Expression of High Affinity Interleukin-4 Receptors on Human Renal Cell Carcinoma Cells and Inhibition of Tumor Cell Growth In Vitro by Interleukin-4

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Abstract

Previously, Puri et al. (Puri, R. K., M. Ogata, P. Leland, G. M. Feldman, D. Fitzgerald, and I. Pastan. 1991. *Cancer Res.* 51:3011–3017) have demonstrated that murine sarcoma and colon adenocarcinoma cells express high affinity interleukin-4 receptors (IL-4R) which are internalized after binding to a chimeric ligand consisting of IL-4 and *Pseudomonas* exotoxin. In the present study, we have tested primary cultures of human renal cell carcinoma (RCC) cells, generated from tumor specimens obtained after nephrectomy, for the expression of IL-4R and their modulation by IL-4. By using iodinated IL-4 in a receptor binding assay, we observed that renal cell carcinoma cells expressed a single class of high affinity IL-4R ranging from 1,425±207 (mean±SEM) to 3,831±299 (mean±SEM) IL-4R molecules/cell with a K_d ranging from 112±11 pM to 283±71 pM. Northern blot analysis for IL-4R gene expression, performed with a cDNA probe to IL-4R, revealed that all RCC cells exhibited a single mRNA species of 4 kb. IL-4 downregulated the surface expression of IL-4R on one RCC tumor cell line. The function of IL-4R expression on RCC tumor cells was further determined by investigating the effect of IL-4 on tumor cell growth in vitro and comparing it with IL-4 effect on growth of normal fibroblast and endothelial cell lines. Tumor cell growth, as measured by [^{3}H]thymidine incorporation, was inhibited by IL-4 from 20 to 68% in a dose-dependent manner. A neutralizing antibody to human IL-4 was able to reverse the growth inhibitory effect of IL-4. Normal human fibroblast and endothelial cell lines also expressed high affinity IL-4R, however, IL-4 did not inhibit their growth in vitro. In fact, IL-4 caused modest stimulation of their growth. Taken together, our findings can help develop strategies for the treatment of RCC in which IL-4R may be used as a target for IL-4 itself, for IL-4 toxin therapy or, alternatively, in gene therapy. (*J. Clin. Invest.* 1993. 91:88–93.) Key words: IL-4 receptors on renal cell carcinoma • modulation of IL-4 receptors • tumor cell growth inhibition

Introduction

IL-4 is a glycoprotein product predominantly of Th2 lymphocytes (1, 2) and mast cells (3). Although it was originally characterized on the basis of its effects on activated B cells (4), IL-4 has since been shown to have a variety of effects on different cell types. It has been shown to promote survival, growth, and differentiation of both B and T lymphocytes (5–8), mast cells (9, 10), and endothelial cells (11). IL-4 affects macrophages by inhibiting the production of tumor necrosis factor, IL-1, and IL-6 (12, 13). More recently, IL-4 has also been shown to have antitumor effects in murine models of malignancy (14–16) and to inhibit proliferation of human tumor cell growth in vitro (17, 18). Although others have shown that the effect of IL-4 on immune cells are mediated through IL-4 receptor (IL-4R) (19, 20), the expression of functional IL-4R on nonlymphoid human tumors has not been described. In a recent study, Puri et al. (21) demonstrated the expression of high affinity IL-4R on murine sarcoma and adenocarcinoma tumor cells. These receptors were capable of internalizing IL-4 after binding to its receptor. In the current study, we have examined primary cultures of human renal carcinoma cells and normal fibroblasts and endothelial cells for the expression of IL-4R. Our results indicate that human renal cell carcinoma (RCC)[1] cells express mRNA for IL-4R and a single class of high affinity IL-4R on the cell surface. Furthermore, IL-4 caused a decrease in the density of IL-4R and tumor cell proliferation suggesting that antitumor effects may be mediated through downregulation of its own receptors. High affinity IL-4Rs were also expressed on normal human fibroblast and endothelial cell lines, however, IL-4 did not inhibit, but caused a modest stimulation of their growth in vitro.

