Expression and Targeting of Interleukin-4 Receptor for Primary and Advanced Ovarian Cancer Therapy

Mitomu Kioi, Satoru Takahashi, Mariko Kawakami, Koji Kawakami, Robert J. Kreitman, and Raj K. Puri

Abstract

Because the most characteristic property of ovarian cancer is i.p. spread, the majority of patients are diagnosed at an advanced stage, leading to limited availability of options for curative therapies. With an intent to identify targeted therapeutic approaches, we have observed that ~60% of 21 ovarian cancer tissue samples express a high density of interleukin-4 receptor (IL-4R), whereas normal ovarian tissues tested (n = 7) expressed no or low levels of IL-4R. To target IL-4R, we have developed IL-4 cytotoxin, which in circularly-permuted IL-4 is fused to a mutated form of Pseudomonas exotoxin. This cytotoxin is specifically and highly cytotoxic to PA-1, IGROV-1, and SK-OV3 ovarian carcinoma cell lines in vitro. In addition, it shows remarkable antitumor activities against established s.c. ovarian tumors in immunodeficient animals. i.p. administration of IL-4 cytotoxin in mice with orthotopically implanted ovarian tumors caused regression of established tumors and prevented these animals from tumor metastasis. Continuous i.p. infusion of IL-4 cytotoxin prolonged survival of tumor-bearing mice even with bulky disease. These results indicate that IL-4R–targeted cytotoxin may be a useful agent for the management of patients with ovarian cancer, and further studies need to be done to evaluate its safety, tolerability, and efficacy. (Cancer Res 2005; 65(18): 8388-96)

Introduction

Ovarian carcinoma is the fifth leading cause of cancer-related deaths in females and the leading cause of death from gynecologic malignancy in the U.S. Approximately 14,700 patients died in the year 2002 alone. The overall 5-year survival rate of ovarian cancer patients is 30% to 50%, as the majority of these patients are diagnosed at an advanced stage (III or IV) of the disease, at which time the primary tumor has metastasized (1, 2). One of the characteristics of the malignancy of ovarian cancers is their invasion and metastatic potential. In most cases, the spread of cancer is through the i.p. route. Therefore, i.p. administration of chemotherapy is a logical approach for ovarian cancer therapy. The first such trial involving i.p. administration of cisplatin-based chemotherapy revealed an improved effect in both survival and toxicity, however, toxicities by the i.p. route were higher compared with the i.v. route (3, 4). Additional therapeutic approaches are needed for effective management of patients with ovarian cancer.

Interleukin-4 (IL-4) is an important Th2-derived cytokine, which is involved in mediating numerous antitumor immune-modulating activities (5). It is a key cytokine involved in IgG, IgE, and MHC class II expression, the generation of dendritic cells, and tumor-reactive CTLs. In addition, IL-4 causes direct inhibition of cancer cell growth in vitro (6, 7). Because of these properties, IL-4 was tested in the clinical trials for hematopoietic and some solid malignancies (8, 9). As IL-4 mediated limited antitumor activity, most clinical trials were aborted. Another characteristic of IL-4 and host interaction through IL-4 receptor (IL-4R) have been explored in recent years as a large number of hematopoietic and solid human tumors express IL-4R in vitro and in vivo (10–12). To target IL-4R on human tumors, we have developed a fusion protein, IL-4 cytotoxin, which is composed of a circularly permuted IL-4 and a mutated form of a powerful bacterial toxin (Pseudomonas exotoxin). After binding to IL-4R on the cell surface, IL-4 cytotoxin internalizes into an endosome with the IL-4R, translocates to the cytosol, and then ribosylates elongation factor 2 to prevent the initiation of protein synthesis, leading to cell death. IL-4 cytotoxin, termed IL-4(38-37)-PE38KDEL, has shown remarkable antitumor activity in vitro and in vivo against a variety of human tumors (12–15). Because of these activities, this molecule has been tested in the clinic in patients with malignant glioma, and advanced solid tumors (16–18). We have also shown that two ovarian cancer cell lines, PA-1 and IGROV-1, express IL-4R on their cell surface (19). However, expression of IL-4R in human ovarian cancer specimens is not known. As IL-4 cytotoxin can be administered by i.p. route, here we have examined whether ovarian cancer specimens and normal ovary tissue samples express IL-4R and whether these receptors can be targeted by IL-4 cytotoxin.

