Interleukin 4 Receptor-directed Cytotoxin Therapy for Human Head and Neck Squamous Cell Carcinoma in Animal Models

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ABSTRACT
Receptors for interleukin 4 (IL-4R) are overexpressed on the surface of various human solid tumors including renal cell carcinoma, glioblastoma, Kaposi’s sarcoma, and head and neck squamous cell carcinoma (SCCHN). On the basis of this preferential receptor overexpression, a novel IL-4R-targeted cytotoxin, IL-4 (38–37)-PE38KDEL, was developed in which circularly permuted IL-4 [IL-4 (38–37)] was fused to mutated form of Pseudomonas exotoxin (PE38KDEL). Despite the recognized expression of the IL-4R on SCCHN, the utility of a receptor-specific fusion protein for the treatment of this disease remains unknown. The purpose of this study was to establish the utility of IL-4 (38–37)-PE38KDEL for the treatment of established SCCHN in animal models of human disease.

Expression of IL-4R in SCCHN was confirmed by immunohistochemistry with eight of eight tissue sections expressing IL-4R. Protein synthesis inhibition assays demonstrated growth inhibition of two cell lines in IL-4 (38–37)-PE38KDEL in a dose-dependent fashion. In two SCCHN s.c. xenografted nude mouse models, i.p. and intratumoral injection of IL-4 (38–37)-PE38KDEL mediated tumor regression with no visible toxicity observed in any of the animals. Subcultured tumor cells after intratumoral treatment with IL-4 toxin did not develop resistance to the drug. These data demonstrate that IL-4 (38–37)-PE38KDEL is effective in mediating significant antitumor effects in SCCHN and may represent an attractive therapeutic option for patients with advanced cancers of the upper aerodigestive tract.

INTRODUCTION
Metastatic SCCHN is difficult to treat by conventional means. One particularly challenging subpopulation of patients to address from a therapeutic perspective is the one with persistent regional disease after maximal surgical/radiotherapy (1–3). Because patients with persistent regional disease after treatment are resistant to traditional forms of therapy, many biological response modifiers are under investigation both to increase the potential for cure and to provide improved palliation. One particularly exciting area of investigation is the use of tumor-specific toxins targeting local-regional disease.

Recent evidence suggests that IL-4R are preferentially expressed on the surface of a variety of solid tumors including SCCHN (4–6). On the basis of this preferential receptor expression, a novel chimeric protein was designed, fusing a truncated form of the PE to a circularly permuted form of IL-4 (7–9). Preliminary studies with Kaposi’s sarcoma, glioblastoma, and breast cancer in nude mice demonstrate tumor-specific regression after toxin administration via intratumoral, i.p., and i.v. routes (10–12). These effects are dose dependent with minimal associated toxicity. Based on these animal data, a phase I/II human trial is currently underway to investigate the utility of this chimeric protein for the treatment of recurrent glioblastoma (13).

The goal of the present work was to confirm the expression of IL-4R in additional clinical SCCHN samples and to perform the preclinical studies to assess the potential utility of IL-4 (38–37)-PE38KDEL in mediating IL-4-specific toxicity in vitro (4). Subsequent studies were designed using nude mouse tumor models to document the utility of IL-4 (38–37)-PE38KDEL for the treatment of SCCHN in vivo and to assess appropriate methods of cytotoxin delivery.

MATERIALS AND METHODS
Cell Lines and Tissue Samples. The 012SCC and WMMSCC cell lines were kindly provided by Drs. Bert O’Malley, Jr., University of Maryland, Baltimore, MD and Suyu Shu from Cleveland Clinic Foundation, Cleveland, OH. The SCC15 is from the American Tissue Type Collection. The KCCT873 cell line was established in the Research Institute.
Kanagawa Cancer Center (Yokohama, Japan; Ref. 4). These lines were grown and maintained in RPMI 1640 cell medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat inactivated fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah), 25 mM HEPES, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (Life Technologies, Inc.). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. Tumor samples from patients undergoing surgical resection of their SCCHN at Mayo Clinic, Rochester, MN, were obtained under an Institutional Review Board-approved research protocol. Frozen tissue samples were stored in liquid nitrogen tanks until use. Frozen tumor tissue pieces were fixed in 10% formalin, paraffin embedded, and 4-μm sections cut for immunohistochemistry assays.