Methods

Tumor cells and normal cell lines. Fresh RCC specimens were obtained from Wayne State University (Harper Hospital, Detroit, MI) and from the Surgery Branch, National Cancer Institute (Bethesda, MD) from patients undergoing nephrectomy as part of their medical care. Single cell suspensions of tumors were prepared by digesting minced tumor pieces with 0.002% deoxyribonuclease, type 1; 0.01% hyaluronidase, type V; and 0.1% collagenase, type IV (Sigma Chemical Co., St Louis, MO), and used to establish primary cultures. The cells were maintained in complete medium consisting of Dulbecco's modified Eagle medium with 4.5 g/liter glucose supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). RCC tumor cells between passages 5 and 20 were used for all experiments. The human Burkitt lymphoma-B cell lines, Molt-4 and Daudi, were kindly provided by Dr. J. A. Hank (University of Wisconsin, Madison, WI). Normal human skin fibroblast cell lines (39-Sk and 969-Sk) and human umbilical vein endothelial (HUVE) cells were obtained from American Type Culture Collection, Rockville, MD.

Cytokines and reagents. Recombinant human IL-4 (sp act 10^7 U/mg protein) and an 836-bp cDNA probe for the human IL-4 receptor were a kind gift from Immunex Corp. (Seattle, WA). Rabbit polyclonal

Dr. Obiri and Dr. Hillman made equal contributions to the study.

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1. *Abbreviations used in this paper:* HUVE, human umbilical vein endothelial; IL-4R, IL-4 receptor; RCC, renal cell carcinoma.

antibody to recombinant human IL-4 was purchased from Genzyme Corp. (Boston, MA).

Radioreceptor binding assay. Recombinant human IL-4 was enzymatically labeled with ^{125}I (Amersham Corp., Arlington Heights, IL) by the enzymobead method according to manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA). The specific activity of the radiolabeled IL-4 was determined by competition for binding sites of labeled IL-4 with unlabeled IL-4 using the IL-4R-positive gibbon lymphoma cell line, MLA 144. The specific activity of ^{125}I-IL-4 was estimated to range from 6×10^9 to 5×10^{10} cpm/mg protein.

For IL-4R assays, equilibrium binding studies were performed by the method previously described (21, 22). Briefly, 2.5×10^6 RCC cells in 126 μl binding buffer (RPMI 1640 containing 0.2% human serum albumin) were incubated with various concentrations of ^{125}I-IL-4 at 4°C in polypropylene tubes in a shaking water bath. For each concentration of ^{125}I-IL-4, nonspecific binding was determined by including 100–200 molar excess of unlabeled IL-4 in duplicate tubes. Cell-bound ^{125}I-IL-4 was separated by centrifugation through a cushion of phthalate oils and counted in a gamma counter. The number of receptors and binding affinities were determined by Scatchard plot analysis of the data (23).

For regulation experiments, PM-RCC tumor cells were cultured with or without IL-4 (20 ng/ml) for 72 h at 37°C. Cells were then harvested and IL-4R determined.

Northern blot analysis. Adherent tumor cells were harvested from tissue culture flasks by brief exposure to versene (Whittaker Bioproducts, Walkersville, MD). After several washes, total RNA was extracted with RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX) in a one-step procedure according to the manufacturer's instructions. 10 μg of total RNA were electrophoresed through 1% agarose/formaldehyde denaturing gel and transferred to a nylon membrane (S and S Nytran; Schleicher and Schuell, Keene, NH) by capillary action (24). The nucleic acid was bound to the membrane by ultraviolet cross-linking (Stratagene Inc., La Jolla, CA). After a 12-h prehybridization at 42°C, the immobilized RNA was allowed to hybridize at 42°C for 12 h with a ^{32}P-labeled human IL-4R cDNA probe (^{32}P-deoxycytidine was obtained from ICN ImmunoBiologicals, Costa Mesa, CA). Filters were exposed to autoradiographic film for 3–10 d.

