Structure, Function, and Targeting of Interleukin 4 Receptors on Human Head and Neck Cancer Cells

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ABSTRACT

Despite advances in diagnosis and treatment, survival rates for patients with head and neck cancer have remained unchanged for the last 30 years. In an attempt to develop novel therapeutic agents, we have observed that a variety of murine and human carcinoma cells expresses high levels of receptors for interleukin 4 (IL-4) in vitro and in vivo. Here, we demonstrate that 17 head and neck cancer cell lines also express surface IL-4 receptors (IL-4R) and IL-4 binds to IL-4R on one cell line studied with low affinity ($K_d = 37.9 \pm 0.4$ nM). The investigation of the subunit structure of IL-4R demonstrated that head and neck cancer cell lines expressed mRNA for IL-4R$\beta$ chain (also known as IL-4R$\alpha$) and IL-13R$\alpha'$ chain (also known as IL-13R$\alpha_1$). However, no cell line expressed IL-2R common $\gamma$-chain, which is known to be shared with IL-4R in immune cells. IL-4R is functional because IL-4 strongly induced activation of signal transducers and activators of transcription 6 (STAT-6) in these cell lines. A fusion protein, IL-4(38-37)-PE38KDEL, containing translocation and enzymatic domains of Pseudomonas exotoxin and a circularly permuted human IL-4 was found to be highly and specifically cytotoxic to IL-4R-positive head and neck cancer cells, as determined by protein synthesis inhibition assay and confirmed by clonogenic assay. IL-4(38-37)-PE38KDEL induced DNA fragmentation and condensation of nuclei indicative of apoptotic cell death. These results establish uniform expression of IL-4R on head and neck cancer cell lines and IL-4 toxin IL-4(38-37)-PE38KDEL as a novel therapeutic agent for the possible treatment of human head and neck cancers.

INTRODUCTION

SCCHN represents approximately 5% of all cancers, and about 70,000 new cases were diagnosed in 1997 alone in the United States (1, 2). Although advances in surgical reconstruction and combined modality therapy have improved functional outcome, there has been no significant improvement in survival in the past 30 yr (3, 4).

We have reported that a wide variety of murine and human carcinoma cells expresses plasma membrane receptors for an immune regulatory cytokine, IL-4, in vitro and in vivo (5–8) and that IL-4 functions by signaling through its receptors (9). We have also studied the subunit composition of IL-4R on a variety of cell types (10–13). It was found that the IL-4R system could exist in three different types. Type I receptors are shown to consist of a major $M_1$ 140,000 protein (IL-4R$\beta$, also known as IL-4R$\alpha$) and IL-2R$\gamma_c$. Type II receptors are composed of IL-4R$\beta$ and IL-13R$\alpha'$ (also known as IL-13R$\alpha_1$) chains. In a third type of IL-4R, all three chains may form an IL-4R complex (11–15). Although the importance of expression of IL-4Rs on solid tumors is not known, we and others have observed that solid human tumors including malignant melanoma, breast carcinoma, ovarian carcinoma, mesothelioma, neuroblastoma, renal cell carcinoma, and AIDS-associated Kaposi’s sarcoma respond to IL-4 (7, 8, 16–19). IL-4, a unique cytokine produced by activated T lymphocytes and mast cells (5, 6, 20), inhibits the in vitro growth of several tumor cell lines. It has also been reported that IL-4 can induce apoptosis in human breast cancer cell lines (21). In contrast, Myers et al. (22) reported that IL-4 could stimulate the growth of 6 of 13 SCCHN cell lines.

Using a chimeric protein composed of circular permuted IL-4 and a truncated form of a powerful bacterial toxin called PE [IL-4 toxin called IL4 (38-37)-PE38KDEL], we have shown that this toxin is highly cytotoxic to IL-4R-positive tumor cells in vitro (23–28) and in vivo (16, 26, 29). In our previous study, we have reported that head and neck cancer cells also express IL-4R in situ (30). However, IL-4R structure, function, and cytotoxic activity of IL-4 toxin in SCCHN have not been investigated. In the present study, we have examined the expression, structure, and function of IL-4R on head and neck cancer cell lines. In addition, we have investigated the cytotoxicity and mechanism of cytotoxicity of IL-4 toxin in SCCHN cell lines.