### Table 1 In situ expression of IL-4 receptors in SCC

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Gender</th>
<th>Histopathology and tumor grade</th>
<th>Tissue source</th>
<th>Recurrent vs. primary IL-4R expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71</td>
<td>Male</td>
<td>Grade 3 (of 4) SCC</td>
<td>Tongue</td>
<td>Recurrent</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>Male</td>
<td>Grade 2 (of 4) SCC</td>
<td>Submandibular gland, soft tissue, mandible</td>
<td>Primary</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>Male</td>
<td>Invasive Grade 3 (of 4) keratinizing SCC</td>
<td>Tongue</td>
<td>Recurrent</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>Male</td>
<td>Invasive Grade 3 (of 4) SCC</td>
<td>Piriform sinus</td>
<td>Primary</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>Male</td>
<td>Invasive Grade 3 (of 4) SCC</td>
<td>Frontal sinus</td>
<td>Recurrent</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>Female</td>
<td>Grade 2 (of 4) SCC</td>
<td>Tongue</td>
<td>Recurrent</td>
</tr>
<tr>
<td>7</td>
<td>76</td>
<td>Male</td>
<td>Invasive Grade 4 (of 4) SCC</td>
<td>Mass extended through mandible and soft tissue</td>
<td>Primary</td>
</tr>
<tr>
<td>8</td>
<td>69</td>
<td>Male</td>
<td>Invasive Grade 3 (of 4) SCC</td>
<td>Mass extended through mandible and soft tissue</td>
<td>Primary</td>
</tr>
</tbody>
</table>

**Fig. 1** Immunohistochemistry of IL-4R α chain in formalin-fixed head and neck cancer tissue sections. Tissue sections were generated from fresh-frozen tumor tissues obtained from patients undergoing surgical resection. Different magnifications are shown from two cases.

Recombinant Cytokine and Toxin. Recombinant human IL-4 was purchased from R&D Systems (Minneapolis, MN). Recombinant IL-4 toxin IL-4 (38–37)-PE38KDEL, containing the circularly permuted IL-4 mutant in which amino acids 38–129 were linked to amino acids 1–37 via a GGNGG linker and then fused to truncated toxin PE38KDEL, consisting of amino acids 253–364 and 381–608 of PE, followed by KDEL, was expressed in *Escherichia coli* and purified by modified procedure as described previously (7–9).

Immunohistochemistry. Tissue was fixed in 10% formalin and then embedded in paraffin. Sections of 4 μm were cut and mounted on slides. To deparaffinize the tissue, slides were washed with xylene and 100% ethanol followed by 95% ethanol and then distilled H₂O. Slides were then placed in target retrieval buffer (DAKO) at 90°C for 30 min. After slides had cooled, antibody staining was performed using the Ventana Medical Systems Basic AEC Detection kit (Ventana Medical Systems). The primary antibody was a monoclonal antibody against the human IL-4R α chain from Immunotech. Briefly, slides were incubated with a 1:20 dilution of the primary antibody for 4 h at 37°C. After a wash in PBS the slides were incubated with biotinylated secondary antibody for 8 min at 37°C then washed with PBS. The sections were then incubated with Streptavidin-HRPO for 8 min at 37°C followed by a wash in PBS. The slides were incubated in AEC solution plus hydrogen peroxide solution for 8 min at 37°C to visualize the antibody complex.

Proliferation Assays. The cytotoxic activity of IL-4 toxin to human SCCHN cell lines (012SCC and SCC15) was tested as described previously (14). In general, protein synthesis inhibition (inhibition of leucine uptake) directly correlates with cytotoxicity or cell death (14). Typically, 10⁴ cells were cultured in leucine-free medium with or without various concentrations of IL-4 (38–37)-PE38KDEL for 20–22 h 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 h. Cells
were harvested, and radioactivity incorporated into cells was measured by the Wallac Trilux 1450 Microbeta counter. Results are reported as percentage of inhibition in comparison with untreated controls.

