Interleukin-4 Cytotoxin Therapy Synergizes with Gemcitabine in a Mouse Model of Pancreatic Ductal Adenocarcinoma

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Abstract
Targeting cell surface receptors with cytotoxins or immunotoxins provides a unique opportunity for tumor therapy. Here, we show the efficacy of the combination therapy of gemcitabine with an interleukin-4 (IL-4) cytotoxin composed of IL-4 and truncated Pseudomonas exotoxin in animal models of pancreatic ductal adenocarcinoma (PDA). We have observed that 42 of 70 (60%) tumor samples from patients with PDA express moderate- to high-density surface IL-4 receptor (IL-4R), whereas normal pancreatic samples express no or low-density IL-4R. IL-4 cytotoxin was specifically and highly cytotoxic [50% protein synthesis inhibition (IC50) ranging from 0.1 to 13 ng/mL] to six of eight pancreatic cancer cell lines, whereas no cytotoxicity (IC50 >1,000 ng/mL) was observed in normal human pancreatic duct epithelium cells, fibroblasts, and human umbilical vein endothelial cells (HUVEC). We also showed that IL-4 cytotoxin in combination with gemcitabine exhibited synergistic antitumor activity in vitro. To confirm synergistic antitumor activity in vivo and monitor precise real-time disease progression, we used a novel metastatic and orthotopic mouse model using green fluorescent protein–transfected cancer cells and whole-body imaging system. The combination of both agents caused complete eradication of tumors in 40% of nude mice with small established PDA tumors. In addition, combined treatment significantly prolonged the survival of nude mice bearing day 14 advanced distant metastatic PDA tumors. Similar results were observed in mice xenografted with PDA obtained from a patient undergoing surgical resection. These results indicate that IL-4 cytotoxin combined with gemcitabine may provide effective therapy for the treatment of patients with PDA. [Cancer Res 2007;67(20):9903–12]

Introduction
Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal human malignancies (32,000 deaths per year). Because of its aggressive growth and rapid metastasis to lymph nodes and liver, only 10% to 15% of patients are found to be resectable at diagnosis (1). Currently, the most common strategy for the treatment of advanced pancreatic cancer is treatment with gemcitabine, although the median survival time continues to be <6 months for these patients (2, 3). Recently, several types of inhibitors targeting the epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, and nuclear factor-kB (NF-κB) have shown their effectiveness in pancreatic cancer in murine models (4–7). In clinical trials, EGF receptor tyrosine kinase inhibitor (Erlotinib, Tarceva) plus gemcitabine enhances 1-year survival for patients with advanced pancreatic cancer (8). However, the difference in median survival between Erlotinib plus gemcitabine group and gemcitabine alone group is <1 month. An effective new approach is needed for management of patients with this disease.

Targeting cell surface receptor with cytotoxins or immunotoxins provides a unique opportunity for tumor therapy. Targeted toxins offer the advantage of enhanced specificity and direct toxicity for tumor cells that overexpress the receptor, thus limiting the potential toxicity to normal tissues (9). Several clinical trials using IL-4 cytotoxin, IL-13 cytotoxin, and recombinant immunotoxin BI22 have shown survival benefits in patients with glioblastoma multiforme, chronic lymphocytic leukemia, and hairy cell leukemia (10–13).

Interleukin-4 (IL-4) is an important Th2-derived cytokine, which is involved in mediating antitumor immune-modulating activities (14). IL-4 has been shown to have a modest but direct inhibitory effect on the growth of several tumor cells in vitro and in vivo (15, 16). Based on these properties, IL-4 was tested in the clinic as a treatment for hematopoietic and solid malignancies, but it showed limited antitumor activity (17). To improve this limited activity, we targeted IL-4 receptor (IL-4R) because a variety of human tumor cells, including pancreatic cancer, express high-affinity receptors for IL-4 (18–22). We have created a circular permuted IL-4, which was fused to a mutated form of Pseudomonas exotoxin [the fusion protein termed IL4(38-37)-PE38KDEL or IL-4 cytotoxin; ref. 23]. After binding to IL-4R, IL-4 cytotoxin internalizes, translocates, and then ribosylates elongation factor 2 to prevent the initiation of protein synthesis, leading to cell death (24). The IL-4 cytotoxin is highly and specifically cytotoxic to several types of tumor cells in vitro and has remarkable antitumor activity in animal xenograft models of a variety of human tumors (18–22). The safety and tolerability of IL-4 cytotoxin was shown in phase 1 clinical trials in patients with advanced solid tumors (25). The efficacy of IL-4 cytotoxin was also shown by long-term survival of patients with recurrent malignant glioma (10, 11, 26).

