Interleukin-4 Receptor-targeted Cytotoxin Therapy of Androgen-dependent and -independent Prostate Carcinoma in Xenograft Models

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Abstract

Prostate cancer is the most commonly diagnosed solid tumors in United States men. Survival with advanced prostate cancer is dismal because of a lack of effective treatments. Overexpression of interleukin 4 receptors (IL-4R) on prostate carcinoma cells makes them suitable targets for the interleukin 4 (IL-4) fused Pseudomonas exotoxin, IL-4 cytotoxin (IL4-CTx). Androgen-dependent (LNCaP) and -independent (DU145) human prostate cancer cell lines overexpress IL-4Rs and are exquisitely sensitive to IL4-CTx. Using LNCaP and DU145 cell lines, IC50 values of 4.5 and 6.5 ± 0.5 ng/ml, respectively, were obtained for IL-4CTx in protein synthesis inhibition assays. Primary cultures established from prostate tumor biopsies were equally sensitive to the cytotoxic effects of IL4-CTx.

Reverse transcription-PCR analysis, although not quantitative, indicated the presence of mRNA for IL-4Ra, a primary subunit of the IL-4R receptor complex in prostate carcinoma cell lines, primary cultures, benign prostatic hyperplasia, and prostate carcinoma tissues. Immunohistochemistry studies revealed the presence of IL-4R in benign prostatic hyperplasia and prostate carcinomas. Five daily (QD) injections of IL4-CTx (100 μg/kg) administered i.v., i.p., or intratumoral (i.t.) caused several complete responses in nude mice with s.c. DU145 and LNCaP tumors. i.t. injections of IL4-CTx elicited tumor regression in a dose-dependent manner with complete responses occurring in 100% of the animals when treated with IL4-CTx (500 μg/kg) given five QD injections. Administration of IL4-CTx i.t. (500 μg/kg) either 10 times QD or six injections on alternate days elicited complete responses in 40% of mice with DU145 tumors that were three times larger (67 mm3) on initiation of treatments. IL4-CTx appeared to be well tolerated. On the basis of these results, combining i.t. injections of IL4-CTx with systemic administration may provide an effective strategy for treating patients with advanced, refractory prostate cancer.

Introduction

Prostate carcinoma has the highest incidence of noncutaneous malignancy in American males, in particular among African-American males (1). According to American Cancer Society, ~189,000 new cases of prostate cancer will be diagnosed among men in the United States, and an estimated 30,200 men are expected to die of the disease in year 2002 alone (2, 3). Prostate cancer-related deaths in the United States has declined in recent past most probably because of awareness and early detection. The surgery and radiation treatment in early stage localized prostate cancer has improved the survival rate (4). When extraprostatic or metastatic disease develops, castration and androgen ablation is used (5); however, the androgen resistance is acquired during the progression of disease and manifests a dismal prognosis warranting new experimental therapies.

Advanced prostate cancer is treated conventionally with hormonal therapy. High response rates were mostly transient and without survival benefits. The limited response to hormonal therapy is because of the emergence of androgen-independent clones (6) and has renewed interest in the use of chemotherapeutic agents for treating HRPC (7). Doxorubicin treatment led responses in >60% of the cases, but accompanied with significant toxicity. A combination with ketoconazole improved PSA response but did not alter the toxicity profile (8). In a clinical trial, Mitoxantrone, an anthracycline derivative, plus hydrocortisone demonstrated symptom improvement but without survival benefit (9). Vinblastine, paclitaxel, and topoisomerase inhibitors such as etoposide had promising effects in a Phase III trial, when used in combination with estramustine but again did not offer a better survival rate (10, 11). Patients with advanced disease treated with hormone therapy and chemotheraphy achieved undetectable PSA level postoperatively, but a goal of pT0 status could not be met.
(12). Moreover, the role of chemotherapy as an adjuvant treatment remains controversial.