Assay for tumor cell growth. Tumor or normal cells were harvested from tissue culture flasks with versene or trypsin and resuspended to a concentration of 5×10^4 cells/ml in complete medium. 100 μl of this suspension were then plated in quadruplicates in a 96-well flat-bottomed microtiter plate and supplemented with 0–1,000 ng/ml IL-4 in 100 μl. The cultures were incubated at 37°C in a 5% CO_2 incubator for 72–96 h and then labeled with 1 μCi/well [^{3}H]thymidine (Amersham Corp.) for 18 h. Cells were harvested onto a glass fiber filter with a harvester (Micromate 196; Packard Instrument Co., Inc., Meriden, CT) using versene. [^{3}H]thymidine uptake was determined with a β direct counter (Matrix 96; Packard Instrument Co., Inc.).

Results

Cell surface expression of IL-4R. To investigate the expression and binding affinity of IL-4R, we incubated RCC tumor cells with increasing concentrations of ^{125}I-IL-4 at 4°C for 2 h. Representative results from two RCC lines (WS-RCC and GW-RCC) are shown in Fig. 1. RCC tumor cells bound IL-4 in a concentration dependent manner (Fig. 1 *A*). Scatchard plot analysis of the binding data suggested that a single class of high affinity IL-4R were expressed on GW-RCC (K_d = 112±11, n = 3), WS-RCC (K_d = 130±0, n = 2) (Fig. 1 *B*) and PM-RCC tumor cells (K_d = 283±71 pM, mean±SEM, n = 4) (not shown). In multiple experiments, the number of IL-4 molecules bound/cell on the PM-RCC tumor cells was 3,831±299 (mean±SEM, n = 4), on WS-RCC 2,269±132 (n = 2), and on GW-RCC tumor cells was 1,425±207 (n = 3). Other RCC tumor cells also expressed similar number of IL-4R (data not shown). Similar numbers of IL-4 receptors with similar binding affinity have been reported on human T and B cell lines (19, 25). Recently we have also observed that human ovarian, breast, and melanoma cell lines express a comparable number and affinity of IL-4R as human RCC cells (unpublished data).

