An Orthotopic Model of Human Pancreatic Cancer in Severe Combined Immunodeficient Mice: Potential Application for Preclinical Studies

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ABSTRACT
Pancreatic adenocarcinoma is one of the most incurable and least understood of all cancers. It is the fourth leading cause of cancer-related mortality in males (after lung, prostate, and colon) in the United States with <2-3% of patients surviving >5 years. In an attempt to search for more effective therapies for this disease, we report here, for the first time, an effective treatment, the combination of gemcitabine and auristatin-phenethylamine (PE), against an orthotopic implantation of a human pancreatic adenocarcinoma cell line (HPAC) in severe combined immunodeficient (SCID) mice. Tumor implantation was performed by injecting 100 μl of the HPAC cell suspension (1 × 10⁶ cells) directly into the pancreas of 5-week-old SCID mice. After implantation, tumor formation was checked twice a week. All palpable tumors were detected within 21 days (100% take rate), and tumors were confirmed histologically to be pancreatic adenocarcinoma. For the subsequent efficacy trial, tumor-bearing SCID mice were randomized into four groups with five mice in each group. One served as a control, the second received gemcitabine alone (2.5 mg/kg/injection i.p.), the third received auristatin-PE alone (2.0 mg/kg/injection i.v.), and the fourth group received the combination of gemcitabine (i.p.) and auristatin-PE (1.5 mg/kg/injection i.v.). All animals were euthanized 7 days after the completion of their treatments, and the pancreases were resected. Histological examination revealed the tumors to be adenocarcinoma. The tumors were composed of diffuse sheets of cells interrupted by glandular spaces containing secretory material. Cytologically, the tumor cells were large, pleomorphic, and hyperchromatic. Many cells contained intracellular lumina containing mucin. Immunohistochemical studies showed strong p21WAF (p21) expression but no immunoreactivity with p53 and Her-2/neu antibodies. The mean pancreatic weight in the gemcitabine/auristatin-PE combination group was significantly (P = 0.014) lower (0.84 ± 0.639 g) when compared with those of the control (2.91 ± 1.19 g) and gemcitabine alone (1.84 ± 0.796 g; P = 0.064) groups. In addition, the mean weight in the combination group approached statistical significance when compared with the auristatin-PE group alone (1.16 ± 0.635 g; P = 0.028). We conclude that the combination of gemcitabine and auristatin-PE is an effective treatment against HPAC tumors in this xenograft model and more effective than treatment with either gemcitabine or auristatin-PE alone and could be considered for future animal studies with pancreas cancer and/or for human clinical trials.

INTRODUCTION
Adenocarcinoma of the pancreas is presently estimated to be the fourth leading cause of cancer death for males (after lung, prostate, and colon) and females (after lung, breast, and colon) in the United States (1–3), with an average survival of approximately 6 months (4). Its etiology is unknown, and no generally effective treatment is presently available. Overall, the incidence is decreasing in whites and increasing in blacks, especially in black females, with probability of death reaching 95%. Present treatment of localized adenocarcinoma of the pancreas is limited to surgical and chemo-radiotherapeutic options; however, these options have not made a significant impact on the course of this disease. Less than 2-5% of patients are alive at 5 years. Systemic chemotherapy usually consists of 5-fluorouracil or 5-fluorouracil-containing regimens (5, 6). However, none of these therapies are effective (curative). Newer chemotherapeutic agents such as gemcitabine hydrochloride (2',2'-difluorodeoxy-cytidine) have shown antitumor activity against a variety of solid tumors (7, 8), including pancreatic tumors, but the prognosis and overall survival remain poor. For example, a recently completed Phase II trial of gemcitabine in 5-fluorouracil-refractory cancer showed a response rate of only 27%, with median...
duration of 14 weeks and median survival of 3.85 months (9). Therefore, the search for new therapies continues.

As part of the National Cancer Institute natural products program, a number of novel agents with anti-solid tumor activity derived from marine products have been identified. The dolastatins represent one such group of novel agents. Dolastatin 10 was isolated from the shell-less marine mollusk Dolabella auricularia (sea hare) in 1984 and reported in 1987 (10). It is a linear tetrapeptide (with three unique amino acids) linked to a complex primary amine (11) that interacts with tubulin to inhibit microtubule polymerization (12). Auristatin-PE is a structural analogue of dolastatin 10 with the dolaphenine unit substituted in SCID or nude mice has proven useful in the study of the preclinical efficacy of novel therapeutic agents in vivo. Although most human malignant tumors grow well as xenografts in nude or SCID mice in the s.c. site, they seldom metastasize, despite their malignant characteristics (14, 15). To mimic the pattern of local growth of pancreatic cancer in the pancreas organ, the implantation of human pancreatic tumor cell line in the pancreas of nude mice (orthotopic implantation) was found to reproduce local and distal dissemination (16–18) with a 100% take rate and retention of tumor metastatic behavior in 50–100% of nude mice (19, 20). However, an orthotopic model of human pancreatic cancer in the SCID mouse has not been documented.

