenesis inhibitors such as TNP-470 might keep the tumor and its metastases dormant (rather than killing it), and co-administration of cytotoxic drugs might kill it^[5,8,21,22]. Many studies have been conducted to evaluate the therapeutic effects of angiogenic inhibitors with in combination with cytotoxic agents. Kato *et al*^[23] reported significant effects of TNP-470 in combination with mitomycin-C, adriamycin, CDDP, and 5-FU in mouse models. The anti-tumor effect of TNP-470 was enhanced by combination chemotherapeutic agents^[7,8,14,25,26]. Shishido *et al*^[6] further showed that the effect of TNP-470 in combination with CDDP against pancreatic carcinomas was enhanced. Although TNP-470 alone and CDDP alone had no effect on tumor growth *in vivo*, 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP had a significant effect. Therefore, angiocytotoxic therapy has been gradually accepted worldwide in recent years.

Recently, a number of new drugs have been developed for treating patients with pancreatic carcinoma. Early studies with gemcitabine suggested a modest antitumor activity with significant improvement in disease-related symptoms. Therefore, gemcitabine has been generally considered to be the first-line therapy for pancreatic cancer, and is now widely used^[26-28]. In 2002, we established the metastatic model of SW1990 by surgical orthotopic implantation in nude mice, which replicated the clinical behaviors of pancreatic carcinoma including local growth and regional and distant metastases. The successful transplantation rate and the metastatic rate for 9 wk were 100% and 93.8% respectively^[29]. Gemcitabine alone had no significant anti-tumor effect on SOI model of pancreatic carcinoma, but was not helpful to reduce tumor metastasis^[30]. However, the effects of angiocytotoxic therapy (TNP-470 in combination with gemcitabine) on pancreatic carcinoma and its mechanism were not elucidated completely.

In the current study, we used the well-established SOI model of pancreatic carcinoma SW1990 cell line to evaluate the effect of angiocytotoxic therapy. We found that TNP-470 30 mg/kg alone had a significant inhibitory effect on the metastasis of pancreatic carcinoma, but had no significant anti-tumor effect. In contrast, gemcitabine (100 mg/kg) alone could significantly inhibit primary tumor growth, but induced severe diarrhea and weight loss, and had no significant anti-metastatic effect. Both groups had no significant improvement in the survival rate of SOI models. While low dose of TNP-470 and gemcitabine alone had no significant inhibitory effect on the tumor growth and metastasis, angiocytotoxic therapy (TNP-470 in combination with low dose of gemcitabine) showed significant anti-tumor, anti-metastatic and survival-improving effects. The inhibitory effect on G50 group was enhanced 2 times in comparison with T30, and 25% of the animals bearing tumors were cured by the combination therapy. It is clear that the mechanism of angiocytotoxic therapy includes both the antiangiogenic effect of TNP-470 (reducing the level of MVD) and cytotoxic effect of

gemcitabine (reducing the level of PI).

In summary, by combining antiangiogenic agents with each other and/or with other modalities in the treatment of cancer, the limitations of each therapeutic approach could be overcome, leading to enhanced efficacy with diminished toxicity^[14]. Angiocytoxic therapy (TNP-470 in combination with low dose of gemcitabine) showed excellent anti-tumor and anti-metastatic effects on SOI model of pancreatic carcinoma, which is very helpful to reduce the dosage of cytotoxic drugs and its side effects. These results suggest that angiocytoxic therapy may provide a new safe and effective strategy for the treatment of advanced pancreatic carcinoma. However, before these agents can be used into clinical practice, a better understanding of their mechanism of action and regulation is needed.

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