

A Novel "Patient-like" Treatment Model of Human Pancreatic Cancer Constructed Using Orthotopic Transplantation of Histologically Intact Human Tumor Tissue in Nude Mice¹

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ABSTRACT

Pancreatic cancer is a disease with essentially no effective treatment. To increase the potential for discovering effective treatment, we have developed a new treatment model whereby a human pancreatic cancer line, PANC-4, was orthotopically transplanted to the pancreas of nude mice as histologically intact tumor tissue. The tumor grew with subsequent invasive local tumor growth and liver and peritoneal metastases. The antitumor activity of 5-fluorouracil (5-FU) and mitomycin C (MMC) against PANC-4 was initially determined in the *in vitro* collagen-sponge-gel supported histoculture drug-response assay with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide end point. Inhibition rates were 5.6% for 5-FU and 39.4% for MMC indicating higher efficacy of MMC than 5-FU against PANC-4. When the antitumor activities of 5-FU and MMC against PANC-4 were determined *in vivo* using the nude mouse orthotopic transplant treatment model, slight local tumor growth inhibition with equivalent incidence of metastases to the liver and the peritoneum as the control were observed in the mice treated with 5-FU, while those treated with MMC had considerably reduced local tumor growth without liver and peritoneal metastases. Thus the histoculture drug-response assay in combination with the orthotopic transplant metastatic models provides for the first time a paradigm for evaluation of agents which may be effective against not only locally growing human pancreatic cancer but resulting metastases as well.

INTRODUCTION

Pancreatic cancer is extremely aggressive and very resistant to currently known systemic treatment. Most patients are found to have metastatic lesions, and for the majority of patients, even with early disease removed surgically, there is no prospect for cure or even effective palliation because of almost inevitable metastases observed soon after surgery (1, 2). New treatment strategies are necessary against metastases of pancreatic cancer and require appropriate models for their development. Transgenic rodent models of pancreatic cancer as well as chemically induced pancreatic cancer in rodents have been useful to study this disease, including possible models of growth control (3). It is important, however, to study clinically relevant metastases and their treatment. Metastases of human pancreatic cancer after s.c. transplantation in nude mice have only occasionally been reported, however (4, 5). Vezeridis *et al.* (6) reported a metastatic model using splenic injection of a fast-growing variant of human pancreatic cancer. Although this was a valuable model for the study of certain steps of the metastatic process, it bypasses invasion and in essence generates colonization rather than metastases (7).

Recently, Tan and Chu (8) and Marnicola *et al.* (9, 10) reported a metastatic model of human pancreatic cancer using orthotopic implantation of tumor-cell suspensions, which resulted in invasive local tumor growth and subsequent metastases. Moreover, Vezeridis *et al.* (7) used tumor tissue for orthotopic transplantation, resulting in ex-

tensive local growth, metastases to liver, lung, and lymph nodes. Furthermore, Fu *et al.* (11) used histologically intact patient specimens of pancreatic cancer for orthotopic transplantation to nude mice to construct a metastatic model of human pancreatic cancer. We report here that this orthotopic transplant approach to pancreatic cancer utilizing histologically intact tissue can be utilized to test treatment efficacy against local and metastatic growth allowing the design of treatment effective against metastases.

MATERIALS AND METHODS

Mice. Male BALB/c nu/nu mice, which originated from the Central Institute for Experimental Animals (Kawasaki, Japan), were obtained from CLEA Japan, Inc. (Tokyo Japan). Animals which were 6-8 weeks old and weighed 20-22 g were used.

Drugs. 5-FU³ and MMC were purchased from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan).

Human Pancreas Cancer Xenograft. PANC-4, a human pancreatic carcinoma xenograft, was provided by Dr. T. Nomura, the Central Institute for Experimental Animals, and was maintained by serial s.c. transplantation into nude mice at Keio University School of Medicine.

Orthotopic Transplantation of Histologically Intact Tumor Tissue. Pancreatic tumor tissues were transplanted orthotopically in nude mice using the method of Fu *et al.* (11) with some modifications. Tumors at the exponential growth phase in nude mice were resected aseptically, necrotic tissues were cut away and the remaining healthy tumor tissues were cut with scissors and were minced into approximately 3 × 3 × 3 mm pieces in Hanks' balanced salt solution containing 100 units/ml penicillin and 100 µg/ml streptomycin. Each piece was weighed and adjusted with scissors to be 50 mg.