To correlate leucine inhibition with cell growth, an analogous experiment was designed in which the absolute number of viable cells was determined at defined intervals based on culture in defined concentrations of IL-4 (38–37)-PE38KDEL. Specifically, 012SCC cells were plated onto a 96-well cell-culture plate at 10^4 cells/well in 100-μl volume. IL-4 toxin was added to each well in serial 1:10 dilutions from a starting concentration of 1000 ng/ml to 0.1 ng/ml concentrations. Each dilution of toxin was performed in triplicate, and a negative medium control was included. Cells in each well were trypsinized and counted at 48 and 96 h.

**Animals.** Athymic nude mice 4 weeks old (~20 g in body weight) were obtained from Frederick Cancer Center Animal Facilities (National Cancer Institute, Frederick, MD). The mice were housed in filter-top cages in a laminar flow hood in pathogen-free conditions with 12-h light/12-h dark cycles. Animal care was in accordance with the guidelines of NIH Animal Research Advisory Committee.

**Tumor Establishment and Treatment.** Human head and neck cancer model was established in nude mice by s.c. injection of 5 × 10^5 KCCT873 or 1 × 10^5 012SCC cells in 150 μl of PBS plus 0.2% human serum albumin into the flank. Palpable tumors developed within 3–4 days. The mice then received injections of excipient (0.2% HSA in PBS) or chimeric toxin with a 27-gauge needle either i.p. (500 μl) or intratumorally (30 μl). Tumor growth was carefully measured at least twice weekly by Vernier calipers in a standard manner as described in various publications (10, 11). Five mice were used in each group. Both cell lines produced consistently palpable tumors within 3–5 days. Mean size of tumors was calculated with SDs. The statistical significance of tumor regression was calculated by Student’s t test.

**Tumor Pathology.** Two days after the treatment of 012SCC tumor-bearing nude mice with one or two intratumoral injections of IL-4 (38–37)-PE38KDEL (5 μg/kg), mice from both control and treated groups were sacrificed. Tumors were resected, fixed in 10% formalin, and 5-μm cryostat sections were fixed in 90% ethanol and stained with H&E.

**RESULTS**

**Expression of IL-4R in Surgical Tissue Samples from Head and Neck Cancer Patients.** We first examined the expression of IL-4R in surgical samples from eight patients with SCCHN. Samples were stained with anti-IL-4R antibody, and the intensity of the staining was evaluated. For controls, no primary antibody was used and all of the controls showed negative staining. For positive control, we used the Daudi cell line, which is known to have IL-4Rs. This cell line stained positive for IL-4Rs with the IL-4R antibody. As shown in Table 1, all eight (100%) of the samples tested showed modest to moderate level of expression of IL-4R. Fig. 1 shows staining of SCCHN from two surgical cases. An intense staining with IL-4R antibody was observed as demonstrated in different magnifications. In the surrounding mucosa, a low level but diffuse staining of IL-4R was also observed (not shown).