In this study, we investigated the antitumor effect of gemcitabine and its combination with auristatin-PE in a SCID mouse orthotopic xenograft model using the HPAC cell line.

**MATERIALS AND METHODS**

**Cell Line and Culture.** The HPAC (21) cell line used in this study was obtained from American Type Culture Collection (Bethesda, MD). The cell line was cultured in DMEM:F12 (1:2) medium supplemented with 5% fetal bovine serum, 15 mM HEPES, 2 μg/ml insulin, 5 μg/ml transferrin, 40 ng/ml hydrocortisone, and 10 ng/ml epidermal growth factor. The HPAC cells were incubated in a humidified 5% CO₂ incubator at 37°C. The medium was replaced with fresh medium as needed, and cells were maintained by serial passaging after trypsinization.

**Tested Agents.** Auristatin-PE is a new structural modification of dolastatin 10, which was isolated from the sea hare Dolabella auricularia (10). Auristatin-PE was dissolved in DMSO at 3–10 mg/ml, and it was used at either 2.0 mg/kg/i.v. or 1.5 mg/kg/i.v. Gemcitabine (Eli Lilly & Co., Indianapolis, IN) was used at 2.5 mg/kg/i.v.

**Laboratory Animals.** Five-week-old female ICR-SCID mice weighing 20–22 g were obtained from Taconic Laboratory (Germantown, NY) and used to develop an orthotopic model bearing the HPAC cells. The mice were acclimatized and housed in a sterile environment where cages, bedding, food, and water were autoclaved.

**Orthotopic Model.** HPAC cells were suspended in serum-free RPMI 1640 at a concentration of 10⁷ cells/ml. The viability of the cells was >90%. Five SCID mice were used to determine the take rate. General anesthesia was performed using ketamine (70 mg/kg i.p.) + xylazine (6 mg/kg i.p.). A left lateral laparotomy was performed, and the spleen and distal pancreas were mobilized. Approximately 100 μl of the HPAC cell suspension (~10⁶ cells) were injected into the pancreas. The abdominal incision was closed using a surgical staple, and analgesia was administered for immediate pain relief. After implantation, the SCID mice were inspected daily for 1 week for any bleeding or wound complications and were checked for tumor formation twice a week by palpation. Palpable tumors were detected after 21 days in all of the mice (100% take rate) and were confirmed histologically to be pancreatic adenocarcinoma.

**Histological Characterization of HPAC Cells.** Tumors grown in the pancreas of untreated SCID mice were excised, fixed in formalin, and embedded in paraffin. Five-μm tissue sections were obtained and stained with H&E for routine microscopic examination. Mucicarmine histochemical stain was used to determine the presence of mucin production by the tumor cells.

Immunohistochemistry was performed using antibodies to Her-2/neu (Zymed, San Francisco, CA; 1:10 dilution), p21WAF1 (Oncogene Research Products, Cambridge, MA; 1:10 dilution), and p53 (DO7; Vector Laboratories, Burlingame, CA; 1:100 dilution) to investigate the expression of these growth factor receptor and cell cycle regulators. For immunohistochemical staining, 5-μm sections from formalin-fixed, paraffin-embedded tissues were mounted on sialinized slides and incubated in a 60°C oven for 60 min. The sections were deparaffinized in xylene, rehydrated in 100% and 95% ethanol, respectively, dipped in 3% hydrogen peroxide to block endogenous peroxidases, and microwaved in a 1-liter citrate buffer for antigen retrieval. The slides were then rinsed in water and in buffered horse serum solution for 10 min to block nonspecific protein binding and subsequently incubated with appropriate antibodies.