We report that a great majority of ovarian cancer specimens (~60%) overexpress IL-4R. In contrast, normal ovary tissue samples do not express IL-4R or express at very low frequency. IL-4R on ovarian cancer cells can be targeted by IL-4 cytotoxin in vitro and in vivo in mouse models of ovarian cancer.

Materials and Methods

Cell culture and reagents. The human PA-1, SKOV-3, and OVCAR-3 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured as described previously (20, 21). The human IGROV-1 and OVCAR-429 cells were kindly provided by Dr. K. Stromberg, Food and Drug Administration, Bethesda, MD. IL-4 cytotoxin, IL-4(38-37)-PE38KDEL, was provided by Neurocrine Biosciences, Inc. (San Diego, CA; ref. 22).

Immunohistochemistry. Immunohistochemistry was done using Vector ABC peroxidase kit according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). Paraffin-embedded tissue sections

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were deparaffinized by xylene and washed with different concentrations of ethanol and PBS. Sections were then incubated with anti-human IL-4 receptor polyclonal antibody (Santa Cruz, Santa Cruz, CA) or isotype control (IgG) for 16 hours at 4°C. Immunohistochemistry slides were evaluated independently by three of the authors (M. Kioi, S. Takahashi, and M. Kawakami). The results were scored on the basis of the density of staining as −, negative; −+/+, weakly positive; +, positive; and ++, strongly positive.

### Semiquantitative and real-time TaqMan reverse transcription-PCR

Semiquantitative reverse transcription-PCR was done as described previously (23). Quantification of IL-4Rα mRNA expression levels in ovarian cancer cell lines was determined by real-time reverse transcription-PCR using a set of IL-4Rα-specific TaqMan probe (5′-FAM, 3′-MGB) and primers (Applied Biosystems, Foster City, CA). Total RNA was reverse transcribed into the cDNA primed with oligo-dT. Quantitative PCR reactions were done using the ABI PRISM 7700 sequence detection system (Applied Biosystems). cDNA (50 ng) was added to a reaction volume (30 μL) containing 1× TaqMan PCR master mix, IL-4Rα-specific probe/primers set, and glyceraldehyde-3-phosphate dehydrogenase or β-actin-specific probe (5′-VIC, 3′-MGB)/primers mix (Applied Biosystems). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase or β-actin before the fold change in gene expression was calculated.

### Protein synthesis inhibition assay

The in vitro cytotoxic activity of IL-4 cytotoxin was measured by the inhibition of protein synthesis (24). Briefly, cells (1×10⁴) were cultured in leucine-free medium with or without various concentrations of IL-4 cytotoxin for 22 hours at 37°C. Then 1 μCi of [³H]leucine (NEN Research Products, Boston, MA) was added to each well and incubated for an additional 4 hours. Cells were harvested and radioactivity incorporated in cells was measured by a β plate counter (Wallac, Gaithersburg, MD). All assays were done in quadruplicate, and the concentration of IL-4 cytotoxin at which 50% inhibition of protein synthesis occurred was calculated (IC₅₀).

### Subcutaneous xenograft ovarian tumor model

All animal experiments were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. PA-1 (5×10⁴), SK-OV3, OVCAR-3, and IGROV-1 cells (1×10⁴) were suspended in 150 μL PBS and inoculated s.c. into the right dorsal flank of 6-week-old female BALB/c nude mice. Tumor growth was monitored using Vernier calipers, and tumor size (mm²) was calculated by multiplying length and width of tumor on a given day. Treatments were started 6 days after inoculation when the tumors reached a mean size of 30 mm³ (5-6 mm diameter). Mice were randomly divided into different therapeutic groups and one control group (six to seven mice per group). Mice were given injections of excipient (0.2% human serum albumin in PBS) or IL-4 cytotoxin by either i.p. (500 μL with 27-gauge needle) or intratumoral (L; 30 μL with microinjection syringe) routes. To determine the sensitivity of regrown PA-1 s.c. tumors to IL-4 cytotoxin, PA-1 tumors treated with 50 μg/kg dose of IL-4 cytotoxin were excised at day 60, digested with collagenase and DNase, and cultured. After two passages, the sensitivity of the cells to IL-4 cytotoxin was determined by protein synthesis inhibition assays.