Mice were anesthetized by i.p. administration (0.3 ml/mouse) of 2.5% solution of a mixture of 2,2,2-tribromoethanol (Aldrich Chemical Company, Inc., Milwaukee, WI) and *tert*-amylalcohol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (1:1). An incision was then made through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed and a tumor piece was transplanted on the middle of the pancreas with a 6-0 Dexon (Davis-Geck, Inc., Manati, Puerto Rico) surgical suture. The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed with 6-0 Dexon sutures. Animals were kept in a sterile environment.

Experimental Chemotherapy. On day 7 after orthotopic transplantation, mice were randomized into control and treated groups. 5-FU and MMC, dissolved in 0.2 ml of physiological saline solution, were administered i.p. as boluses. The doses of the drugs used were 180 mg/kg for 5-FU and 6 mg/kg for MMC, which were determined as maximum tolerated doses in nude mice in our previous studies (12). On the 90th day after orthotopic transplantation, the tumors growing in the peritoneal cavity and the liver were removed from each mouse, weighed, and then examined histologically after careful macroscopic examination. Since it required at least 90 days in the initial experiments for PANC-4 to express its metastatic potential after orthotopic transplantation (data not shown) some mice died in this period due to extensive local tumor growth. Therefore, only the mice which survived 90 days were evaluable.

Histoculture Drug Response Assay with the MTT End Point. The HDRA was performed using the MTT end point as reported previously (13). The MTT end point is a simple and convenient colorimetric method. The

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³ The abbreviations used are: 5-FU, 5-fluorouracil; HDRA, histoculture drug-response assay; MMC, mitomycin C; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

inhibition of the number of viable tumor cells correlates well with inhibition of total succinate dehydrogenase activity. Succinate dehydrogenase activity is measured by optical density at A_{540} resulting from the DMSO-extracted formazan crystals produced by MTT reduction (14, 15). Special collagen gels manufactured from pig skin were purchased from Health Design, Inc. (Rochester, NY). The gels were removed from their sterile packages and cut with scissors into 1-cm³ pieces, and 1 piece was placed in each well of several 24-well plates. The antitumor drugs were dissolved in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 20% fetal calf serum (GIBCO, Grand Island, NY), 100 units/ml penicillin and 100 μ g/ml streptomycin. One ml/well of the solutions was added to each well which reached but did not cover the upper part of the gel. The cutoff concentrations of the drugs used, or concentrations that allowed *in vitro* evaluation of tumor drug response, were 300 μ g/ml for 5-FU and 7.5 μ g/ml for MMC since these concentrations allowed high correlation with *in vivo* response (12).

Tumor pieces divided into 50-mg units as described above were further scissor-minced into pieces about 1 mm in diameter. The tissue was further minced into 5–10 pieces about 0.5 mm in diameter, which were then placed on each of the prepared collagen surfaces in 24-well plates. The plates were incubated for 7 days at 37°C in a humidified atmosphere containing 95% air/5% CO₂.

After incubation, 100 μ l of Hanks' balanced salt solution containing 0.1 mg/ml collagenase (Worthington Biochemical Co., NJ) and 100 μ l of MTT (Dojindo Laboratories, Kumamoto, Japan) solution, dissolved in 5 mg/ml phosphate-buffered saline containing 0.1 mol/l of sodium succinate and filtered through a 0.45- μ m membrane filter (Millipore, Bedford, MA), were added to each well and incubated for an additional 8 h. The medium was then aspirated completely from each well by careful use of micropipets. One ml of DMSO (Nacalai Tesque, Inc., Kyoto, Japan) per well was added to dissolve the formazan product. After 2 h the solutions were transferred to 96-well microtiter plates (100 μ l/well) and the absorbance of the solution in each well was read at 540 nm on a Model EAR 340 AT reader (SLT-Labinstruments). The absorbance/g of each tumor was calculated from the mean absorbance of 4 wells and the initial tumor weight which was estimated prior to the culture.

The inhibition rate was calculated using the formula:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{\text{Mean } A_{540} \text{ of DMSO-extracted formazan of treated tumors/g}}{\text{Mean } A_{540} \text{ of DMSO-extracted formazan of control tumors/g}} \right) \times 100$$

Each drug concentration was tested in triplicate wells and the experiment was repeated three times. The *in vitro* test was considered to be evaluable for tumor drug response at the cutoff drug concentrations which were determined as 300 μ g/ml for 5-FU and 7.5 μ g/ml for MMC in our previous study, since these concentrations allowed high correlation with *in vivo* response (13). The concentration of each drug which lowered tumor cell MTT reduction activity by 50% was calculated using concentration effect data with the steepest slope determined by linear regression.