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<tr>
<th>Table 1</th>
<th>Efficacy of gemcitabine and its combination with auristatin-PE in orthotopic xenografts of the HPAC cell line</th>
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<tr>
<td>Agent</td>
<td>Dose (mg/kg/injection)</td>
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<tr>
<td>Diluent</td>
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<td>Gemcitabine</td>
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<td>Auristatin-PE</td>
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<td>Gemcitabine</td>
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\[3^\text{The abbreviations used are: PE, phenethylamine; SCID, severe combined immunodeficient; HPAC, human pancreatic adenocarcinoma.}\]
at room temperature for 30 min, rinsed three times with modified PBS, and reincubated with biotinylated secondary antibody, rinsed again, and incubated with avidin-peroxidase solution for 20 min at room temperature. Finally, the slides were rinsed in water, stained in AEC substrate solution for 20 min, counterstained with hematoxylin for 3–5 min, and blued in ammonia water (0.2% ammonium hydroxide) for 10 s.

**Efficacy Trial Design.** For the subsequent drug efficacy trials, the mice were randomly removed and assigned to four treatment groups after 7 days from implantation (before implanted cells developed into palpable tumors) as shown in Table 1. Five mice served as the control, and five mice were treated with gemcitabine (2.5 mg/kg/injection) given i.p. daily 5 days/week for 2 weeks. The third group was treated with auristatin-PE alone (2.0 mg/kg/injection) given i.v. (via the tail vein) every other day for a total of three injections, and the fourth group was treated with the combination of gemcitabine (i.p.) plus auristatin-PE at 1.5 mg/kg/injection (Table 1). Doses were determined based on previous studies with these agents (22). It is important to note that the drug doses of auristatin-PE were reduced from 2.0 mg/kg/injection to 1.5 mg/kg/injection for combination treatment to avoid toxicity. All animals were euthanized 1 week after the completion of the treatment. The whole pancreas containing tumors were surgically removed from all animals, and their weights were recorded. Tumors were divided into two halves; one-half was frozen, and the other half was fixed in neutral buffered formalin for pathological examination.

**RESULTS**

**Establishment and Characterization of the HPAC Orthotopic Xenografts.** All SCID mice, injected (implanted) with HPAC cells, developed palpable tumors (100% take rate) within 21 days after implantation (Fig. 1), which were found to be adenocarcinoma. The tumors excised from the mice were examined initially under routine light microscopy. Histologically, the tumor cells formed viable nodular masses with variable sizes. They were relatively well circumscribed with pushing borders, compressing the adjacent pancreas (Fig. 2A). Uninvolved spleen and pancreas were unremarkable, with no evidence of local or distant metastases. The tumors were composed of diffuse sheets of cells, frequently interrupted by glandular spaces that contained secretory material (Fig. 2B), a common finding in adenocarcinomas. Cytologically, the cells were large, pleomorphic, and hyperchromatic, and the chromatin was clumped and irregular, all features of malignant cells. Many cells contained intracellular lumina containing mucin as shown by the Mucicarmine stain (Fig. 3A). This stain also highlighted the abundance of the glands within the tumor. In contrast, the tumors grown s.c. showed better differentiation, as reported in our previous study (22). They showed multilobular, cystic growth pattern with occasional villous, papillary projections. Cytologically, the cells were blend, elongated (cigar shaped), and well polarized. Chromatin was relatively fine, and the nucleoli were less prominent. They also had abundant cytoplasm. The overall appearance of these cells lining the cysts was more akin to that of intestinal-type adenocarcinoma (data not shown). The p21WAF1 immunostaining of orthotopic tumors showed strong and diffuse positivity of the tumor cell nuclei (Fig. 3B), whereas no immunoreactivity was observed for p53 and Her-2/neu.

Microscopic examination of the cell line grown in culture also showed sheets of epithelial cells with focal clumping, reminiscent of gland formation. In these foci of clumping, tumor cells also showed distinctive vacuolization of the cytoplasm (Fig. 4A), some of which contained mucin (Fig. 4B), as assessed by Mucicarmine stain, indicating glandular differentiation as indicated above.

**Orthotopic Xenografts and in Vivo Drug Efficacy.** Drug efficacy trials were conducted on orthotopic xenografts implanted with HPAC cells 7 days after surgery. Table 1 shows...
the antitumor activity of gemcitabine, auristatin-PE, and their combinations, compared with that of the control mice (receiving only diluent), *in vivo*. Results indicate that gemcitabine inhibited the growth of the HPAC tumor significantly with mean tumor weight and SD of $1.84 \pm 0.796$ g ($P = 0.15$), compared with the control group $2.91 \pm 1.19$ g ($P = 0.028$). Similarly, the mean tumor weight in the auristatin-PE-treated group was lower ($1.16 \pm 0.635$ g, $P = 0.028$) compared with untreated control. Moreover, when gemcitabine (i.p.) was administered with auristatin-PE (i.v.) in combination, the tumor weight significantly...
Fig. 3  A, Mucicarmine histochemical stain shows the glandular spaces, and intracellular lumina contain abundant mucin (red), a pathognomonic finding of adenocarcinomas. Adjacent acinar pancreatic tissue is devoid of mucin, as expected.  