Several immunotherapy and gene therapy approaches are also being explored in hormone-refractory prostate cancer (13, 14). It has been suggested that HER-2 protein-expressing prostate cancer cells can be effectively killed by HER-2-specific antibody PE fusion protein in vitro (15). Two Phase II trials using trastuzumab, a monoclonal antibody against HER-2 both as monotherapy or in combination with either paclitaxel or estramustine are also ongoing. The neutralizing antivascular endothelial growth factor antibody could suppress primary tumor growth and inhibited metastatic tumor dissemination in a DU145 prostate tumor xenograft (16), but its use in a clinical trial did not show a decline in PSA level (17). Many metastases and primary prostate tumors express platelet-derived growth factor receptor and were targeted with a receptor inhibitor, SU101, in an efficacy trial, producing no encouraging results (18). In another receptor-directed therapy, bFGF receptors on prostate tumor cells were targeted with bFGF-saporin chimera producing a significant antitumor activity in a xenograft model of DU145 tumors (19). Epidermal growth factor, transforming growth factor, insulin-like growth factor, and IL-6 receptors were also investigated as potential targets for prostate carcinoma therapy (13, 20). In a tumor vaccine approach, patients with HRPC were injected with dendritic cells pulsed with two HLA-A2-specific prostate-specific membrane antigen peptides resulting in 30% positive responders (21). Despite all of these advancements, the survival of patients with HRPC has not significantly improved.

In the search of novel growth factor/cytokine receptor for targeted therapy, we have identified the overexpression of IL-4Rs on several solid tumor cells including renal cell carcinoma (22), glioblastoma (23, 24), AIDS-associated Kaposi’s sarcoma (25), head and neck (26), and breast cancers (27). These overexpressed IL-4Rs can be effectively targeted in vitro and in vivo with a PE-based IL-4 chimeric protein, IL-4-CTX (28). In one of our clinical trials, IL-4-CTX was found to be safe in the patients with recurrent glioblastoma multiforme (29). A multicenter international clinical trial is under-going to explore its efficacy. In the current study, we demonstrate that IL-4Rs are overexpressed in hormone-dependent and -independent prostate carcinoma cell lines, primary cultures established from fresh prostate tumors, and prostate tumor specimens. We additionally show that the xenograft tumors developed from DU145 and LNCaP cells were completely regressed by IL4-CTx in the IL-4R-directed therapy.

Materials and Methods

Cell Culture and Reagents. Human prostate carcinoma cell lines, DU145 and LNCaP, were purchased from American Type Culture Collection (Manassas, VA), and cultured in Eagle’s Modified Essential Medium or RPMI 1640, respectively supplemented with 10% heat-inactivated fetal bovine serum, 1 mM L-glutamine, penicillin (100 µg/ml), streptomycin (100 µg/ml), and 50 µg/ml gentamicin (all from BioWhittaker, Walkersville, MD). The prostate epithelial cell cultures, CRI1527CP, CRI568CP, and CRI570CP, were obtained from Dr. Robert Bright (Franz Cancer Research Center, Chiles Research Institute, Portland, OR). These cell cultures were generated from primary adenocarcinoma of the prostates resected from patients in culture conditions described elsewhere (30). Recombinant human IL-4 was a gift from Schering Corporation (Kenilworth, NJ). BPH and malignant prostate tissue specimens were obtained from The CHTN, Charlotteville, VA, and CHTN, Philadelphia, PA.

Recombinant IL-4 Cytotoxin. The chimeric IL4-CTx toxin was prepared by fusing a truncated PE gene encoding PE38KDEL 3’ to a circularly permuted IL-4 mutant gene encoding IL-4 amino acids 38–129, the linker GGNNG, and IL-4 amino acids 1–37. Resulting chimeric protein, IL-4(38–37)PE38KDEL binds with high affinity and induces potent cell killing (31).