### Orthotopic xenograft ovarian tumor model

For the orthotopic model, tumor chunks obtained from s.c. growing tumors were minced and 1×1 mm pieces were sutured and implanted in the right ovary under anesthesia. Tumor volume (V) was calculated using the formula: V (mm³) = L×W²×π/6, where L is the length of the tumor in millimeters, and W is the width of the tumor in millimeters (25). Treatments were started either 5 or 15 days after inoculation when the tumors were established or started to spread their invasion, respectively. Mice were randomly divided into different therapeutic groups and one control group. Animals were given i.p. injections of excipient (0.2% human serum albumin in PBS) or IL-4 cytotoxin by continuous infusion pump with 50 or 500 μg/kg/d dosage for 14 days.

### Statistical analysis

The statistical significance of data was calculated by Student’s t test. All statistical tests were two-sided. Survival probabilities were estimated by Kaplan-Meier methods. For analysis of statistical significance between the therapeutic group and control group in survival assay, log-rank test was used.

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### Results

**Expression of IL-4 receptor in ovarian tumor tissues.** Tissue sections from 7 normal ovary and 21 ovarian tumor samples were analyzed by immunohistochemical analysis for the expression of IL-4R. The expression of IL-4Rα chain, which is a predominant IL-4 binding protein, was analyzed (26). Some of the tumor specimens showing high levels of IL-4Rα expression (sample numbers 1 and 21) are presented in Fig. 1A. In contrast to cancer tissues, normal ovary tissue specimens showed no staining to weakly positive (+, +/−) staining for IL-4Rα chain (Fig. 1A, sample number 22). The expression level of IL-4Rα chain and histology of ovarian cancer are summarized in Table 1. Approximately 60% of ovarian tumor tissues expressed IL-4Rα chain at high density (+++) and 33% at moderate density (+).

### Ovarian cancer cell lines expressing IL-4R are sensitive to IL-4 cytotoxin

We next determined the expression of IL-4Rα mRNA by semiquantitative reverse transcription-PCR and TaqMan real-time reverse transcription-PCR analyses in five ovarian cancer cell lines. The expression of mRNA for IL-4Rα chain was not detected in OVCA-429 cell line, but all other ovarian cancer cell lines showed varied density of IL-4Rα mRNA expression by conventional semiquantitative reverse transcription-PCR analysis (data not shown). Real-time reverse transcription-PCR confirmed conventional reverse transcription-PCR results and showed that SK-OV3 cell line expresses highest level of IL-4Rα mRNA. IL-4 cytotoxin showed moderate cytotoxicity in this cell line. With this exception, the expression level of IL-4Rα mRNA correlated with sensitivity to IL-4 cytotoxin in all other ovarian cancer cell lines.

**Intraperitoneal administration of IL-4 cytotoxin inhibits ovarian tumor growth.** We investigated the effect of IL-4 cytotoxin in s.c. xenografted human ovarian tumor in nude mice. We injected IL-4 cytotoxin (50 or 200 μg/kg × 5 days) i.p. twice daily in PA-1, SK-OV3, or OVCAR-3 tumor-bearing mice (n = 6). The IL-4 cytotoxin treatment started on day 6 as palpable tumors developed within 3 to 4 days. As shown in Fig. 2A, IL-4 cytotoxin mediated significant antitumor activity against both PA-1 and SK-OV3 s.c. tumors in a dose-dependent manner, whereas the growth of OVCAR-3 s.c. tumors was not affected by IL-4 cytotoxin treatment. By day 45, administration of 200 μg/kg dose of IL-4 cytotoxin resulted in a 75% reduction in tumor size in PA-1 s.c. tumor.