**IL-4 (38–37)-PE38KDEL Inhibits the Growth of Head and Neck Cancer Cell Lines in Vitro.** Protein synthesis inhibition assays were performed to characterize the effects of IL-4 (38–37)-PE38KDEL on the growth of two SCCHN cell lines. As shown in Fig. 2a, although a cytotoxic effect was found to be superior in 012SCC cells, IL-4 toxin was cytotoxic to both 012SCC and SCC15 cell lines in a dose-dependent manner. This effect was dependent on the IL-4R specifically, because competitive inhibition by the addition of IL-4 resulted in normalization of leucine uptake. Inhibition of leucine uptake correlated with absolute cell number measured at defined time intervals (Fig. 2b). These data clearly demonstrate that IL-4 (38–37)-PE38KDEL is effective in mediating specific cytotoxicity to SCCA cell lines in vitro.
i.p. Administration of IL-4 (38–37)-PE38KDEL Inhibits the Growth of Head and Neck Tumor in Nude Mice. To explore IL-4 toxin-mediated antitumor activity to SCCHN tumors, we s.c. injected nude mice with 012SCC or KCCT873 tumor cells, and then IL-4 (38–37)-PE38KDEL was administered twice daily for 5 days from day 4 to 8 (total of 10 injections). As shown in Fig. 3A, all of the control mice showed rapid KCCT873 tumor growth requiring sacrifice on day 25 because of large tumor burden (168 mm²). On the other hand, tumors treated i.p. with IL-4 toxin started regressing during the treatment, and one tumor treated with a higher dose (200 µg/kg) of IL-4 toxin completely disappeared by day 10. In mice treated with 100 µg/kg of IL-4 toxin, by day 25 the mean size of the tumors was significantly smaller than tumors in control mice, and reduction in tumor size was 62% (P < 0.0002) compared with control tumors. In mice treated with 200 µg/kg of IL-4 toxin, the mean size of the tumors on day 25 remained the same as on the first day of IL-4 toxin treatment (39 mm²), and the reduction in tumor size was 87% (P < 0.0001) compared with control tumors.

In the 012SCC-tumor model, all of the tumors started regressing during the IL-4 toxin treatment (200 µg/kg), and by day 7 two tumors had completely regressed (Fig. 3B). Thereafter, the tumors started growing gradually; however, the size remained significantly smaller compared with control tumors, including one tumor that disappeared. Tumors in control mice injected with vehicle only continued to grow exponentially, and these mice were sacrificed on day 26. The reduction in tumor size in the treated group on day 25 was 74% (59 mm²; P < 0.0006) compared with tumors in the control group (225 mm²). No visual evidence of toxicity was observed in any of the animals treated with i.p. administration of IL-4 (38–37)-PE38KDEL. In our previous studies, we have shown preclinical toxicity of IL-4 toxin. Up to 200 µg/kg dose given i.v. every alternate day for three doses showed modest elevations of hepatic enzymes (8).

Intratumoral Administration of IL-4 (38–37)-PE38KDEL Leads to Complete Regression of Head and Neck Tumor in Nude Mice. We also assessed the efficacy of intratumoral administration of IL-4 toxin against KCCT873 tumors, because intral esional administration of any biological agent is a viable option for this malignancy. As shown in Fig. 4, treatment of KCCT873 tumors with intratumoral IL-4 (38–37)-PE38KDEL (200 or 500 µg/kg/day for 5 days) from day 4 to 8 dramatically inhibited tumor growth. Three of five tumors in the 200 µg/kg treatment group completely regressed by day 8, and all five of the tumors in the 500 µg/kg treatment group disappeared by day 10. Although a palpable tumor appeared in some of the tumor-regressed mice, one mouse in the 200 µg/kg group and two mice in the 500 µg/kg group remained tumor-free until the day they were sacrificed (day 26).

Histological Evidence of Tumor Necrosis after Intratumoral IL-4 (38–37)-PE38KDEL Treatment. To assess the histological evidence of tumor necrosis, 012SCC tumors were...
treated with one or two intratumoral injections of either 5 μg of IL-4 (38–37)-PE38KDEL or saline. Mice were sacrificed 2 days after the final injection. As shown in Fig. 5, tumors treated with only saline had histological evidence of a poorly differentiated SCCHN with infiltration of skeletal muscle and surrounding fibrous tissue and minimal evidence of necrosis. On the other hand, animals treated with one or two injections of IL-4 toxin showed large areas of necrosis. Importantly, even in the treated tumors, viable tumor cells remained on the periphery of the specimen suggesting that distribution of this drug may be critical for the powerful antitumor activity to be revealed.