In this study, we examined expression of IL-4R in samples derived from PDA and the efficacy of IL-4 cytotoxin, gemcitabine, and combination of both in primary and metastatic tumor models. To mitigate aggressive clinical situation and to monitor precise real-time disease progression, we used a novel metastatic and orthotopic advanced pancreatic cancer model using retroviral green fluorescent protein (GFP)–transfected pancreatic cancer cell
line and whole-body imaging system (27). Together, our study shows that IL-4 cytotoxin synergizes with gemcitabine, significantly inhibiting the growth of primary and metastatic tumor lesions, prolonging the survival time, and completely eradicating tumors in 40% of mice in an early pancreatic cancer model.

Materials and Methods

Cell culture, reagents, and tissue specimens. Cell lines were obtained from the American Type Culture Collection and Scincell. Human pancreatic duct epithelium (HPDE) cells were cultured routinely in keratinocyte serum-free medium supplemented with bovine pituitary extract and epidermal growth factor (Life Technologies; ref. 28). IL-4 cytotoxin [IL4(38-37)-PE38KDEL] was produced as described previously (23). Fifteen paraffin-embedded tissue sections and tissue arrays containing 70 tumor specimens were obtained from Cooperative Human Tissue Network and U.S. Biomax, respectively. Gemmacebine was procured through the pharmacy of the clinical center (NIH).

Immunohistochemistry and flow cytometry. Immunohistochemistry was done as described previously (24). Deparafinized tissue sections were incubated with anti-human IL-4Rα polyclonal antibody (Santa Cruz Biotechnology) or isotype control (IgG). The results were scored on the basis of the density of staining, 0% to 10%, 11% to 50%, 51% to 100% as negative, weak, moderate, and strong, respectively. Tissue sections for IL-4Rα were evaluated by Dr. Satoru Takahashi who is a pathologist at Nagoya City University in Japan.

Expression of IL-4Rα on pancreatic cancer cell lines and HPDE cells was assessed by flow cytometry using phycoerythrin-conjugated anti-IL-4Rα monoclonal antibody as previously described (29). Staining with isotype-matched IgG served as control.

Protein synthesis inhibition assay and assessment of synergism or antagonism. The in vitro cytotoxic activity of IL-4 cytotoxin, gemcitabine, and their combination was measured by the inhibition of protein synthesis (18).

Drug interaction between IL-4 cytotoxin and gemcitabine was assessed at a concentration ratio of 1:1, using the combination index (CI), where CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively (30). On the basis of the isobologram analysis for mutually exclusive effects, the CI value was calculated as follows:

\[
CI = \frac{(D_1)}{(D_1)_{100}} + \frac{(D_2)}{(D_2)_{100}}
\]

where \((D_1)_{100}\) and \((D_2)_{100}\) are the concentrations of IL-4 cytotoxin and gemcitabine, respectively, required to inhibit cell growth by 50%, and \((D_1)\) and \((D_2)\) are the drug concentrations in combination treatments that also inhibit cell growth by 50% (isosensitive compared with the single drugs).

Semi quantitative and real-time TaqMan reverse transcription-PCR. Semi quantitative reverse transcription-PCR (RT-PCR) was done as described previously (31). Quantification of IL-4Rα mRNA expression levels in pancreatic cancer cell lines was determined by real-time RT-PCR using a set of IL-4Rα-specific TaqMan probe (5′-FAM, 3′-MGB) and primers (Applied Biosystems; ref. 24). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase or β-actin before the fold change in gene expression was calculated.

Retroviral transduction and selection of high-GFP-expressing MIA PaCa-2 pancreatic cancer cells. MIA PaCa-2 cells expressing GFP were established using a 1:1 precipitated mixture of retroviral supernatants of the HEK293 cells and RPMI 1640 (Life Technologies, Inc.), as described previously (32).