**Intratumoral administration of IL-4 cytotoxin eradicates ovarian tumors.** We next evaluated the effect of IL-4 cytotoxin in three different ovarian tumor models, selected based on their sensitivities to IL-4 cytotoxin in vitro. s.c. xenografted tumor-bearing mice (six mice per group) were treated i.t. with three injections of IL-4 cytotoxin. Interestingly, IL-4 cytotoxin treatment (125 or 500 μg/kg/d) on alternate days for 3 days eradicated PA-1 s.c. tumors in two out of six mice and in four out of six mice at low and high dose, respectively (Fig. 2B). The residual small tumors in the higher or lower dose groups did not show regrowth by day 44 of follow up. However, the tumors in control mice continued to grow exponentially, and mice were sacrificed on day 49 due to large tumor burden. The overall reduction in tumor size was 89% in the 125 μg/kg
IL-4 cytotoxin treatment did not cause complete regression of SK-OV3 s.c. tumor by i.t. administration, all mice receiving 125 or 500 μg/kg doses showed a significant regression of tumors (60%; P < 0.001 or 80%; P < 0.0001 at low and high doses, respectively; compared with controls). On the other hand, OVCAR-3 tumor-xenografted mice were resistant to IL-4 cytotoxin treatment, and average tumor sizes in both treatment groups were not significantly different from control group (P = 0.23). The efficacy of IL-4 cytotoxin by either i.p. or i.t. administration in three different xenografts seemed to correlate well with their cytotoxicity in vitro protein synthesis assays. Because both dosages (125 and 500 μg/kg) of IL-4 cytotoxin mediated remarkable efficacy against PA-1 tumors, we next tested whether lower dosages of IL-4 cytotoxin could also mediate antitumor effects. As shown in Fig. 2C, IL-4 cytotoxin at the 50 μg/kg dose still mediated strong antitumor activity, and one out of six mice showed complete regression of tumor. In addition, a 5 μg/kg dose also significantly inhibited the tumor growth as compared with the control group (P < 0.001). After 45 days of observation, tumors in the 50 μg/kg IL-4 cytotoxin dose group started to regrow slowly. To explore whether regrowth was caused by heterogeneity in IL-4R expression, development of resistance, or poor accessibility of IL-4 cytotoxin, PA-1 tumors in either control or IL-4 cytotoxin-treated groups were resected on day 60 and cultured in vitro, and their sensitivity to IL-4 cytotoxin was tested in vitro. As shown in Fig. 2D, tumor cells from control and IL-4 cytotoxin-treated mice maintained equal sensitivity to IL-4 cytotoxin. These results suggest that partial regression of tumors in PA-1 tumor model could be due to inadequate IL-4 cytotoxin distribution within the tumor mass rather than heterogeneity or tumor resistance to IL-4 cytotoxin.

**Prevention of tumor metastasis by IL-4 cytotoxin therapy in orthotopic ovarian tumor model.** The antitumor effect of IL-4 cytotoxin and the survival rate of mice orthotopically implanted with ovarian tumors were investigated to determine the efficacy of IL-4 cytotoxin in a model system that simulated a clinical situation for aggressiveness of cancer. PA-1 and IGROV-1 tumor chunks were orthotopically implanted on ovaries, and then tumor-bearing mice were injected with either excipient (control) or IL-4 cytotoxin (200 μg/kg, twice a day for 5 days from days 5 to 9) through i.p. route. All tumor-bearing animals (n = 5) without treatment showed an aggressive tumor growth, invasion, and spread to other organs (Fig. 3). As determined by macroscopic and microscopic analyses, ovarian tumors invaded other organs including liver, small intestine, ipsilateral kidney, and contralateral ovary in control mice (Fig. 3A-D and I-L). The status of ovarian cancer spread to other organs 4 weeks after implantation of tumor is summarized in Table 2A. Untreated animals showed tumor metastasis outside the pelvic cavity. At the terminal stage, all untreated control tumor-bearing mice showed ascites, which contained tumor cells as confirmed by Giemsa stain (Fig. 3M and N). In contrast, no metastasis was observed in the IL-4 cytotoxin treatment group (Table 2A; Fig. 3E-H; H&E stain not shown). In addition, tumor volumes at the tumor-implanted right ovaries were significantly smaller compared with the control group (P < 0.001) 4 weeks after implantation of tumor (Fig. 3O).