Prostate Carcinoma Xenografts and Their Treatment. Four to 5-week-old male athymic nude mice (20–22 g) were obtained from Frederick Cancer Center Animal Facilities, Frederick, MD. Animals were housed in filter-top cages in a laminar flow hood. Human prostate tumors were established in nude mice by s.c. injection of 5 × 10⁶ DU145 cells in 150 µl of PBS plus 0.2% HSA into the flank. Mice were then injected with 22-gauge needle i.v. (200 µl), i.p. (500 µl), or i.t. (30 µl) with excipient (PBS containing 0.2% HSA) or IL4-CTX at indicated days. For i.t. administration, IL4-CTX was injected slowly (10 µl/min) in a total volume of 30 µl excipient into left and right sides of tumor (15 µl at each side) at each day of injection (25). Tumor sizes were calculated by multiplying length and width of tumors. LNCaP cells (2 × 10⁶) in 100 µl of excipient were admixed with 50 µl reconstituted basement membrane (Matrigel; Collaborative Biomedical Products, Bedford, MA) and were injected s.c. into the flank of nude mice. Both cell lines produced palpable tumors within 4–5 days. Animal care was taken in accordance with the guidelines established by NIH Animal Research Advisory Committee.

Radioreceptor Binding Assay. Recombinant IL-4 was radiolabeled with 125I supplied as sodium iodide (Amersham, Arlington Heights, IL) by the Iodo-gen (Pierce, Rockford, IL) method as described earlier (31). The number of IL-4 molecules bound per cell and their binding affinity was calculated by LIGAND curve-fitting program (32).

RT-PCR Analysis. To detect the mRNA expression of IL-4Rα chain in prostate cancer cells, total RNA was isolated using TRIZOL reagent (Life Technologies, Inc., Grand Island, NY), then RT-PCR analysis was performed using the same primers as described previously (31). The PCR product (10 µl) was run on 2% agarose gel for UV analysis.

Total RNA extracted from the paraffin-embedded tissue sections (30 µm) using paraffin block RNA isolation kit (Ambion, Inc., Austin, TX) was analyzed for IL-4Rα chain mRNA expression by RT-PCR as described earlier (31).

Immunohistochemistry. Immunohistochemistry was performed using the Vector ABC peroxidase kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacture’s instructions as described earlier (33). Immunohistochemical assays were performed twice independently with similar results, and slides were assessed by two independent investigators, and scored 0, +, or 2+ based on the staining.
intensity. A field was defined as a field viewed under ×200 or ×400 magnification. The percentage of positive fields was counted in a blinded manner by viewing the ductal lesion of prostate under the same magnification.

**Protein Synthesis Inhibition Assay.** The in vitro cytotoxicity of IL4-CTx was measured by inhibition of protein synthesis by the incorporation of [3H]leucine into prostate cancer cells following procedures described previously (34). For competition studies, cells were preincubated with IL-4 (2 μg/ml) for 45 min at 37°C before the addition of IL4-CTx to the cells. The cells were harvested on fiberglass filtermats using a Skatron semiautomatic cell harvester (Lier, Norway) and counted on a Beta Plate Counter (Wallac, Gaithersburg, MD). All of the assays were performed in quadruplicates and the concentration of IL4-CTx at which IC50 protein synthesis occurred was calculated.

**Evidence of Necrosis and Apoptosis followed by i.t. Injections of IL4-CTx.** Nude mice were implanted s.c. with DU145 (5 × 10^6) cells, and tumors were allowed to grow for 25 days to reach an average size of 56.8 ± 16.5 mm^2. Tumors were then injected i.t. either with excipient, or 50 or 100 μg/kg/day IL4-CTx using different schedule. Tumors were excised 72 h after the last injection and fixed in 10% formalin. Sections (5-μm thick) were cut through middle of tumors for staining with H&E or terminal deoxynucleotidyl transferase-mediated nick end labeling assay using ApopTag in situ hybridization detection kit (Intergen, Purchase, NY; Ref. 35).

**Statistical Analysis.** Group-wise comparisons of tumor areas in individual groups were made by Student's t test. All of the statistical tests were two-sided.

### Results

**IL-4R Expression on Prostate Carcinoma Cells**

The receptor binding data analysis (32) revealed that the androgen-independent prostate cancer cell line (DU145) expressed IL-4R, and unlabeled IL-4 displaced the [125I]IL-4 binding indicating receptor specificity (Fig. 1A). Scatchard plot analysis of binding data suggested that a single class of high affinity large numbers (12,000) of IL-4R were expressed on DU145 cells with a dissociation constant of 266 pM. (Fig. 1B). It has been reported previously that normal cells including basophils, resting T and B cells, monocytes, fibroblast, and endothelial cells express lower number of IL-4R compared with tumor cells (34).