MATERIALS AND METHODS

Reagents. Recombinant human IL-4 and IL-13 were expressed and purified in our laboratory, as described previously (31, 32).

Recombinant IL-4 Toxin. The IL-4 toxin IL4(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 253–536 and 381–608 of PE, followed by KDEL, was expressed in Escherichia coli and purified as described previously (24–26).

Cell Lines. Human head and neck cancer cell lines (KB, A253, RPMI 2650, and HEP-2) were purchased from American Type Culture Collection (Manassas, VA). The WSU-HN12 (termed HN12) cell line was a kind gift from Dr. Andrew Yeudall (National Dental and Craniofacial Research Institute, NIH, Bethesda, MD; Ref. 33). Twelve head and neck squamous cell cancer cell lines were established in the Department of Otolaryngology, Yokohama City University School of Medicine or Research Institute, Kanagawa Cancer Center (Yokohama, Japan; Ref. 34). The origin of the each cell lines is shown in Table 1. Cells were cultured in Eagle’s Modified Essential Medium (KB, A253, RPMI 2650, HEP-2, and HN12) or RPMI 1640 (the other cell lines) containing 10% fetal bovine serum (BioWhittaker Inc., Walkersville, MD), 1 mM HEPES, 1 mM nonessential amino acids, 100 $\mu$g/ml penicillin, and 100 $\mu$g/ml streptomycin (BioWhittaker Inc.).

Radioreceptor Binding Assays. Recombinant human IL-4 was labeled with $^{125}$I (Amersham Corp.) using IODO-GEN reagent (Pierce Chemical Co., Rockford, IL), as described previously (7). The specific activity of the radiolabeled IL-4 was estimated to be 39 $\mu$Ci/ $\mu$g protein. For binding experiments, $1 \times 10^6$ cells in 100 $\mu$l of binding buffer (RPMI 1640 containing 0.2% human serum albumin and 10 mM HEPES) were incubated with 200 $\mu$l 125I-IL-4 with or without various concentrations (1 ppt to 100 nm) of unlabeled IL-4 or IL-13 at 4°C for 2 h. Cell-bound 125I-IL-4 was separated by centrifugation through a phthalate oil gradient, and radioactivity was determined with 125I (Amersham Corp.) using IODO-GEN reagent (Pierce Chemical Co., Rockford, IL), as described previously (7). The specific activity of the radio-labeled IL-4 was estimated to be 39 $\mu$Ci/ $\mu$g protein. For binding experiments, $1 \times 10^6$ cells in 100 $\mu$l of binding buffer (RPMI 1640 containing 0.2% human serum albumin and 10 mM HEPES) were incubated with 200 $\mu$l 125I-IL-4 with or without various concentrations (1 ppt to 100 nm) of unlabeled IL-4 or IL-13 at 4°C for 2 h. Cell-bound 125I-IL-4 was separated by centrifugation through a phthalate oil gradient, and radioactivity was determined with a gamma counter (Wallac, Gaithersburg, MD). In some experiments, the number of IL-4Rs and binding affinities were calculated by the LIGAND program (35).
Northern Analysis. Total RNA was isolated using TRIZOL reagent (Life Technologies, Grand Island, NY). Equal amounts of total RNA were electrophoresed through an 0.8% agarose, formaldehyde-denaturing gel, transferred to a nylon membrane (S&S Nytran; Scheicher and Schuell, Keene, NH) by capillary action, and immobilized by UV cross-linking (Stratagene, La Jolla, CA). The cDNA for human IL-13Rα, common γc or glyceraldehyde-3-phosphate dehydrogenase, was labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham, Arlington Heights, IL). Membrane-bound RNA was prehybridized for 30 min at 37°C and then hybridized with 32P-labeled cDNA probes for 1 h at 37°C in ExpressHyb hybridization solution (Clontech Laboratories, Inc., Palo Alto, CA). The membranes were washed and subsequently exposed to an X-AR film for 12–72 h at 270°C to obtain an autoradiogram.