Animals. Severe combined immunodeficient (SCID) mice and nude nu/nu mice between age 5 and 6 weeks were maintained in a barrier facility on HEPA-filtered racks. All animal studies were conducted under an approved protocol in accordance with the principles and procedures outlined in the NIH Guideline for the Care and Use of Laboratory Animals.

Whole-body imaging. The tumor-bearing mice were periodically examined in a fluorescence light box illuminated by fiberoptic light at 440/20 nm wavelength (Lighttools Research, Inc.). Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology) on a Hamamatsu C5810 3-chip cooled color charge coupled device camera (Hamamatsu Photonics Systems). Real-time determination of tumor burden was done by quantifying fluorescent surface area as described previously (32).

Surgical orthotopic implantation of MIA PaCa-2-GFP tumors. MIA PaCa-2-GFP cells were injected s.c. into the right dorsal flank of nude mice. Pancreatic tumors, grown s.c. in nude mice, were cut with scissors and minced into ~3 × 3 × 3-mm pieces. For orthotopic surgery, the pancreas was carefully exposed, and tumor chunks were transplanted on the middle of the pancreas with a 6-0 Dexon surgical suture (Davis-Geck, Inc.). The pancreas was then returned to the peritoneal cavity, the abdominal wall, and the skin was closed with 6-0 Dexon sutures.

Experimental design and treatment. For early pancreatic cancer model, primary tumor lesions were detected by external whole-body imaging on day 4 after transplantation. Once the tumors were visualized, mice were randomized into four groups of 10 each. Treatment was initiated on day 5. For advanced pancreatic cancer model, primary and metastasis tumor lesions were detected by external whole-body imaging on day 14 posttransplantation of tumor chunk and randomized into four groups of 10 mice each. Treatment was initiated on day 15.

Primary and orthotopic pancreatic cancer model using a clinical sample. Primary pancreatic cancer specimens were obtained from a patient undergoing radical pancreatectomy at National Cancer Institute under institutional review board-approved protocol. Viable tumor tissue from specimen was cut into small pieces (3 × 3 × 3 mm) and implanted in the pancreas of 5- to 6-week-old male SCID mice. Primary xenografts were propagated continuously in SCID mice for in vivo testing. Clinical sample–bearing mice were also treated after day 31 by the same protocol as described above.

Statistical analysis. The mean tumor volume in therapeutic and control groups was analyzed by ANOVA. Survival curves were generated by Kaplan-Meier method and compared by using the log-rank test.

Result

Expression of IL-4Rα in PDA tissues. Tissue sections from 15 normal pancreas and 70 PDA specimens were analyzed by immunohistochemical analysis for the expression of IL-4Rα (data not shown). As shown in Fig. 1A, tumor specimens showed weak to strong staining for IL-4Rα in PDAs. Only weak staining was observed in tumor-infiltrating stromal fibroblasts and endothelial cells. When the proportion of IL-4Rα-positive cancer cells was counted, 23 of 70 primary tumors classified into strong expression group, 19 into moderate expression group, 11 into weak expression group, and 17 into the negative expression group. Thus, 42 of 70 (60%) PDA samples expressed moderate to high-density IL-4Rα. In contrast, only 2 of 15 normal pancreas samples showed weak staining for IL-4Rα in normal acinar and ductal cells (Fig. 1B).