B, immunohistochemical stain with p21 antibody shows strong and diffuse positivity (brown) of the tumor cell nuclei. Mitotic figures were prominent (arrows).

decreased to 0.84 ± 0.639 g (P = 0.014) when compared with the control, but it was statistically insignificant when compared with either gemcitabine alone (P = 0.44) or auristatin-PE alone (P = 0.064). It is important to note that the concentration of auristatin-PE was higher in the single-treatment group compared with the treatment combination group. Although there was a different range of tumor weights in controls (1.4–4.3 g), gemcitabine (0.9–2.8 g), auristatin-PE (10.6–2.2 g), and gemcitab-
Fig. 4  This photomicrograph of the cell line shows a diffuse sheet of epithelial cells with focal clumping. At the center of the slide (A) is a large malignant cell with distinctive vacuolization of the cytoplasm, evidence of abortive glandular differentiation. Some cells in the clumped foci contain intracellular mucin (B, arrow), further evidence of glandular differentiation (adenocarcinoma; mucinous stain).

Orthotopic Model of Human Pancreatic Adenocarcinoma

In an attempt to search for more effective therapies for the cancer of the pancreas, we report the results of a preliminary study using a combination of gemcitabine and auristatin-PE against an orthotopic implantation of a HPAC cell line in a SCID mouse model. Gemcitabine given concurrently with auristatin-PE resulted in significantly higher antitumor activity compared with control or gemcitabine alone.

In this study, histological examination of all tumors from control and treated pancreas showed carcinoma (Fig. 2) with focal gland formation (Fig. 2B) and intracellular as well as luminal mucin production (Fig. 3A), characteristic features of adenocarcinomas, including pancreatic ductal adenocarcinoma. Microscopic examination of the cultured cell line also showed features of adenocarcinoma with focal vacuolization of the tumor cells (Fig. 4A) reminiscent of intracellular lumina (gland) formation and mucin-positive globules (Fig. 4B). The present study was designed to develop an orthotopic model in SCID mice with human pancreas cancer cells that grow in culture and show local growth when implanted in the orthotopic site without metastasis as a cleaner system prior to addressing the issue of metastasis. Therefore, these tumors did not show any local or distant metastases, which is due to the nature of the HPAC cell line rather than the orthotopic site. Similar observations have been reported recently by Reyes et al. (20). Although p53 and Her-2/neu were negative, p21WAF1 showed diffuse and strong positivity (Fig. 3B) of the tumor cell nuclei, which correlates with the high-grade appearance of tumor cell nuclei (23). The tumors grown in the s.c. site, however, showed a different growth pattern and differentiation. Cyst and gland formations were much more prominent, and the cytology of the cells was of well-differentiated adenocarcinoma, as demonstrated earlier (22). In contrast, tumors grown orthotopically (Fig. 2) appeared poorly differentiated, strongly suggesting the effects of tropic factors in tumor growth.

The primary purpose of our study was to develop more effective treatment modalities by evaluating novel therapeutic agents against human pancreatic cancer using an orthotopic model. This model is believed to be more clinically relevant for drug evaluation compared with other models with the s.c. implantation of pancreatic cells. Although most malignant cells grow s.c. as xenografts in animals, they seldom metastasize, despite the malignant nature of the original human tumors (14, 15). The tumors grown in the s.c. site, however, showed a different growth pattern and differentiation. Cyst and gland formations were much more prominent, and the cytology of the cells was of well-differentiated adenocarcinoma, as demonstrated earlier (22). In contrast, tumors grown orthotopically (Fig. 2) appeared poorly differentiated, strongly suggesting the effects of tropic factors in tumor growth.