**BPH and Malignant Prostate Cells Express IL-4R Transcripts**

Both androgen-dependent (LNCaP) and -independent (DU145) cell lines, 3 primary prostate cell cultures, 7 BPH tissues, and 4 malignant prostate tissues were analyzed for the expression of IL-4Rα, a primary subunit of IL-4R by RT-PCR assay. As shown in Fig. 2, mRNA for IL-4Rα chain was detected in all of the samples examined. PM-RCC, a renal carcinoma cell line known for the expression of IL-4Rα, was used as a positive control (36). Because of the paucity of RNA from tissue specimens, the level of IL-4R mRNA expression could not be compared by quantitative RT-PCR analysis. The data indicate that malignant tissues like prostate tumor cell lines also express mRNA for IL-4R.

**BPH and Prostate Carcinoma Tissues Express IL-4R**

The IL-4R expression patterns of BPH and prostate tumor specimens (n = 11) were examined by immunohistochemistry using M57, a IL-4Rα monoclonal antibody. None of the patients had received radiation or chemotherapy before resection of tissues. The median age of the patients...
was 62.7 years (range, 48–84 years). The representative photomicrograph of sections from 1 BPH and 2 prostate cancer specimens are shown in Fig. 3. The IL-4R antibody staining in ductal lesions was higher in adenocarcinoma tissue specimens (78T1–3 and 87T1–2) compared with BPH tissues (87T1–11). Neither BPH nor prostate cancer tissues stained with IgG isotype control antibody (data not shown). Two of the 3 adenocarcinoma tissues stained with double (2+) intensity compared with BPH tissue (+). No rigorous quantitative assessment of level of IL-4R expression could be made because of the low number of tissue specimens available.

Both Androgen-dependent and -independent Cell Lines, and Primary Cultures Are Equally Sensitive to IL4-CTx

Because androgen-dependent (LNCaP) and -independent (DU145) prostate tumor cell lines express mRNA for IL-4R (Fig. 2), and many IL-4Rs were detected on cell surface of DU145 (Fig. 1), we decided to use them as a target for IL4-CTx therapy. LNCaP and DU145 cells were extremely sensitive to IL4-CTx in a protein synthesis inhibition assay, and IC_{50} observed were 4.5 ± 2.0 and 6.5 ± 0.5 ng/ml, respectively (Fig. 4A). The protein synthesis inhibitory effect was abrogated by excess of IL-4 (2 μg/ml) indicating that cytotoxicity occurred through IL-4R binding. We have demonstrated previously that human normal cells e.g., endothelial cells that express lower levels of IL-4R compared with tumor cells are not sensitive to IL4-CTx (31, 34). These in vitro studies suggest that both prostate tumor cell lines were equally sensitive irrespective of their androgen sensitivity.

Because it is known that cell lines cultured for an extended period compromise their phenotype and may not be true representatives of tumors in a clinical situation, it was necessary to test the sensitivity of IL4-CTx in primary cell cultures established from prostate tumors. Three primary cultures (CRI527CP, CRI568CP, and CRI470CP) were exposed to various concentration of IL4-CTx in a protein synthesis inhibition assay. All three of the cultures were extremely sensitive, and IC_{50} were <5 ng/ml (Fig. 4B). Like cell lines, excess of IL-4 neutralized the cytotoxic effect of IL4-CTx confirming the IL-4R-mediated cytotoxicity. Thus far, all of the in vitro data indicate that IL-4R-expressing prostate cells are extremely susceptible to IL4-CTx.