**Intraperitoneal IL-4 cytotoxin prevents cachexia and prolongs survival of mice implanted orthotopically with ovarian tumors.** We also evaluated overall survival of IL-4 cytotoxin-treated ovarian tumor-bearing mice. Mice orthotopically implanted with PA-1, SK-OV3, or IGROV-1 tumors (five mice per group in PA-1 and IGROV-1, eight mice per group in SK-OV3) were treated with excipient only (control) or IL-4 cytotoxin (50, 100, or 200 μg/kg twice daily for 5 days from days 5 to 9). As shown in Fig. 3P and Q, survival of mice was significantly extended in all
treated groups (PA-1, \( P < 0.01 \); SK-OV3, \( P < 0.001 \); IGROV-1, \( P < 0.05-0.0001 \), compared with controls). At the highest IL-4 cytotoxin dose (200 μg/kg), all IGROV-1 tumor-bearing mice survived for as long as we followed these animals (>200 days), whereas all untreated mice died in <100 days.

Orthotopically implanted IGROV-1 ovarian tumor–bearing mice showed substantial body weight loss and tumor progression with excipient treatment, whereas IL-4 cytotoxin–treated mice showed weight gain and tumor regression (Fig. 3R). Mice body weight were measured at least once a week, and showed persistent weight loss in control mice along with tumor progression and invasion. After 4 weeks of implantation, body weight of control mice did not increase, gradually stabilized, and after 8 weeks, loss in body weight occurred despite the growth of tumors (Fig. 3R). The IGROV-1 orthotopic tumor-harboring mice treated with 100 or 200 μg/kg dose of IL-4 cytotoxin (Fig. 3Q) showed linear increase in body weight for the 12 weeks that we followed these animals. Interestingly, the 100 μg/kg dose of IL-4 cytotoxin did not completely cure tumor-bearing animals, nevertheless, these animals also showed linear increase in body weight (Fig. 3R).

**IL-4 cytotoxin eradicates large s.c. ovarian tumors and has antitumor activity against orthotopically implanted ovarian cancer in an advanced tumor model.** To evaluate the efficacy of IL-4 cytotoxin in advanced ovarian cancer models, we treated tumor-bearing mice (six mice per group) with IL-4 cytotoxin by i.p. or i.t. routes starting on day 19 when the PA-1 s.c. tumors grew to \( f \leq 75 \text{ mm}^2 \). Twice a day i.p. (200 μg/kg) or daily i.t. (500 μg/kg) injections of IL-4 cytotoxin for 10 days (5 days/wk), followed by a 7-day rest, formed the first cycle of treatment (Fig. 4A). The tumors began to regress just after the first treatment cycle in both i.p. and i.t. treatment groups. Both groups of animals also received a second cycle of treatment on days 25 to 29 by the i.t. route and on days 32 to 36 by the i.p. route. The tumor regression continued after treatment was completed, and four out of six mice showed complete regression of their tumors in the i.t. treatment group. i.p. treatment also showed statistically significant antitumor activity (\( P = 0.0003 \), versus control); however, complete regression of tumors was not observed.

Encouraged by the therapeutic effect of IL-4 cytotoxin in s.c. and orthotopic ovarian tumor models, we next determined the efficacy of IL-4 cytotoxin in advanced orthotopic ovarian cancer model. We started the injection of IL-4 cytotoxin 15 days after implantation of IGROV-1 ovarian tumor on the right ovary. As shown in Fig. 4B, i.p. treatment of IL-4 cytotoxin caused regression of tumor growth in implanted right ovary. At 500 μg/kg dose, a dramatic reduction in tumor volume was observed. The average tumor volumes in 50 and

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**NOTE:** --, negative staining; --/+, weak staining; +, positive staining; and ++, strongly positive staining.