**Tumor Outgrowth in Treated Animals Is Not Secondary to the Development of Resistance to IL-4 (38–37)-PE38KDEL.** To evaluate whether tumor outgrowth was secondary to the development of resistance to IL-4 (38–37)-PE38KDEL, a leucine inhibition assay was performed on freshly isolated tumors with persistent growth after an initial response. Tumors (012SCC) were grown in nude mice and treated with intratumoral administration of IL-4 (38–37)-PE38KDEL 7.5 μg on days 4, 6, and 8 (three times). Viable tumors after treatment were resected, minced, and treated with 10 μg/ml collagenase, 1 mg/ml hyaluronidase, and 0.5 mg/ml DNase (Sigma Chemical Co., St. Louis, MO), washed with PBS for two times, and cultured in medium containing 10% FBS. After in vitro passage, contaminating tumor debris and blood cells were removed, and a leucine inhibition assay was performed. As shown in Fig. 6, IL-4 toxin effectively inhibited leucine uptake in these subcultured 012SCC cells, and the sensitivity to IL-4 toxin was similar to control 012SCC cells that remained in culture in vitro. These data suggest that observed tumor outgrowth after IL-4 (38–37)-PE38KDEL administration in vivo is not secondary to the development of drug resistance.

**DISCUSSION**

In this study we demonstrate that IL-4R-directed cytotoxic has pronounced antitumor activity against SCCHN in animal models of human disease. This antitumor activity was evident against established human tumors in immunodeficient athymic nude mice. i.p. administration of IL-4 (38–37)-PE38KDEL produced remarkable antitumor activity with occasional complete responses. Intratumoral administration produced more pronounced effects with several animals demonstrating complete tumor regression. The dose that produced profound antitumor activity did not cause toxic reactions in any animal, because all of the mice appeared healthy and survived during and after IL-4...
(38–37)-PE38KDEL therapy. The lack of toxicity at efficacious doses of IL-4 (38–37)-PE38KDEL confirm our previous observations in human glioma, breast cancer, and AIDS-associated Kaposi’s tumor models (10–12). Previously we have reported that IL-4 toxin could be safely given i.v. to immunocompetent monkeys at high doses with reversible hepatic toxicity (15).

The profound antitumor activity of IL-4 (38–37)-PE38KDEL in SCCHN correlated with the expression of IL-4R in vitro. Eight of eight SCCHN tumor sections showed specific staining for IL-4R. These data agree with our and other previous reports that SCCHN cells express IL-4R on their cell surface and confirm that IL-4Rs are expressed in situ. (4, 5, 16). Because previous evidence suggests those human squamous cell carcinomas preferentially express the IL-4R in comparison with histologically normal mucosa (5), our current study provides preclinical data to support the hypothesis that preferential expression of the IL-4R in SCCHN might provide a target for directed therapy using the IL-4-PE conjugated fusion protein [IL-4 (38–37)-PE38KDEL].

The critical findings of this study are that IL-4 (38–37)-PE38KDEL mediates cytotoxicity in vitro, which is competitively blocked by the addition of IL-4, suggesting that the observed effects are mediated through binding of IL-4 (38–37)-PE38KDEL to the IL-4R. Translation of these findings to a preclinical animal model demonstrates that IL-4 (38–37)-PE38KDEL is effective in mediating the regression of established tumors after either local or systemic therapy. Leucine inhibition assays performed on tumors, which continued to proliferate after initial regression, remained sensitive to IL-4 (38–37)-PE38KDEL in vitro. Additionally, histological data from treated animals and saline-injected controls demonstrated tumor necrosis in the treatment group. Despite therapy, microscopically viable tumor remained on the edges of the histological section. These data suggest that the mechanism for tumor escape after treatment is not secondary to drug resistance but rather results from inadequate drug distribution throughout the tumor bed. From a clinical perspective, this is an important distinction, because it supports retreatment with potential additional therapeutic benefit.

Previous studies have demonstrated that IL-4–PE can mediate apoptotic cell death of head and neck cancer cells in vitro (4). Similarly, various chemotherapeutic agents that are being used for head and neck cancer have shown apoptotic cell death (17–19). Whether a combination of standard drugs with IL-4–PE may mediate optimal apoptosis and enhances antitumor activity remains unknown. IL-4–PE may be useful for managing microscopic disease when administered during or after tumor resection. Future studies will address these possibilities.

REFERENCES


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