Pancreatic cancer cell lines expressing IL-4Rα are sensitive to IL-4 cytotoxin. We examined the expression of IL-4Rα mRNA by RT-PCR and real-time RT-PCR in eight pancreatic cancer and one normal HPDE cell lines. Six of eight cancer cell lines showed varied density of IL-4Rα mRNA expression, whereas HPAF-II, PK-1, and HPDE cell lines showed no expression (data not shown). Real-time RT-PCR analysis confirmed conventional RT-PCR results and showed that MIA PaCa-2 and SW1990 cell lines expresses highest level of IL-4Rα mRNA, followed by Capan-1, ASPC-1, Panc-1, and HST766T cell lines (Fig. 1C). Flow cytometric analysis confirmed mRNA expression data and showed that IL-4Rα is expressed on the cell surface of three pancreatic cancer cell lines but not in normal HPDE cells (Fig. 1D).
Next, we determined the sensitivity of pancreatic cancer cell lines to IL-4 cytotoxin by protein synthesis inhibition assay, which has been shown to be directly proportional to cell death (19). IL-4 cytotoxin inhibited protein synthesis of pancreatic cancer cell lines in a concentration-dependent manner. MIA-PaCa-2 and SW1990 cell lines were extremely sensitive to the cytotoxin (IC50 0.08 and 0.36 ng/mL, respectively), followed by Capan-1 (IC50 7 ng/mL) and HS766T (IC50 13 ng/mL; Fig. 2A). IC50 in Panc-1 and ASPC-1 cell lines was <10 ng/mL (data not shown). Consistent with the lack of IL-4Rα mRNA expression, HPAF-II and PK-1 cell lines were not sensitive to IL-4 cytotoxin (IC50 > 1,000 ng/mL; data not shown). The cytotoxic activity of IL-4 cytotoxin was neutralized by incubation with an excess of IL-4, suggesting specific cytotoxicity through binding of IL-4 cytotoxin to IL-4Rα (data not shown). We also examined the cytotoxicity of IL-4 cytotoxin in fibroblast, HUVEC, and HPDE cell lines, because some of the specimens revealed weak expression of IL-4Rα in nontumor cells. However, IL-4 cytotoxin was not found to be cytotoxic to these cells (IC50 ≥ 1,000 ng/mL; Fig. 2B). The IL-4 cytotoxin cytotoxic activity correlated with extent of IL-4Rα expression. For example, MIA-PaCa-2 cells showed lowest IC50 and highest density IL-4R expression as determined by flow cytometric and real-time PCR analyses whereas PK-1 cell line showed highest IC50 as this cell line showed undetectable level of mRNA expression. We also used another cytotoxin IL-13 Pseudomonas exotoxin, an IL-13 receptor-specific fusion protein (12), to assess the cytotoxicity to pancreatic cancer cell line. However, IL-13 cytotoxin was not cytotoxic to HPAF-II cells (IC50 ≥ 1,000 ng/mL; data not shown).

Synergistic cytotoxicity of IL-4 cytotoxin and gemcitabine in pancreatic cancer cell lines. Gemcitabine alone mediated a dose-dependent inhibition of protein synthesis with IC50 of 22 nmol/L in MIA-PaCa-2 cells, 3.2 nmol/L in Capan-1 cells, 1,000 nmol/L in SW1990 cells, and 14 nmol/L in HS766T cells (Table 1). When it was combined with IL-4 cytotoxin, the protein synthesis inhibition in...
MIA-PaCa-2 cells was greatly enhanced: IC$_{50}$ of IL-4 cytotoxin became 0.012, 0.001, and 0.00004 ng/mL by adding 0.03, 0.3, and 3 nmol/L gemcitabine, respectively (Fig. 2C). These same phenomena were also observed in SW1990 and Capan-1 cells, but not in HS766T cells (data not shown). The combination index at IC$_{50}$ and IC$_{75}$ (concentration of drug causing 75% inhibition of protein synthesis) in MIA-PaCa-2, SW1990, and Capan-1 cells was <1 at all concentrations of gemcitabine (0.03–30 nmol/L) was assessed by protein synthesis inhibition assay.

In vivo whole-body optical imaging of PDA. We developed pancreatic cancer models to investigate antitumor effects of IL-4 cytotoxin and showed its correlation with imaging studies in vivo. Pancreatic cancer cells were transfected with GFP. Our transfection technique using retroviral vector revealed consistent bright GFP fluorescence of MIA-PaCa-2 cells. There was no significant difference in morphology, growth rate, and sensitivity to IL-4 cytotoxin between parent and GFP-transfected cells (data not shown). GFP-transfected MIA-PaCa-2 tumor chunks were orthotopically transplanted to pancreas of nude mice. These tumor pieces were derived from MIA-PaCa-2-GFP cells transplanted s.c. As shown in Figs. 3A and 4A, GFP fluorescence enabled real-time and sequential whole-body imaging of tumors. Noninvasive quantitative measurements of external visible fluorescent area enabled the construction of in vivo tumor growth curves, which seem to correlate with visible tumor growth (Figs. 3B and 4B).