RT-PCR Analysis. To detect the expression of IL-4Rβ chain in head and neck cancer cell lines, we performed RT-PCR analysis. Total RNA (2 μg) was incubated for 30 min at 42°C in 20-μl reaction buffer containing 10 mM Tris-HCl (pH 8.3), 5 mM MgCl2, 50 mM KCl, 1 mM each of dNTPs, 1 unit/μl RNase inhibitor, 2.5 μM random hexamer, and 2.5 units/μl of MMLV RT (Perkin-Elmer Corp., Norwalk, CT). A 10-μl aliquot of RT reaction was amplified in a final 100-μl volume of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 2 mM MgCl2, 50 mM KCl, 2.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp.), and 0.1 μg of specific primer (5′ primer 5′-GACCTGGAGCAACCGTATC-3′ and 3′ primer 5′-CATAGCACAA-...
Electrophoretic mobility shift assay. After incubation with IL-4 (50 ng/ml) for 10 min, cells were washed with cold extraction buffer [1 mg/ml leupeptin, 5 mg/ml pepstatin A, 2 mg/ml aprotinin, 20 mM HEPES (pH 7.0), 10 mM KCl, 300 mM NaCl, 0.5 mM DTT, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na$_3$VO$_4$, and 20% glycerol]. DNA-protein interactions were assessed by electrophoretic mobility shift assay using a Bandshift kit (Pharmacia Fine Chemicals, Piscataway, NJ). Briefly, 50 μg of sample proteins were incubated in 20 μl of binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 0.05% NP-40, and 0.05 mg/ml poly(dI-dC)$_2$] for 20 min at room temperature with 1 ng of 32P-labeled double-stranded oligonucleotide probe SBE1. SBE1 is a STAT-binding element (5′-gatcGCTCTTCTTCCCAGGAACTCAATG-3′), which is from the region flanking the transcription start site of the human $sIL-1R$ antagonist gene that is necessary for response to IL-4 (36). A 10× loading dye (2 μl) was added to samples that were then applied to a 4% nonreducing polyacrylamide gel and run at 150 V for 2.5 h. Gels were dried for 2 h and autoradiographed overnight at room temperature.

Protein Synthesis Inhibition Assay. The cytotoxic activity of IL-4 toxin was tested as described previously (37). Typically, 10$^5$ cells were cultured in leucine-free medium with or without various concentrations of IL4(38-37)-PE38KDEL for 20–22 h at 37°C. For blocking experiments, cells were preincubated with IL-4 or IL-13 (2 μg/ml) for 1 h before the addition of IL-4 toxin. 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 a β plate counter (Wallac).

Detection of Apoptotic Cells. Apoptotic cells were assessed morphologically by fluorescent microscopy. KB cells (2 × 10$^6$) were plated in 100-mm Petri dishes with 7 ml of medium and were allowed to attach for 24 h. The cells were exposed to IL-4 toxin (1000 ng/ml) or IL-4 (100 ng/ml) for 48 h, then washed, fixed with 1% glutaraldehyde, and stained with 1 mM bis-Benzimide (Hoechst no. 33342; Sigma Chemical Co., St. Louis, MO). The samples were examined by fluorescent microscopy under an UV filter.

DNA Fragmentation Assay. After a 48-h incubation of KB cells (2 × 10$^6$) with IL-4 toxin, the cell extracts containing fragmented DNA were incubated with 0.5 mg/ml RNase A at 37°C for 60 min, then with 0.5 mg/ml proteinase K at 37°C for 60 min. After incubations, fragmented DNA was precipitated by isopropanol and dissolved in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5% glycerol, and 0.05% bromphenol blue. DNA fragments (2 μg), separated by 1% agarose gel electrophoresis, were stained with ethidium bromide and photographed on a UV transilluminator.