Comparison of Efficacy of IL4-CTx Delivered through Various Routes

Androgen-independent Tumors. The delivery of a chemotherapeutic drug or a biological agent at a solid tumor site has been a major impediment in the successful development of therapeutic drugs. The route of administration of a drug plays a critical role in accumulating optimal concentration of drug at tumor site to exert its efficacy. Here, we delivered IL4-CTx via various routes and compared its antitumor efficacy in xenograft models. Nude mice were implanted with androgen-independent DU145 prostate tumor cells on day 0. The treatment was initiated on day 4 when average tumor size reached to 23.9 ± 0.8
mm² (mean ± SD). The IL4-CTx (100 μg/kg) was administered QD for 5 consecutive days via i.v., i.p., or i.t. routes (Fig. 5A). In the i.v.-treated group, the tumors in all of the mice began to respond just after first injection and continued to regress during the course of treatment. By the last day of injection (day 8), tumors were regressed ~50% (P < 0.005) compared with control group tumors on the same day. Thereafter, tumors began to regrow, and no significant (P = 0.861) difference was observed between the tumor sizes of i.v.-treated and control groups on day 71. In the i.p.-treated group, tumors continued to decrease even after the treatment cycle was completed. By day 22, tumors decreased to an average size of 6.3 ± 5.9 mm² including two durable CRs compared with control tumors (37.4 ± 10.5 mm²; P < 0.00004). However, tumors in partial responders began to regrow, but the average size was still significantly smaller (P < 0.0027) than control tumors on day 71. Thus far, data indicate that i.p. treatment was better than i.v. in decreasing tumor burden as well as yielding CRs. Because prostate tumor is a localized disease, it was of our interest to determine whether i.t. delivery of IL4-CTx would lead to an improved antitumor activity. The i.t. treatment with similar dose was very effective, and by the day of the last injection, tumors in all of the mice were completely eliminated and mice remained tumor-free until day 32. Two of six CRs showed regrowth and attained a size of 25 and 35 mm² each on day 71. The average tumor size in the control group was 144 mm².

**Androgen-dependent Tumors.** To assess the antitumor activity of IL4-CTx in an androgen-dependent prostate cancer xenograft model, the identical dose with similar routes and schedule were administered in LNCaP implanted nude mice on day 5. The tumors in i.v.-treated mice continue to decrease during and after the treatment cycle. By day 26, a 55% growth inhibition occurred compared with control tumors (Fig. 5B). Unlike DU145 tumors, 1 of 5 mice displayed CR on day 26. Later, the tumors in the remaining 4 mice grew somewhat slower and stayed smaller (P < 0.02) than control on the last day of the experiment (day 76). The tumors in control mice injected with vehicle continue to grow although slower than DU145. i.p. injections were more effective in diminishing the tumor sizes. An impressive tumor regression (60%) occurred after 10 days of last injection (day 19), and the antitumor effect continued. By the end of the experiment, 3 of 6 mice appeared as CR, and an 80% reduction (P < 0.00005) in tumor sizes was noted. In i.t.-injected mice, LNCaP tumors showed a dramatic response and a first CR.
appeared on day 12. After 3 additional weeks, 5 of 6 mice showed complete remission. None of CRs suffered relapse of tumors. The tumor in the lone partial responder grew to a size of 25 mm² by day 70, but average size of all of the tumors was 93% reduced than control tumors. Overall, all of the mice tolerated IL4-CTx therapy well except for 1 death because of unexplained reasons in the i.v.-treated LNCaP group.

Dose-dependent CRs by i.p. IL4-CTx in DU145 Xenografts

Because i.p. IL4-CTx (100 μg/kg/dose) afforded fewer CRs in Fig. 5, we reasoned that a higher number of injections (from QD to BID) and doses (up to 200 μg/kg) may raise the number of CRs. The mice with s.c. DU145 tumors (23.3 ± 0.9 mm²) were injected i.p. BID with 50, 100, or 200 μg/kg/dose on days 4–8 (a total of 10 injections) after tumor implantation (Fig. 6). By day 12, IL4-CTx administration resulted in a 73–77% reduction in tumor size in all of the treated groups. All three of the doses (50, 100, and 200 μg/kg) induced CRs of tumors in 1 of 6, 2 of 6, and 3 of 6 mice, respectively. However, the tumors in the control group injected with vehicle only continued to grow exponentially. The CRs remained tumor-free until day 54. Thereafter, tumors recurred in 1 each of the CRs in the 100 and 200 μg/kg dose groups. Although the average size of tumors in treated groups (28.7 ± 21.5, 38.3 ± 37.2, and 28.8 ± 30.2 mm²) was not significantly different from each other on day 54, they exhibited a dose-dependent antitumor activity in producing the CRs.