Complete eradication of tumors by combination of IL-4 cytotoxin and gemcitabine in an early tumor model. Small primary tumor lesions on day 4 after transplantation were observed in all mice by the real-time whole-body imaging (average fluorescent area 26.17 ± 4.19 mm$^2$). Imaging studies on days 14 and 24 confirmed the significant primary tumor growth and metastasis in nontreatment group (Fig. 3A). In contrast, gemcitabine or IL-4 cytotoxin treatment group showed a reduction in the rate of tumor growth, compared with nontreatment group (Fig. 3A and B). The IL-4 cytotoxin treatment group showed no tumor lesions in 6 of 10 mice on day 14, although tumor recurred by day 34. Remarkably, the combination treatment group revealed significant suppression of tumor growth of primary tumor lesions. Tumor lesions were undetectable in all 10 mice on day 14. By day 44, 6 of 10 mice showed local recurrence and distant metastasis as shown in the bottom row of Fig. 3A. The rest of the four mice showed complete eradication of tumor and mice remained tumor-free through day 94 when the experiment was terminated.

Synergistic increase in survival of mice treated with a combination of IL-4 cytotoxin and gemcitabine in an early tumor model. As shown in Fig. 3C, median survival time of animals was 27 days in nontreatment group, whereas it was significantly increased to 54, 64, and 92 days in gemcitabine group (P < 0.0001), IL-4 cytotoxin group (P < 0.0001), and their combination group (P < 0.0001) compared with nontreatment group, respectively. Compared with gemcitabine group, significant prolonged survival time was observed in IL-4 cytotoxin group (P = 0.017) and the combination group (P < 0.0001). Increase in significant survival advantage correlated with tumor area as detected by GFP fluorescence. Prolonged survival time in the combination group was 341% compared with the nontreatment group. In addition, we did not observe any organ toxicity in heart, liver, lung, kidney, and spleen of IL-4 cytotoxin-injected mice evaluated by histologic examination (data not shown).

Real-time imaging of tumor growth of the primary and metastasis lesion in an advanced in vivo model. As ~85% patients with PDA are diagnosed at an advanced stage at initial diagnosis, an advanced PDA in vivo model needs to be established to imitate the clinical situation and to monitor the disease and treatment effect (33). Fluorescence imaging on day 14 posttransplantation confirmed the tumor growth of primary lesions in all mice and also detected the metastasis lesions to liver, lymph nodes, and peritoneal locations in 40 of 62 mice. Six mice showed metastatic lesions to liver or lymph nodes around hepatoduodenal ligament, 8 showed metastasis lesions corresponding to peritoneal locations, and 26 with both metastasis lesions. We did not include mice with the GFP spot at spleen as a metastasis group. Forty mice with confirmed primary and metastasis tumor lesions on day 14 posttransplantation were divided into four groups and treated as described in Materials and Methods (average fluorescent area 94.67 ± 8.31 mm$^2$). The real-time whole-body imaging of tumor growth confirmed the significant primary tumor growth and metastatic spread on days 14, 21, and 28 after transplantation of tumor in nontreatment control group (Fig. 4A). Gemcitabine and IL-4 cytotoxin treatment group showed a reduction in the rate of
tumor growth compared with the nontreatment group (Fig. 4B). Especially, the combination treatment group revealed significant suppression of tumor growth at primary and metastasis tumor lesions. The reduction in tumor size on day 28 was 39.8% in the gemcitabine group ($P < 0.001$), 71.2% in the IL-4 cytotoxin group ($P < 0.001$), and 79.6% in the combination group ($P < 0.001$) compared with the no treatment group.

**Combination of IL-4 cytotoxin and gemcitabine prolongs the survival of mice with advanced orthotopic pancreatic tumor.** We examined the efficacy of IL-4 cytotoxin on survival of animals in an advanced PDA model. As shown in Fig. 4C, median survival time of animals was 28 days in nontreatment group, whereas it was significantly increased to 34, 43, and 52 days in gemcitabine group ($P = 0.0089$), IL-4 cytotoxin group ($P < 0.0001$), and their combination group ($P < 0.0001$), respectively. Compared with gemcitabine group, significant prolonged survival time was also observed in the IL-4 cytotoxin group ($P = 0.0047$) and the combination group ($P = 0.0002$). Prolonged survival time in the combination group was 186% compared with the nontreatment group. Increase in significant prolongation of survival correlated with tumor area as detected by whole-body imaging.