RESULTS

Orthotopically transplanting PANC-4 human pancreatic cancer to the pancreas of nude mice resulted in local invasive growth (Fig. 1A), invasion of the duodenum (Fig. 1B), and liver metastasis (Fig. 1C). The incidence of metastases observed in the untreated control mice were 6 and 7 of 18, for liver and peritoneal metastases, respectively (Table 1).

Table 2 shows the *in vitro* results of antitumor activity of 5-FU and MMC against PANC-4 determined in the HDRA with the MTT end point. The mean concentrations of 5-FU and MMC which lowered tumor cell MTT reduction activity by 50% were over 10- and 3-fold above the cutoff drug concentrations, respectively, and the mean inhibition rates at the cutoff concentrations were 5.6 and 39.4%, respectively, indicating higher efficacy of MMC than 5-FU against PANC-4.

Table 1 shows the *in vivo* results of antitumor activity of 5-FU and MMC against PANC-4 after orthotopic transplantation in nude mice. Local tumor growth was slightly inhibited in mice treated with 5-FU, resulting in 80% of the mean actual tumor weight in treated mice

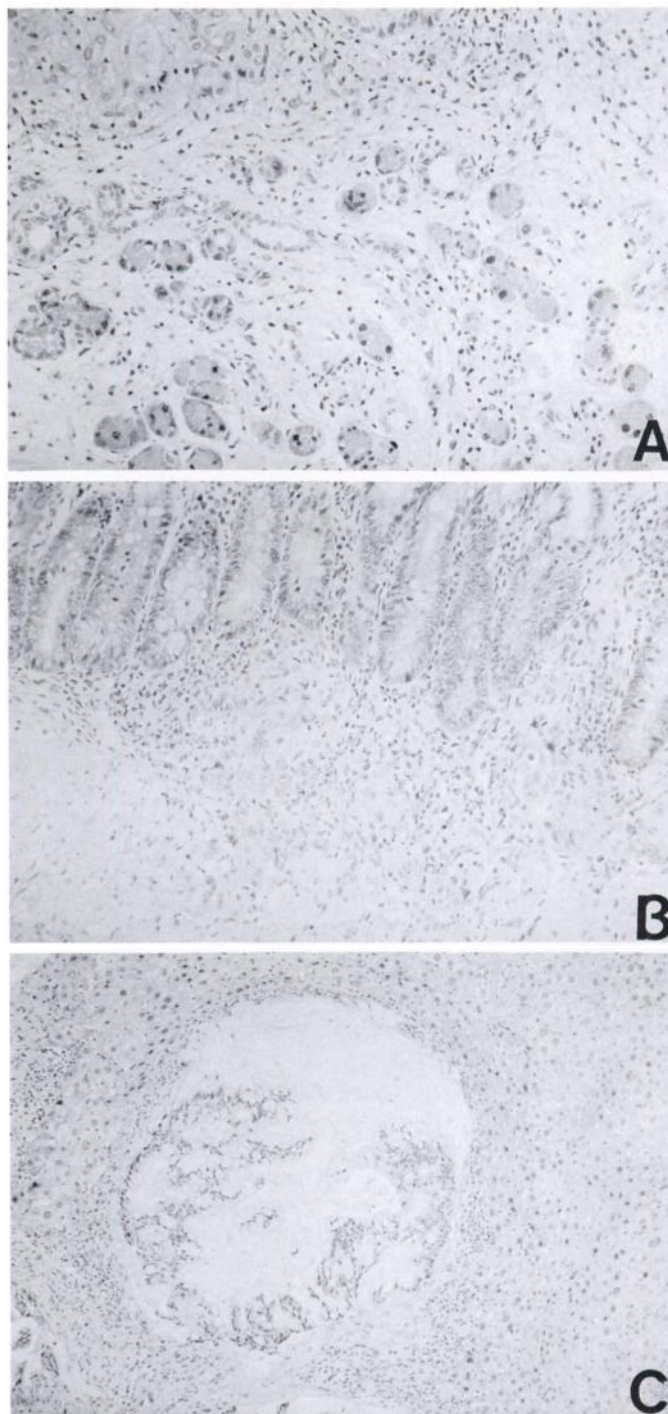


Fig. 1. Microscopic views of local invasive growth into the pancreatic ducts (A), invasion to the duodenal submucosal layer (B), and liver metastasis of the PANC-4 human pancreatic cancer observed after orthotopic transplantation of histologically intact tumor tissue in nude mice (C). Mice were transplanted with PANC-4 on day 0 and then examined on day 90.

relative to that in control mice (T/C). The incidence of metastases to the liver and the peritoneum in the mice treated with 5-FU was 2 of 6 each, equivalent to that observed in the control mice. On the other hand, MMC demonstrated a considerable antitumor activity on local tumor growth, resulting in a 46% T/C value in actual tumor weight, with a statistically significant difference from the control. Most importantly, none of 8 mice treated with MMC developed liver or peritoneal metastases. A loss of body weight was observed for the majority of the mice which correlated with tumor growth (data not shown).