i.t. IL4-CTx Eradicates Tumors Completely

Not all of the mice displayed complete disappearance of tumors when injected i.t. with a 100 μg/kg dose of IL4-CTx (Fig. 5). To achieve absolute remission, we examined a dose-dependent profile of antitumor activity of IL4-CTx. The IL4-CTx (50, 250, and 500 μg/kg/dose) was injected directly into tumors for 5 successive days (days 3–7). Each group had 6 mice. The DU145 tumors in all of the treated xenografts began to decrease just after the initiation of treatment cycle (Fig. 7A). By fifth injections (day 7), the highest dose (500...
μg/kg) regressed tumors in 100% of mice. The intermediate dose (250 μg/kg) was effective in yielding 4 CRs. The 2 CRs also appeared in the lowest dose (50 μg/kg) on day 12. We obtained an additional CR in each group by day 14. Then, partially reduced tumors in both the intermediate- and low-dose groups began to enlarge but slower than the unrestricted tumor growth in control mice. The average tumor sizes were 76% (P < 0.011) and 95% (P < 0.0009) lower than control tumors in 50 and 250 μg/kg doses, respectively, on day 60. The CRs were extremely durable, because long-term monitoring for 8 months (234 days) after treatment showed no sign of tumor recurrence (data not shown). One animal in the 500-μg/kg group was found dead on day 60 most probably because of the infection. The i.t.-injection studies clearly suggest that IL4-CTx can cure androgen-independent tumors for an extended period of time, yielding disease-free survival.

**Efficacy of i.t. IL4-CTx in Mice with Large Tumor Burden**

Encouraged with the 100% CRs by i.t. administration in DU145 xenografts (Fig. 7A), we additionally evaluated whether relatively larger prostate tumors can be treated with IL4-CTx. We grew DU145 tumors to a size three times larger (∼70 mm³) than the previous experiments (Fig. 7B). Each treatment group consisted of 5 mice and control of 6 mice. The i.t. treatment was initiated on day 27 with 500 μg/kg/day dose of IL4-CTx either by QD injections for 10 days (5 injections/week) or 6 injections on alternate days (3 injections/week). The tumors began to regress as soon as the first treatment cycle was initiated. To our surprise, 1 CR appeared in each treated group by the last day of injections (day 38). The regression of tumors continued even after injection cycles were over and an additional CR appeared in each group on day 57. These 40% CRs remained tumor-free until the day of their sacrifice (day 85). The partial responders had tumors (16–36 mm²), which were roughly 2–4-fold less than the tumor size at the time of initiation of the treatments (∼70 mm³). The control tumors injected with excipient only grew to a size of 158 mm² and had to be sacrificed on day 64 because of ethical reasons. These data show that IL4-CTx can render some of the animals tumor-free even with heavy tumor burden.

**i.t. Injections of IL4-CTx Cause Necrosis and Apoptosis in Prostate Tumors**

We have shown previously that i.t. injections of IL4-CTx (250 μg/kg) in head and neck xenografts caused large areas of necrosis (26). To assess whether similar necrosis occurs in prostate tumors, large DU145 tumors (56.8 ± 16.5 mm²) were injected i.t with IL4-CTx (50 μg/kg, QOD × 3 or QD × 5) and 100 μg/kg (QOD × 3). Control tumors injected with excipient only (Fig. 8A) showed no evidence of necrosis. However, tumors treated with 50 μg/kg, QD × 5 IL4-CTx (Fig. 8B) showed large vacuoles as shown at the right side of the panel, which were created by the clearance of necrotic cells. At lower magnification, several of these large vacuoles were observed in IL4-CTx-treated tumors confirming necrotic tumor cell death process. In addition, treated tumors stained with ApopTag (Fig. 8D) showed apoptotic cells (green fluorescence), whereas control tumors (Fig. 8C) injected with excipient did not exhibit any apoptotic cells. Similar results were observed in tumors treated with 50 or 100 μg/kg (QOD × 3) of IL4-CTx (data not shown). These data demonstrate that direct injection of IL4-CTx into a tumor bed causes cell death through necrosis and apoptosis pathways.