**Expression of IL-4R in a clinical sample and development of orthotopic xenograft tumor model.** We obtained a tumor tissue sample that was surgically resected at Surgery Branch at NIH and pathologically diagnosed as moderately differentiated adenocarcinoma (data not shown). This tumor section showed strong staining for IL-4Rα in the ductal adenocarcinoma cells and faint staining of fibroblasts (Fig. 5A). We also established tumor and fibroblast cells cultured from this sample to examine the antitumor activity of IL-4 cytotoxin. The cancer cells expressing IL-4R were highly sensitive to IL-4 cytotoxin ($IC_{50}$ 0.32 ng/mL), whereas fibroblast cells were not sensitive ($IC_{50} \geq 1,000$ ng/mL; Fig. 5B).

**IL-4 cytotoxin, gemcitabine, and their combination significantly prolonged survival of mice transplanted with a clinical pancreatic cancer sample.** The clinical sample was orthotopically transplanted on the pancreas of SCID mice and when tumors grew, they were harvested and then orthotopically propagated in the next set of SCID mice. All mice showed growth of primary tumor and metastasis to lymph nodes in peritoneum, hepatoduodenum ligament, and para-aortic areas. Seventy-five percent of these mice showed the metastasis lesion to liver when mice were sacrificed 30 days after tumor implantation (Fig. 5C). To assess the effect of IL-4 cytotoxin in an advanced metastasis model, a third set of SCID mice were orthotopically implanted with tumor pieces obtained from the second set of mice. These mice, when advanced disease developed, were divided into four groups on day 31 and treated as described in Materials and Methods. As shown in Fig. 5D, median survival time of animals was 62 days in the nontreatment group, whereas it was significantly increased to 86, 102, and 134 days in the gemcitabine group ($P = 0.0081$), IL-4 cytotoxin group ($P = 0.0006$), and combination group ($P < 0.0001$), respectively. Compared with gemcitabine, significant prolonged survival time

### Table 1. Cytotoxicity of IL-4 cytotoxin, gemcitabine, and their combination in pancreatic cancer cell lines

<table>
<thead>
<tr>
<th>Cancer cell line</th>
<th>Drug combination</th>
<th>IC$_{50}$</th>
<th>IC$_{75}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIA-PaCa-2</td>
<td>IL-4 cytotoxin + gemcitabine 0.03 nmol/L</td>
<td>0.153</td>
<td>0.563</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 0.3 nmol/L</td>
<td>0.0336</td>
<td>0.094</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 3 nmol/L</td>
<td>0.137</td>
<td>0.0138</td>
</tr>
<tr>
<td>Capan-1</td>
<td>IL-4 cytotoxin + gemcitabine 0.003 nmol/L</td>
<td>0.287</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 0.03 nmol/L</td>
<td>0.026</td>
<td>0.0713</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 0.3 nmol/L</td>
<td>0.096</td>
<td>0.0603</td>
</tr>
<tr>
<td>SW1990</td>
<td>IL-4 cytotoxin + gemcitabine 0.003 nmol/L</td>
<td>0.938</td>
<td>0.401</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 0.3 nmol/L</td>
<td>0.5</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 0.03 nmol/L</td>
<td>0.27</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 0.3 nmol/L</td>
<td>0.021</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 30 nmol/L</td>
<td>0.014</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>IL-4 cytotoxin + gemcitabine 300 nmol/L</td>
<td>0.03</td>
<td>0.11</td>
</tr>
</tbody>
</table>

NOTE: CI $< 1$, CI = 1, and CI $> 1$ indicate synergistic, additive, and antagonistic effects, respectively.

* Fifty percent protein synthesis inhibition.

† Seventy-five percent protein synthesis inhibition.

CI values were calculated using the formula described in Materials and Methods.
was observed of IL-4 cytotoxin–treated mice ($P = 0.0037$) and the combination group ($P < 0.0001$). Prolonged survival time in the combination group was 216% compared with the nontreatment group.