Table 1 Metastases observed after orthotopic transplantation of PANC-4 and *in vivo* antitumor effects of 5-FU and MMC against PANC-4

PANC-4 was transplanted into nude mice on day 0 and maximum tolerated doses of 5-FU or MMC were administered i.p. bolus on day 7. Three separate control groups were run at different times. On day 90, only the surviving mice were evaluated.

Treatment	Survivors after 90 days	Actual ^a tumor weight	T/C value of local tumor (%)	Liver metastases	Peritoneal metastases
Control 1	7/13	1.687 ^b		2/7	3/7
Control 2	7/9	(0.614) [0.232]		3/7	2/7
5-FU	6/8	1.353 ^b (0.652) [0.266]	80.2	2/6	2/6
Control 3	4/8	1.945 ^c (0.306) [0.153]		1/4	2/4
MMC	8/8	0.894 ^c (0.751) [0.266]	46.0	0/8	0/8
Control (total) ^d	18/30			6/18	7/18

^a Data are shown as mean tumor weight in grams. Numbers in parens, SD. Numbers in brackets, SEM.

^b Not significant.

^c $P < 0.025$ by Student's *t* test.

^d Summary of control 1, 2, and 3.

Table 2 *In vitro* chemosensitivity of PANC-4 determined by the histoculture drug-response assay with the MTT end point

The inhibition rate (%) was calculated as shown in "Materials and Methods." The IC₅₀ values of drugs was calculated using concentration effect data with the steepest slope determined by linear regression. Cutoff concentration used were 300 and 7.5 µg/ml for 5-FU and MMC, respectively (please see text for details). Data are shown as mean (SD) from triplicate determinations.

Drug	IC ₅₀ ^a (µg/ml)	Inhibition rate (%) at cutoff
5-FU	>3000	5.6 (2.8)
MMC	21.6 (4.2)	39.4 (8.8)

^a IC₅₀, concentration of each drug which lowered tumor cell MTT reduction by 50%.

DISCUSSION

Orthotopic transplantation of histologically intact PANC-4 tumor tissue resulted in extensive local growth, invasion to surrounding organs, and metastases to liver and peritoneum. In other orthotopic transplant models developed by us for colon (16, 17), stomach (18, 19), bladder (20, 21), lung (22), and prostate (23), transplantation of histologically intact tissue results in greater metastatic potential in immunodeficient mice than injection of cell suspensions (17, 18, 20). In this study, we applied this model to experimental treatment, which seemed advantageous compared with other nude mouse models of metastatic human pancreatic cancer.

In routine *in vivo* chemosensitivity assays using s.c. tissue transplantation in nude mice (12), both 5-FU and MMC demonstrated only negligible effects against PANC-4 (data not shown). In the model using orthotopic transplantation, both drugs failed to score positive effects against the local tumor, according to currently used criteria which determine a positive effect as that lowering the mean local tumor weight by 42% (12). However, MMC showed preventive effects against liver and peritoneal metastases, which might be produced from more rapidly growing and therefore possibly more chemosensitive cell subpopulations of this line (24), possibly resulting in an improved survival rate of the mice treated with MMC. In contrast, 5-FU showed only minimal antitumor effect on local tumor growth, resulting in 80% of control growth in terms of actual tumor weight, and mice treated with 5-FU had essentially an equivalent incidence of liver and peritoneal metastases in comparison with the control mice. Therefore, drugs selected according to the results of the *in vitro* HDRA seemed to have potential to prevent metastasis of human

pancreatic cancer in our orthotopic transplant model. This response could not have been evaluated with the currently used s.c. implantation nude mouse models, where metastasis rarely occurs.

Thus, the metastatic model of human pancreatic cancer using orthotopic transplantation of histologically intact tumor tissue in conjunction with the HDRA *in vitro* model provides a paradigm for treatment of pancreatic cancer both at the level of local growth and metastasis.

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