*Paraffin-embedded tissue sections were obtained from the Cooperative Human Tissue Network and stained with anti-IL-4R antibody.
500 μg/kg/d doses were 59% \((P < 0.02)\) and 99% \((P = 0.009)\) of controls, respectively, on day 43. The status of ovarian cancer spread to other organs 6 weeks after implantation of tumor is summarized in Table 2B. Ovarian tumors invaded the liver, small intestine, and ipsilateral kidney in all control mice (three mice per group). In contrast, no metastasis was observed in the 500 μg/kg/d IL-4 cytotoxin–treated group. An IL-4 cytotoxin dose of 50 μg/kg/d showed metastasis to liver and kidney, but not in all animals.

Consistent with the regression of established tumors, the survival of advanced orthotopic ovarian tumor–bearing mice (nine mice per group) was also extended in 500 μg/kg/d IL-4 cytotoxin–treated animals compared with controls \((P < 0.001)\). The 50 μg/kg/d dose showed tumor regression, however, this regression was not enough to statistically prolong significant survival from aggressive tumor growth (Fig. 4C). Median survival time was 85 days (range, 56-95 days; \(n = 9\)) in the control group, 93 days (range, 72-112 days; \(n = 9\)) in the 50 μg/kg dose group, and 137 days (range, 91-180, experimental period, days; \(n = 9\)) in the 500 μg/kg dose of IL-4 cytotoxin. These data indicate that i.p. administration of IL-4 cytotoxin is effective in the regression of large ovarian tumors as well as in the prevention of metastasis of advanced orthotopic ovarian tumors.

**Discussion**

Despite advances in surgery and chemotherapy, long-term survival of patients with stage III or IV ovarian cancer is limited due to i.p. spread of tumor cells at diagnosis, for which no curative therapy is available (27). To develop novel therapeutic approaches, in this study, we show that ~60% ovarian tumor samples

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**Figure 2.** Antitumor activity of IL-4 cytotoxin by i.p. and i.t. administration in s.c. xenografted ovarian tumors. **A**, tumor cells \((5 \times 10^5 \text{ or } 1 \times 10^7)\) were inoculated in the right flank of nude mice \((n = 6\) per group), and then starting on day 6, IL-4 cytotoxin was injected i.p. at dose of 50 or 200 μg/kg twice a day for 5 days. Tumors were measured on indicated days. Tumor sizes (mm²) shown are means ± SD minimum of six mice per group. **B** and **C**, ovarian tumor cells \((5 \times 10^5 \text{ or } 1 \times 10^7)\) were s.c. inoculated in the right flank of nude mice (six mice per group), and then starting on day 6, IL-4 cytotoxin was injected at doses of 125, 500 (B), 5, or 50 (C) μg/kg/d on alternate days (days 6, 8, and 10). Tumor nodules were measured on indicated days. Tumor sizes (mm²) shown are means ± SD minimum of six mice per group. **D**, PA-1 tumors treated with 50 μg/kg dose of IL-4 cytotoxin were excised, digested by collagenase and DNase I, and cultured. The sensitivity of the cells to IL-4 cytotoxin was determined by protein synthesis inhibition assays.
overexpress IL-4R at high levels and 33% of ovarian tumor samples at moderate levels. As normal ovarian tissues expressed no or low levels of IL-4R, our results indicate that IL-4R might be a tumor-associated target for ovarian tumor therapy. We exploited this property of epithelial ovarian tumors and observed that IL-4R-positive ovarian tumor cell lines are highly sensitive to the cytotoxic effect of IL-4 cytotoxin \textit{in vitro}, which targets IL-4R.

The sensitivity of these tumor cell lines to IL-4 cytotoxin \textit{in vitro} approximately correlated positively with the density of IL-4R mRNA expression. This promising antitumor activity of IL-4 cytotoxin was also tested \textit{in vivo} in animal models of human ovarian tumors. We selected three tumor cell lines expressing high, moderate, and low levels of IL-4R. Consistent with varied receptor expression, these cell lines showed high, intermediate, and low sensitivity to IL-4 cytotoxin \textit{in vitro}. Antitumor effect \textit{in vivo} was first analyzed in s.c. tumor models. We observed that s.c. administration of IL-4 cytotoxin to tumor-bearing mice mediated dramatic antitumor effects by causing regression of tumors in a dose-dependent manner. Similar to \textit{in vitro} effects, IL-4 cytotoxin mediated the highest antitumor activity against PA-1 tumors followed by SK-OV3 tumors. Limited antitumor activity was seen against OVCAR-3 tumors. These results show that IL-4 cytotoxin can mediate profound antitumor effects in ovarian tumors.