**Discussion**

We show that 60% PDA samples express moderate- to high-density surface IL-4Rs, whereas normal pancreas express no or very low levels of IL-4R. IL-4 cytotoxin is highly cytotoxic to pancreatic cancer cell lines; however, it was not cytotoxic to HPDE cells, fibroblasts, and HUVEC, which express no or low levels of IL-4R. We also show that IL-4 cytotoxin synergizes with gemcitabine in mediating cytotoxic activity in pancreatic cancer cell lines in vitro, and in animal models of human pancreatic cancer in vivo. A significant prolonged survival effect of IL-4 cytotoxin and its combination with gemcitabine was shown in mice with early disease. Forty percent of mice that received combination therapy showed complete eradication of pancreatic tumors. In addition, this significant survival benefit was also confirmed in animals implanted with the clinical pancreatic cancer sample.

Gemcitabine (Gemzar) is a widely accepted first-line therapy for advanced pancreatic cancer, although the median survival time continues to be <6 months for these patients (2, 3). As most studies using single agent show low response rate and little effect on patient survival in advanced adenocarcinoma, several clinical trials using a combined approach of radiotherapy and/or molecular target therapy with gemcitabine have been initiated (33). In vitro studies have reported synergistic effect of gemcitabine with cisplatin, fluvastatin hydroxymethylglutaryl-CoA reductase inhibitor, CpG-oligodeoxynucleotides, EGFR, PDGF, and vascular endothelial growth factor inhibitor targeting drugs (6, 34–37). In
addition, immunotoxins were shown to exert synergistic effect with chemotherapeutic drugs, for example, doxorubicin plus anti-B4-blocked ricin, Ara-C plus granulocyte macrophage colony-stimulating factor fused to truncated diphtheria toxin (DT388-GM-CSF), and fludarabine with rituximab saporin-S6 conjugated protein (38–40). These studies support our observations of gemcitabine synergizing with IL-4 cytotoxin. Despite synergistic effect with gemcitabine, few combinations have shown clinical advantage (4–7). For example, although EGFR inhibitor showed synergistic antitumor effect in preclinical models, the survival benefits for patients with advanced pancreatic cancer seem very modest at best. It was later found that mutations in the EGFR gene, which correlate with clinical response, are found in <5% of pancreatic cancer patients (8, 41). Therefore, new effective therapies that do not depend on receptor mutation are needed. As our results show the survival benefit by IL-4 cytotoxin when combined with gemcitabine in both early and advanced pancreatic cancer models, it is possible that this novel approach will afford better tumor responses than previously observed.

The precise mechanism of synergistic effect of gemcitabine with IL-4 cytotoxin is not known. Gemcitabine is a synthetic pyrimidine antimetabolite structurally related to cytarabine (42). Gemcitabine inhibits DNA synthesis through inhibition of ribonucleotide reductase and depletion of deoxynucleotide pools. On the other hand, IL-4 cytotoxin inhibits protein synthesis after internalization into an endosome. In addition, we have previously shown that IL-4 cytotoxin can cause apoptotic cell death of cancer cells regardless of the cell cycle status (43). It is possible that gemcitabine enhances apoptotic cell death induced by IL-4 cytotoxin. Because apoptosis is a prominent mechanism of cancer cell death, the combination therapy of these drugs, which act through different mechanism, may be a beneficial treatment option for patients with PDA.

We studied two types of advanced pancreatic cancer models to show the antitumor activity of IL-4 cytotoxin and gemcitabine. In orthotopic model, the freshly resected clinical tumor was implanted to pancreas of SCID mice. It has been reported that this model recapitulates the natural history of the clinical disease.