**Discussion**

The cytotoxin or immunotoxin composed of tumor-selective cytokine/growth factor or antibody coupled to toxin moiety are a new class of molecular targeting agents. A number of these agents are being tested in xenograft models and in the
clinic for hematological and solid neoplasms (29, 33, 37). For example, IL-6 fusion toxin is being tested for prostate carcinoma, because IL-6 receptor mRNA was detected in 78% of BPH, 100% of prostate carcinomas, and three established prostate carcinoma cell lines (DU145, LNCaP, and PC-3). These cell lines were found to be sensitive to the cytotoxic action of IL-6 toxin (38). In another report, a subset of LNCaP cells containing PR1 antigen were shown to be quite sensitive in vitro to a recombinant immunotoxin composed of the monoclonal antibody PR1 and PE. Because PR1 antigen was detected in prostate carcinoma and normal prostate, it was suggested that this immunotoxin may be useful in human prostate cancers (39). Prostate tumors are also being targeted by the bFGF receptor-directed chimeric toxin, bFGF-saporin (19). In our study, we targeted IL-4R for prostate cancer therapy.

We observed that DU145 and LNCaP prostate cancer cells express mRNA for the IL-4Rα chain. In an earlier report, both cell lines were shown to express IL-13 receptor α1 chain mRNA (40). Thus, it is reasonable to hypothesize that prostate carcinoma cells express type II IL-4R where IL-4R complex is made of IL-4Rα and IL-13 receptor α1 subunits (36). We also provide the evidence for the first time that IL-4Rs are overexpressed in both androgen-dependent and independent prostate carcinoma cell lines. Furthermore, we demonstrate that IL-4R-positive prostate cancer cell lines are highly sensitive to the cytotoxic effect of IL4-CTx. The IC50 for LNCaP and DU145 cell lines were 4.5 and 6.5 ng/ml, respectively (Fig. 4). It is encouraging to note that the androgen-resistant cell line was equally sensitive to IL4-CTx. In addition, primary prostate tumor cell cultures were equally sensitive (<5 ng/ml) toward IL4-CTx (Fig. 5). However, the status of androgen sensitivity could not be confirmed in the patients from whom the primary cultures were established. Previous studies have demonstrated that IL4-CTx can cause complete regression of IL-4R-positive tumors (23, 25), because these cells were sensitive to IL4-CTx in vitro with similar pM range activity. Thus, it was postulated that IL4-CTx may possess significant antitumor activity in prostate tumor xenografts. Our hypothesis was correct, as both androgen-dependent and independent tumor xenografts showed a remarkable sensitivity to IL-CTx when injected locally or systemically. Although not quantitative, at least the detection of IL-4R on BPH and prostate carcinoma tissues encouraged us to assume that IL4-CTx will have a significant antitumor activity in the clinic. We believe that IL4-CTx therapy may overcome the hurdles encountered in the treatment of hormone-refractory prostate carcinomas. To additionally identify the expression of IL-4R in a larger population of prostate cancer patients, it would be necessary to analyze the prostate cancer tissue arrays on slides.

Depending on the route of administration, IL4-CTx mediated a different frequency of complete responses. Although i.v. injections of IL4-CTx caused tumor growth arrest in LNCaP-bearing mice, only 1 of 5 treated mice displayed CR. This limited response may be attributed to short serum half-life (t1/2 = 10 min) of IL4-CTx (41). However, similar treatment via the i.p. route proved to be better to provide 33% and 50% CR in DU145 and LNCaP xenografts, respectively. To additionally improve the availability of IL4-CTx, BID i.p. administration of IL4-CTx was explored in the DU145 xenograft model (Fig. 6). IL4-CTx (100 µg/kg; BID × 5 days) did not increase the number of CRs (2 of 6) compared with 100 µg/kg, QD × 5 schedule (Fig. 5A). However, on day 60, the
average tumor size were 68% less in BID schedule than 58% less in QD schedule. The highest dose (200 μg/kg; BID × 5 days) in the DU145 model improved CR to 50%. These data suggest that a threshold level of IL4-CTx could not be achieved either by i.v. or i.p delivery to obtain 100% CRs. To additionally improve the efficacy of systemic IL4-CTx, the circulation time of the drug in blood may need to be increased. This may be achieved by pegylation of IL4-CTx or by delivering drug continuously using micropumps. These possibilities are currently being contemplated.