Because ovarian tumors metastasize to the peritoneal cavity, local therapy as a first line or as an adjuvant may be a useful approach. i.p. administration of targeted agents such as IL-4 cytotoxin may cause a therapeutic impact without toxicity to normal organs. To test this hypothesis, we developed the orthotopic tumor model by surgically implanting PA-1 tumor chunks on the peritoneal cavity of mice. Orthotopic ovarian tumors were treated i.p. with excipient only (control) or IL-4 cytotoxin (200 μg/kg, twice a day for 5 days from days 5 to 9). After 4 weeks, tumors and other organs including surrounding tissues were evaluated. Local ovarian tumor (A, E, and J), spread to contralateral ovary (B, F, and L), ipsilateral kidney (C, G, and K), and liver (D, H, and L) in control (A-D) and IL-4 cytotoxin treatment (E-H) groups (five mice per group). Histology of organs in control groups stained with H&E (I-L); arrow, tumor mass; bar, 5 mm. Cancer cells were confirmed in the ascites fluid of PA-1 (M) and IGROV-1 (N) orthotopic ovarian cancer–bearing mice. O, average of tumor volume (mm$^3$) in control and IL-4 cytotoxin–treated group. P, mice harboring PA-1 ($n=5$) and SK-OV3 ($n=8$) tumors were treated with excipient only (control) or IL-4 cytotoxin (200 μg/kg, twice a day × 5 days). Q, survival of animals bearing IGROV-1 tumor ($n=5$) after IL-4 cytotoxin (0, 50, 100, or 200 μg/kg, twice a day × 5 days) treatment. R, mice in each group were weighed at least once a week, and the average body weight was calculated.
After 5 days of implantation, these animals were treated with intermediate doses of i.p. IL-4 cytotoxic for 5 days. IL-4 cytotoxic mediated dramatic reduction of primary established tumor. Remarkably, mice that were treated with IL-4 cytotoxic showed no metastasis to any organs and did not show any ascites. These observations indicate that IL-4 cytotoxic not only causes regression of established orthotopic ovarian tumor but it also prevented ascites formation and tumor metastasis. This is a remarkable observation very rarely seen with other therapeutic approaches.

The remarkable anti–ovarian tumor effects of IL-4 cytotoxic were further confirmed by enhanced survival of treated animals. When treated with IL-4 cytotoxic, the most sensitive tumor-bearing hosts showed statistically significant (P < 0.05) survival compared with control animals in a dose-dependent manner. At the highest dose, all treated animals survived for as long as we followed these animals. Less sensitive tumor (SK-OV3)–bearing hosts also showed statistically significant survival compared with untreated control animals. Although the PA-1 tumor cell line showed the highest sensitivity in vitro and in vivo s.c. tumor model to IL-4 cytotoxic, these orthotopic tumor–bearing mice showed statistically significant prolonged survival compared with control, but not longer expected survival compared with other tumor-bearing mice (SK-OV3 and IGROV-1). One of the possible reasons might be the extreme aggressiveness of this tumor. All mice bearing PA-1 tumors in the control group died within 60 days of implantation compared with SK-OV3 and IGROV-1 tumor–bearing mice which died in >80 days.

For clinical application of IL-4 cytotoxic, several considerations in terms of feasibility of delivery and toxicity are of paramount importance. Previous studies of ours as well as others have shown that IL-4R is expressed on normal immunologic and nonhematopoietic cells. However, IL-4 cytotoxic is not very cytotoxic to these cells as they express no or low levels of IL-4R (14, 28, 29). Preclinical toxicity studies in mice have shown that IL-4 cytotoxic is well-tolerated up to 475 μg/kg dose given i.v. (30). As human IL-4 does not bind murine IL-4R, IL-4 cytotoxic was also injected to cynomolgus monkeys, whose IL-4R binds human IL-4. In these animals, IL-4 cytotoxic was reasonably tolerated up to a dose of 200 μg/kg given i.v. every alternate day for three injections. Reversible liver enzyme elevation and injection site inflammatory reactions were observed. These reversible preclinical observations were also observed in a small phase I clinical study, in which IL-4 cytotoxic caused dose-dependent hepatic enzyme elevation when given i.v. (18). As normal ovarian cells do not express high density of IL-4R and IL-4 cytotoxic does not mediate any other toxicity, including renal toxicity, it is possible that i.p. administration of IL-4 cytotoxic in patients with ovarian tumors would be better tolerated. In that regard, it is important to note that in two phase I clinical trials in which IL-4 cytotoxic was injected intracranially by convection enhanced delivery directly into glioma tumors, this molecule was tolerated with acceptable neurologic and no systemic toxicities (16, 17).