**Figure 4.** Sequential whole-body imaging, quantification of tumor volume, and real-time analysis of pancreatic tumor growth in an advanced tumor model. A, sequential in vivo imaging of tumor progression over time in advanced pancreatic cancer model. Selective tumor GFP fluorescence facilitated real-time visualization of tumor burden in live animals. Panels depict a representative mouse from each of four groups. Each group had 10 mice. Treatment was done after the confirmation of metastasis lesions on days 14. Images are captured every week after day 14. Group 1 served as the negative control and did not receive any treatment. Group 2 received gemcitabine (150 mg/kg) by i.p. administration twice a week as long as the experiment lasted. Group 3 received IL-4 cytotoxin (100 μg/kg) by i.p. route twice a day for 5 d. Group 4 animals were treated with the combination of gemcitabine and IL-4 cytotoxin. B, quantification of primary and metastatic tumor GFP fluorescence similar to Fig. 3B. C, Kaplan-Meier survival curves (n = 10 mice) with advanced PDA after treatment with gemcitabine (150 mg/kg, twice a week), IL-4 cytotoxin (100 μg/kg, twice a day during days 15–19), and their combination.
including the invasive and metastatic pattern (44). Accordingly, the peritoneal organs, lymph nodes, liver, and spleen of mice in our model showed tumor metastasis and invasion 1 month after transplantation. IL-4 cytotoxin and gemcitabine showed remarkable antitumor effects in this model. In future studies, it will be of interest to determine whether metastatic lesions to various organs express IL-4R, and after treatment with IL-4 cytotoxin these receptor levels decrease along with disappearing tumor. In other orthotopic tumor model, tumor pieces developed from MIPaCa-2-GFP cells by s.c. is implanted to pancreas of nude mice. In this model, IL-4 cytotoxin as well as gemcitabine caused profound antitumor effects. These data are compatible with our previous report that showed the survival benefit by IL-4 cytotoxin alone in orthotopic early and advanced animal models using Panc-1 and BxPC-3 pancreatic cancer cell lines (19). Although we did not test IL-4R-negative tumor in vivo models, our previous studies have shown that non–small cell lung cancer cell line expressing no or low IL-4R are not sensitive to IL-4 cytotoxin in vivo (20). Similar conclusions were drawn in squamous cell carcinoma of head and neck tumor models (45). Thus, IL-4 cytotoxin and gemcitabine show better survival benefit compared with either agent alone in two pancreatic tumor models, one derived from clinical sample and the other derived from MIPaCa-2 cell line.

The whole-body imaging of host visualizes the real-time tumor growth at the primary site and tumor development at metastasis sites without the invasive procedures, surgery, anesthesia, or use of contrast medium. Due to the fact that whole-body imaging has the potential of high correlation with MRI in quantifying tumor volume, the precise evaluation of tumor growth rate, metastatic situation, and effectiveness of drugs could all be monitored without sacrificing animals (32, 46). In addition, imaging may identify biomarker of tumor response in preclinical models that can be validated in the clinical trial (47). A recent article reported that red fluorescent protein showed brighter and less background image compared with GFP, when animals were imaged (48). In our study, we used GFP-transfected cells. Therefore, it is possible that we were not able to detect micrometastasis lesions. Nevertheless, we could show that mice developed spontaneous tumor metastasis within the short time after orthotopic transplantation, which correlated with short survival time. In addition, our model showed that IL-4 cytotoxin reduced the rate of tumor growth, including primary and metastasis lesions for 15 and 9 days after treatment in early and advanced model, respectively.

Although IL-4 cytotoxin mediated remarkable antitumor effects in vivo, no visible signs of toxicity and features such as weight loss and inactivity were observed in mice receiving optimal doses of IL-4 cytotoxin and/or gemcitabine (data not shown). These results are compatible with previous studies related to both agents (data not shown; refs. 19, 24, 49). Previous studies have shown that low-density IL-4R are expressed on normal immunologic and non-hematopoietic cells (22). Consequently, IL-4 cytotoxin is not cytotoxic to these cells. Preclinical toxicity studies in mice have shown that IL-4 cytotoxin is well tolerated up to 475 μg/kg dose given i.v. (50). As human IL-4 does not bind murine IL-4R, IL-4...
cytotoxicity has also been administered to cynomolgus monkeys, whose IL-4R binds human IL-4. In these animals, IL-4 cytotoxicity was reasonably tolerated up to a dose of 200 μg/kg given i.v. every alternate day for three injections (21). In a phase 1 clinical trial, reversible elevation of liver enzymes and injection site inflammatory reactions were reported after i.v. administration of IL-4 cytotoxic at 0.027 mg/ml (25). As our study shows synergistic effects when IL-4 cytotoxic is combined with gemcitabine against pancreatic cancer in vitro and in vivo, lower doses of IL-4 cytotoxic may be effective for the treatment of patients with PDAC when combined with gemcitabine.

In conclusion, these studies provide a novel approach for monitoring tumor response by whole-body imaging of the host. Further studies should be done to evaluate the safety, tolerability, and efficacy of IL-4 cytotoxic when combined with gemcitabine in various pancreatic cancer models. In addition, because of their synergistic effect, IL-4 cytotoxic in combination with gemcitabine should be tested in patients with PDAC.

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References


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