The primary purpose of our study was to develop more effective treatment modalities by evaluating novel therapeutic agents against human pancreatic cancer using an orthotopic model. This model is believed to be more clinically relevant for drug evaluation compared with other models with the s.c. implantation of pancreatic cells. Although most malignant cells grow s.c. as xenografts in animals, they seldom metastasize, despite the malignant nature of the original human tumors (14, 15). Most of the orthotopic transplantation of human pancreatic cancer used either primary pancreatic carcinomas taken from patients or established cell lines to study local and distant metastases (14–20) in nude mice; however, no such studies are reported in SCID mice. Malignant human tumors implanted s.c. into SCID mice rarely metastasize; thus, it may not be a suitable model for drug efficacy trials prior to human therapy, although Marincola et al. (17) showed that adoptive immunotherapy with human lymphokine-activated killer cells and human recombinant interleukin 2 is an effective therapy against human pancreatic cancer growing s.c. in nude mice. Thus, the development of an orthotopic model of human pancreatic tumor is desirable, and future clinical trials using the combination of gemcitabine with auristatin-PE.
our preliminary results are very encouraging. Because most if not all human pancreas cancer show evidence of local or distant metastases at the time of diagnosis, orthotopic tumors of HPAC cells may not be the ideal model of human pancreas cancer. Similar results were also discussed in a recent report (20), where 50% of the primary tumors implanted orthotopically showed local and distal metastases, although all tumors used in this study caused disseminated disease in patients. Because the present study was designed to have a system without complications of metastases, our future studies will focus on the development of an orthotopic SCID mouse tumor model with human tumors that will show local and distant dissemination for clinical therapy prior to human clinical trials with novel chemotherapeutic agents.

Among the rapidly increasing number of marine invertebrate-derived antineoplastic agents against solid tumors, dolastatin 10 has been selected for future clinical development. Auristatin-PE is an improved analogue that is closely related structurally and in which the dolaphenine unit is replaced by a PE group (13). Dolastatin 10 is very effective in inhibiting tubulin polymerization at a lower concentration compared with vincristine (24) and has additional mechanisms of action on tubulin that are quite different from those caused by Vinca alkaloids (12, 25). Because of the promising preclinical data of gemcitabine and that of auristatin-PE (data not shown) against the KCI-MOH1 pancreatic xenograft model (22), we attempted to study the antitumor activity of gemcitabine alone, auristatin-PE alone, and their combinations in a SCID orthotopic model bearing HPAC tumors. Our data clearly reveal that auristatin-PE and gemcitabine alone inhibit the growth of the HPAC tumor significantly with a mean weight of the pancreas by about 60% (tumor weight of 1.16 ± 0.635 g and 1.84 ± 0.796 g in treated groups, respectively, versus 2.91 ± 1.19 g in the untreated control group). Moreover, when gemcitabine was administered (i.p.) concurrently with auristatin-PE (i.v.), the weight significantly decreased by 72% (0.84 ± 0.639 g in the treated group versus 2.91 ± 1.19 g in the control group), indicating a greater efficacy of the combination therapy.

An inherent question in any preclinical model is its relevance to the treatment of the actual human disease and whether animals with s.c. implantation respond to treatment differently than in the orthotopic implantation. Previously, our group (22) reported that gemcitabine treatment (2.5 mg/kg/injection) was active against a human pancreatic cell line (KCI-MOH1) implanted s.c. However, all animals bearing KCI-MOH1 tumors died after the 10th injection of auristatin-PE. In this study, we reduced the dose and changed the injection schedule of the auristatin-PE for both single and combination treatment groups. In addition, we chose to give gemcitabine i.p. rather than i.v., based on our many trial experiments where administration of gemcitabine and auristatin-PE i.v. with multiple injections caused severe technical difficulties (tail vein obstruction), and thus the experiments were terminated. It is well accepted that gemcitabine is an effective agent when administered i.p., which showed promising results in our present study. However, studies are under way in our laboratory to address this issue, and we should be able to design future studies to administer gemcitabine and auristatin-PE through i.v. injections to validate our preliminary results in this orthotopic model.

In conclusion, our preliminary data suggest that the combination of gemcitabine and auristatin-PE is an effective treatment against HPAC cells grown as xenografts in this SCID mouse model and that the combination treatment is more effective than treatment with gemcitabine or auristatin-PE alone. Undoubtedly, further animal studies are needed where orthotopic tumor size can be measured effectively (by magnetic resonance imaging or other imaging techniques) prior to therapy to establish the full efficacy of treatment in the survival of animals. In addition, orthotopic implantation of tumors from patients showing local and distal dissemination should be an ideal orthotopic model for evaluating novel therapeutic strategy for the successful treatment of human pancreatic tumors. Based on our preliminary results, we suggest that auristatin-PE could be considered for clinical trials in pancreatic cancer and might optimally be given in combination with gemcitabine. However, further chemotherapeutic studies are needed in this animal model using human tumor cell lines showing local and distant metastases prior to human therapy.

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