Regarding the route of administration, perhaps i.p. delivery by catheter, similar to Tenckhoff, would be a feasible approach. To address whether this route would be tolerated, we delivered IL-4 cytotoxic continuously by i.p. pump for a period of 14 days and

<table>
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<th>Table 2. Metastasis of PA-1 and IGROV-1 advanced ovarian tumor upon orthotopic implantation in the ovary of mice</th>
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<tr>
<td>(A) PA-1 orthotopic ovarian tumors</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>Site</td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>Lung</td>
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<td>Liver</td>
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<td>Ovary</td>
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<td>Small intestine</td>
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<td>NOTE: A, metastasis of PA-1 orthotopic ovarian tumors into other organs was observed by macroscopic and microscopic analysis 4 weeks after implantation onto the right ovary. B, metastasis of IGROV-1 orthotopic ovarian tumors into other organs was observed macroscopically and microscopically 6 weeks after implantation onto the right ovary. *-+, negative metastasis; +, positive metastasis.</td>
</tr>
</tbody>
</table>
survival of IGROV-1 tumor–bearing mice were followed. None of these animals showed any visible signs of toxicity, however, statistically significant ($P < 0.001$) prolonged survival of IL-4 cytotoxin–treated animals at higher dose was observed. This observation is important, providing a proof of principle for continuous i.p. delivery of IL-4 cytotoxin for metastatic ovarian tumor.

Several molecules targeting ovarian cancer are under development. These targets include epidermal growth factor receptor (EGFR), p53 pathway, TRAIL, BCL-2 family, and vascular endothelial growth factor (31–35). Clinical trials are ongoing, evaluating agents that inhibit vascular endothelial growth factor or EGFR signaling pathways in ovarian cancer (36, 37). Ovarian carcinomas (30-70%) express high levels and EGFR expression correlates with the survival of these patients (38, 39). Because the frequency of IL-4R expression in human ovarian cancer specimens archived is comparable to the expression of EGFR, it is possible that IL-4R may also serve as a biomarker for prognosis or as a target for other approaches other than IL-4 cytotoxin. Additional tumor samples need to be evaluated to confirm this hypothesis.

It is important to note that IL-4 cytotoxin treatment also prevented development of cachexia in tumor-bearing animals. A persistent weight loss in IGROV-1 orthotopic tumor–harboring mice was observed along with the progression of tumor growth and invasion. In sharp contrast, tumor-bearing mice treated with IL-4 cytotoxin showed linear increase in body weight in a dose-dependent manner along with tumor regression. These observations suggest that body weight may serve as a biomarker for tumor response in ovarian tumor–bearing animals. Further studies are needed to confirm this hypothesis. However, thus far, few agents (except IL-4 cytotoxin) have shown a convincing antitumor effect in advanced ovarian cancer models. Thus, IL-4 cytotoxin may be expected to be useful for patients with advanced ovarian cancer.

In conclusion, our studies show that IL-4 cytotoxin has notable antitumor activity against ovarian tumor that express IL-4R. To our knowledge, this is the first report in which orthotopic ovarian tumors were completely eradicated by IL-4R-targeted therapy in animal model. Because in vitro cytotoxicity of IL-4 cytotoxin has revealed positive correlation with in vivo antitumor activity in animal models, it is expected that the efficacy of IL-4 cytotoxin may be realized in clinical situations.

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Expression and Targeting of Interleukin-4 Receptor for Primary and Advanced Ovarian Cancer Therapy

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