Clonogenic Assay. The in vitro cytotoxic activity of IL4(38-37)-PE38KDEL on KB cells was also determined by colony-forming assay. The cells were plated in triplicates in 100-cm$^2$ Petri dishes with 7 ml of medium containing 20% fetal bovine serum and allowed to attach for 20–22 h. The number of cells/plate was chosen such that >100 colonies were obtained in the control group. The cells were exposed to different concentrations of IL-4 toxin (0–100 ng/ml) or IL-4 (0–100 ng/ml) for 9 days at 37°C in a humidified incubator. The cells were washed, fixed, and stained with crystal violet (0.25% in 25% alcohol). Colonies consisting of >50 cells were scored. The percentage
of colony survival was determined from the number of colonies formed in the control and treated groups.

RESULTS

IL-4 Binding on Head and Neck Cancer Cells. We first determined the expression and binding affinity of IL-4R on SCCCHN cell lines by $^{125}$I-IL-4 binding assays. Five different cell lines were labeled with $^{125}$I-IL-4 in the absence or presence of 200-fold molar excess of IL-4. As shown in Fig. 1A, $^{125}$I-IL-4 bound to SCCCHN cells at varying degrees and excess of unlabeled IL-4 displaced the binding of $^{125}$I-IL-4. Because IL-4R and IL-13R share two chains with each other, we also examined whether IL-13 can displace the IL-4 binding in SCCCHN cells (14, 15). As shown in Fig. 1A, IL-13 also displaced for $^{125}$I-IL-4 binding, however, IL-4 was slightly superior to IL-13 in displacing $^{125}$I-IL-4 binding.

To further characterize the IL-4R in head and neck cancer cells, we performed Scatchard analysis on the YCUM862 cell line (Fig. 1, B and C). YCUM862 cells bound IL-4 in a concentration-dependent manner. Scatchard analysis of the binding data showed a single type of receptor with a $k_d$ value of 37.9 ± 0.4 nM. The number of IL-4Rs was calculated as 476,000 ± 5,000 IL-4 molecules bound/cell (mean ± SD, n = 2).

Subunit Structure of IL-4R on Head and Neck Cancer Cells. Seventeen head and neck cancer cell lines were examined for the expression of various putative IL-4R subunits. By Northern analysis, we found that mRNA for IL-13Ra chain was uniformly present in all of the cell lines examined. However, no SCCCHN cell lines showed presence of $\gamma_c$ mRNA (Fig. 2A). H9 T lymphoma cells that express $\gamma_c$ mRNA served as a positive control. We also examined the mRNA expression of IL-4Rb chain by RT-PCR analysis and found that all of the cell lines examined expressed IL-4Rb chain (Fig. 2B).

Activation of STAT6 in Response to IL-4 in Head and Neck Cancer Cells. To determine whether IL-4R expressed on cancer cells are biologically functional, we analyzed STAT6 activation in response to IL-4 in four SCCCHN cell lines. It has been shown that for signaling IL-4 can activate STAT6 protein in various cell types (12, 13, 38). Fig. 3 shows that IL-4 induced STAT6-DNA interaction in KB, A253, and YCUM862 cell lines (Lanes 2, 4, and 6), whereas STAT6 activation was not detectable in HEp-2 cells that expressed a low number of IL-4Rs (Lane 8).