Because sustenance of a drug at a tumor site is of critical importance in chemotherapy as well as in receptor/antigen-targeted therapy, we delivered our drug directly into the tumor by i.t. injections. The prostate tumor being a localized disease, it is practical to adopt this approach in the clinic. A similar approach of convection-enhanced delivery of IL4-CTx was adopted in our Phase I clinical trial in recurrent disease, it is practical to adopt this approach in the clinic. A similar approach of convection-enhanced delivery of IL4-CTx was adopted in our Phase I clinical trial in recurrent glioblastoma patients (29). In contrast to i.v. and i.p. routes of administration, a higher concentration of IL4-CTx (up to 500 μg/kg; QD × 10) could be administered through i.troute without systemic toxicity (Fig. 7B). A significant level of toxin could be accomplished at tumor bed when injected directly into a tumor (42). In our contralateral tumor model of glioblastoma, we demonstrated that i.t. IL4-CTx remained restricted inside the tumor and did not escape to the bloodstream (23). The higher drug accumulation at the tumor site caused remarkable antitumor efficacy without nonspecific toxicity often associated with systemic immunotoxins. The number of CRs obtained by i.t. treatment was dose-dependent. The highest dose (500 μg/kg; QD × 5) was the optimum dose to yield 100% CR (Fig. 7A). It is interesting to note that all of the CRs were extremely durable, and mice lived healthy and free of disease for at least >8 months. Another significant advantage of i.t therapy was that the animals with even heavy tumor burden (~225 mm³) also showed 40% CR. To elucidate the mechanism of tumor cell killing by IL4-CTx, we injected the drug directly into tumors, and 3 days after the last injection tumor sections were analyzed. As shown in Fig. 8, i.t injection of IL4-CTx mediated necrosis and apoptosis at the site of injection. As necrosis and apoptosis are prominent mechanisms of cell death, our data indicate that tumor killing is a direct consequence of IL4-CTx administration.

Because apoptosis is implicated in the bystander mechanism, it is also possible that some bystander effect is operational in IL4-CTx-induced tumor killing.

Targeted antigens or receptors are generally also present on normal cells and may cause unwanted toxicities. However, compared with normal cells, solid tumor cells often express high levels of IL-4R (23). In the case of benign prostate tissues, IL-4Rs are also expressed; therefore, IL4-CTx may cause some toxicity. But, the damage to prostatic hypertrophic tissues may not be an adverse clinical problem, because the entire prostate gland not being an essential organ, is removed during prostatectomy. Because targeted toxins offer the advantage of tumor specificity, it is proposed that IL4-CTx may play a significant role in prostate tumor therapy.

The purpose of our study was to demonstrate that human prostate tumors grown in nude mice can be effectively targeted with human IL4-CTx. Because human IL-4 does not bind murine IL-4R expressed on normal cells or tissues (43), our preclinical efficacy studies did not provide evidence of therapeutic index. To address this issue, we have shown previously that human IL4-CTx can be safely injected systemically to cynomolgus monkeys whose IL-4R binds human IL-4 (24). Up to 200 μg/kg doses of human IL4-CTx could be given with only reversible hepatic toxicity. Therefore, therapeutic systemic doses given in a mouse model of prostate cancer in our present study provides a therapeutic window for targeting prostate cancer by IL4-CTx.

In summary, IL4-CTx is a highly active IL-4R targeting agent and causes durable complete remission of s.c. prostate tumors in a mouse model without undesirable toxicity. IL4-CTx therapy may be pursued as an alternative therapy in chemotherapy-resistant and hormone-refractory prostate carcinoma. For advanced prostate carcinoma, the i.t. treatment with a high dose of IL4-CTx may be a realistic approach for localized tumor followed by low-dose systemic administration for disseminated disease to other organs. An orthotopic model of prostate carcinoma is being developed to mimic the clinical condition.

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