Cytotoxic Effect of IL4(38-37)-PE38KDEL on Head and Neck Cancer Cells. Because SCCCHN cell lines expressed functional IL-4R, it was presumed that IL-4 toxin will be cytotoxic to these cells. To test this hypothesis, we evaluated cytotoxic activity of IL4(38-37)-PE38KDEL in human head and neck cancer cell lines. Table 1 shows the IC$_{50}$s (the protein concentration required for the inhibition of protein synthesis by 50%) in the 17 cell lines studied that ranged from 0.1–600 ng/ml. Fifteen of 17 cell lines were extremely sensitive to IL-4 toxin in which the IC$_{50}$s were <10 ng/ml. The cytotoxic activity of IL4(38-37)-PE38KDEL was neutralized by excess IL-4 and IL-13 in the cell lines examined (Fig. 4). Extremely high numbers of IL-4R...
on YCUM862 cells might account for the high sensitivity to IL-4 toxin (IC_{50} = 0.75 ng/ml).

**IL4(38-37)-PE38KDEL Induces Apoptosis in SCCHN Cells.** Apoptotic cells were assessed morphologically by staining with bis-Benzimide after treatment with IL-4 or IL4(38-37)-PE38KDEL. As shown in Fig. 5A, IL-4 toxin induced pronounced programmed cell death as evidenced by fragmented and condensed nuclei, whereas no apoptotic cells could be detected after incubation with IL-4. Fig. 5B shows DNA fragmentation in the KB cell line after treatment with various concentrations of IL4(38-37)-PE38KDEL. Inter-nucleosomal DNA fragmentation, a biochemical feature of the apoptotic process, was observed when cells were treated with 100 or 1000 ng/ml of IL-4 toxin (Lanes 5 and 6). Lower concentrations of IL-4 toxin also showed slight DNA smears (Lanes 2–4). However, IL-4 at concentrations up to 100 ng/ml did not cause DNA fragmentation (Lane 7 and data not shown for lower concentrations).

**Inhibition of Colony Formation of SCCHN Cells by IL4(38-37)-PE38KDEL.** To confirm IL4(38-37)-PE38KDEL-mediated cell death, we performed a colony formation assay using the KB cell line. Five hundred KB cells were plated in Petri dishes and incubated with various concentrations of IL4(38-37)-PE38KDEL. For comparative purposes, KB cells were treated similarly with IL-4. After 9 days of culture, the percentages of colonies formed in control, IL-4 toxin, or IL-4 groups were compared. As shown in Fig. 6B, IL4(38-37)-PE38KDEL inhibited colony formation in KB cells in a concentration-dependent manner. These results were comparable with the dose-kinetics seen in the cytotoxicity assay (Fig. 6A). In both assays IL-4 had no effect on proliferation of KB cells.

**DISCUSSION**

The aims of this study were to investigate the expression, structure, function and targeting of IL-4R on a variety of head and neck cancer cell lines. The studies described here extend our previous observations and demonstrate that all human head and neck cancer cell lines examined expressed IL-4Rs (30). Although Northern and RT-PCR analyses do not directly show the expression of the IL-4Rβ and IL-13Rα′ chains, our studies imply that IL-4R complex on SCCHN is of type II form where the IL-4Rβ chain forms a complex with the IL-13Rα′ chain. The common γ c chain was not identified in these cells. This receptor structure is similar to that observed on a variety of human solid tumor cell types, e.g., colon cancer, renal cell carcinoma, AIDS-associated Kaposi’s sarcoma, glioblastoma multiforme, and breast carcinoma (8, 10, 12, 16, 27). Thus, it is reasonable to assume that all solid cancers that express IL-4R may express type II receptors. The IL-4R on SCCHN seemed to be related to the IL-13R because IL-13 was able to displace 125 I-IL-4 binding and competed for IL-4 toxin-mediated cytotoxicity.

Typically, a ligand must bind to the extracellular domain of cytokine receptors to generate a biological response. In the case of IL-4, such interaction in immune cells (e.g., B cells and T cells) induces growth stimulation through the binding to type I IL-4Rs in which the IL-4Rβ chain forms a functional complex with the common γ c and through STAT 6 activation. However, unlike other solid cancer cells, including some head and neck cancer cell lines in which IL-4 has been shown to modulate growth, IL-4 did not modulate growth of head and neck cancer cell lines studied in our laboratory (30). Nevertheless, IL-4 was able to signal through activation of STAT6 protein in these cells. Thus, our studies indicate that IL-4 uses a similar distal pathway of signaling in cancer cells and immune cells and that IL-4Rs are functional on head and neck cancer cells.

The IL-4R expressed on head and neck cancer cells served as an efficient target for a cytotoxic agent. As seen in several solid cancers,
IL-4 TARGETS TOXINS TO HEAD AND NECK CANCER CELL LINES

Fig. 6. Inhibition of protein synthesis and colony formation by IL-4 toxin. A, KB cells were cultured with various concentrations (0–1000 ng/ml) of IL4(38-37)-PE38KDEL or IL-4, as described in “Materials and Methods.” The data were obtained from the mean of quadruplicate determinations. ●, IL4(38-37)-PE38KDEL; ○, IL-4. Data are means; bars, SD. B, Five hundred KB cells were allowed to adhere in Petri dishes, and medium was replaced with medium containing various concentrations of IL4(38-37)-PE38KDEL or IL-4. Cells were cultured for 9 days, and colonies consisting of at least 50 cells were scored after staining with crystal violet. ▲, IL4(38-37)-PE38KDEL; ●, IL-4. Data are means of triplicate determinations; bars, SD.

IL4(38-37)-PE38KDEL was also highly and specifically cytotoxic to head and neck cancer cells (23–27). Fourteen of 17 cell lines exhibited remarkable sensitivity to the cytotoxic activity of IL-4 toxin. The cytotoxic activity was further confirmed in a clonogenic assay. Furthermore, the IC₅₀ was similar in protein synthesis inhibition and clonogenic assays. The cytotoxic activity of IL4(38-37)-PE38KDEL was mediated through apoptotic cell death, whereas IL-4 had no apoptotic activity. Others have reported that B3(Fv)-PE38 immunotoxin (in which Lewis² is recognized by the Fv fragment of an antibody connected to a mutated form of PE) induces apoptosis in breast cancer cells through the caspase pathway (39). Our study confirms these observations. However, in contrast to previous studies, IL-4 by itself did not cause apoptosis in SCCHN cell lines (21). Similarly, in contrast to previous studies, our study has not identified growth stimulatory effects of IL-4 in four SCCHN cell lines (e.g., KB, A253, RPMI 2650, and HEP-2; Ref. 22, 30). The reason for this differential effect is not known; however, it is possible that the IL-4 effect is tumor specific representing the heterogeneous nature of this cancer.

Our previous studies have demonstrated that in vitro sensitivity to IL-4 toxin correlates with in vivo antitumor activity in brain tumor, AIDS-associated Kaposi’s sarcoma, epidermoid carcinoma, and breast tumor models in nude mice (16, 26, 29, 40). On the basis of these observations, it is reasonable to predict that IL4(38-37)-PE38KDEL will have significant antitumor activity in SCCHN in vivo in athymic nude mice with s.c.-growing tumors. We are currently examining the antitumor activity of IL4(38-37)-PE38KDEL by systemic administration in a SCCHN xenograft model.

Various innovative approaches, including gene transfer, are being tested for SCCHN. Although these techniques seem to be promising, currently no approach seems to be more effective. In addition, these approaches are limited due to vector-related toxicities and suboptimal gene transfer. Because IL4(38-37)-PE38KDEL is a small molecule and does not involve an indirect mechanism of tumor cell kill, we believe that it may have superior antitumor activity without producing unknown virus or plasmid-related toxicities.

In conclusion, we demonstrate that 100% of SCCHN tumor cell lines examined express surface IL-4Rs that seem to be biologically functional. Because IL-4 toxin IL4(38-37)-PE38KDEL has profound cytotoxic activity against all of the tested cell lines, we conclude that IL-4R can serve as a unique target for the delivery of a cytotoxic agent to SCCHN. Additional studies must be performed to reveal the antitumor activity of IL-4 toxin in animal models, and perhaps a Phase I clinical trial should be undertaken to study its antitumor activity.

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