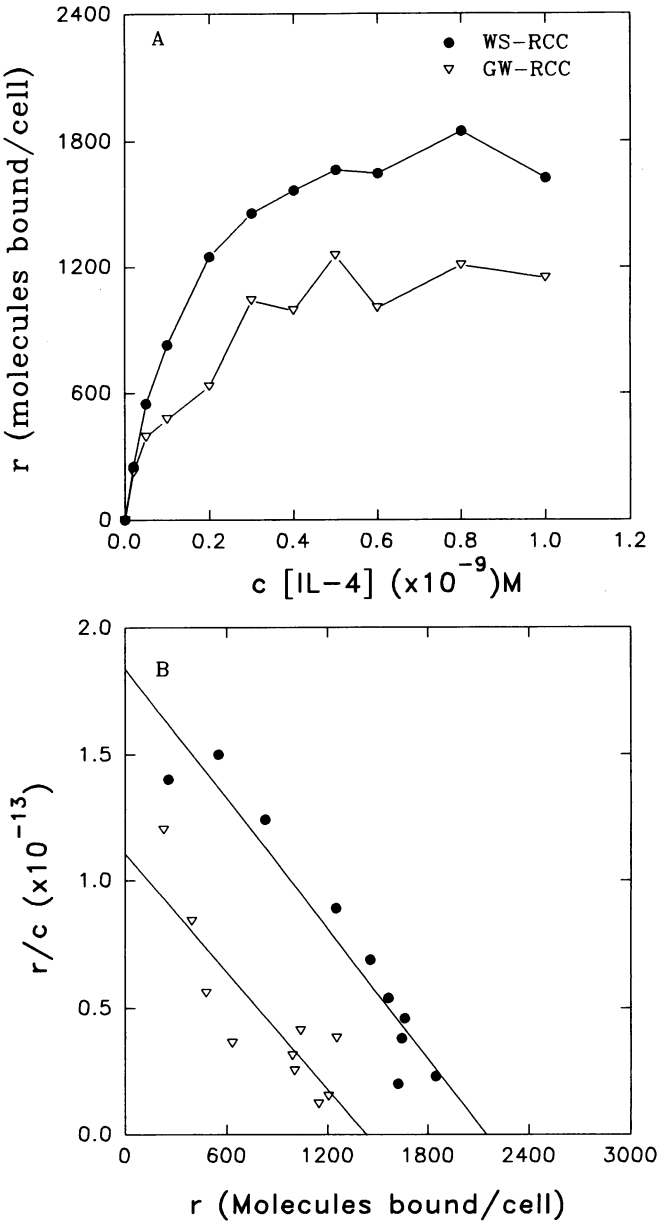

Figure 1. Binding of ^{125}I-IL-4 to human RCC cells. Tumor cells were incubated with 50 pM-1 nM ^{125}I-IL-4 for 2 h at 4°C. In duplicate tubes, 100–200-fold molar excess unlabeled IL-4 was included to determine nonspecific binding. The number of specifically bound ^{125}I-IL-4 molecules/cell was determined and used to plot the binding curve shown in *A* and the Scatchard plot shown in *B*. Data are represented as a single experiment performed in duplicate, representative of two or three separate experiments.

Normal HUVE and skin fibroblast cell lines (969-Sk, 39-Sk), as well as Daudi and Molt-4 lymphoblastoid cell lines, were also examined for the expression of IL-4R. The number of IL-4 molecules bound/cell and K_d on these cells are listed in Table I. Normal skin fibroblast, HUVE, and Daudi cells expressed varying numbers of IL-4R, while on Molt-4 cells, IL-

Table I. Expression of IL-4 Receptors on Human Normal and Lymphoblatoid Cell Lines

Cell type	IL-4 molecules bound/cell	K_d ($\times 10^{-12}$) M
HUVE	1,457±62*	ND‡
39 Sk (fibroblast)	331	300
Daudi (lymphoblastoid)	45	214
Molt-4 (lymphoblastoid)	Undetectable	Undetectable

* IL-4 molecules bound/cell were determined by using single saturating concentration of ^{125}IL-4 (800 pM). ‡ ND, not done.

4R were not detectable. The affinity of IL-4R on fibroblast and Daudi cell lines was similar to that observed on RCC-tumor cells (Table I).

IL-4R gene expression. Northern blot analysis of IL-4R gene expression was performed on RCC tumor cells. RNA from CTLL-2 cells, a mouse cytotoxic T cell line, was included as a negative control while RNA from CTLL-2 cells transfected with the human IL-4R cDNA (CTLL-T22-8) (20) was used as a positive control. RNA from CTLL-2 cells did not hybridize with the hIL-4R cDNA probe (Fig. 2 *A*, lane *1*) while RNA from the IL-4R transfected cell line did (Fig. 2, lane *2*). All tested RCC tumor cell lines expressed a high density of single species of mRNA (size: 4.0 kb) for hIL-4R (Fig. 2 *A*, lanes *3–5*).

We also examined the mRNA expression for IL-4R on Daudi and Molt-4 cells. As depicted in Fig. 2 *B*, only CTLL-2-T22-8 cells (lane *1*) and Daudi cells (lane *2*) expressed the mRNA for IL-4R, however, we did not observe any hybridization with Molt-4 RNA (Fig. 2 *B*, lane *3*).

Effect of IL-4 on tumor cell growth. To determine the functional significance of the expression of high affinity IL-4R on RCC tumor cells, we tested the effect of IL-4 on the growth of these cells in tissue culture. We found that IL-4 caused a direct antiproliferative effect of varying degree on RCC tumor cells isolated from several patients. This tumor growth inhibition mediated by IL-4 was dose dependent (Fig. 3 *A*). Maximum inhibition of PM-RCC and GW-RCC tumor cell growth (~ 50%) was observed at IL-4 concentration of 10–100 ng/ml (Fig. 3 *A*), while no further inhibition was observed at higher concentrations (100–1,000 ng/ml) (Fig. 4). IL-4-mediated growth inhibition was reproducible and observed in other RCC tumor lines. IL-4 at 10 ng/ml inhibited the growth of RG-RCC, TR-RCC, and SJ-RCC, by 68, 31, and 39%, respectively. Although Daudi cells have been shown to express IL-4R (19, 25), the growth inhibitory effect of IL-4 was not observed in these cells (Fig. 3 *A*) indicating that the IL-4 effect is cell type specific.

The level of inhibition of RCC tumor cell growth as determined by cell counts at the end of 7-d culture with IL-4 was comparable to that observed with [^{3}H]thymidine uptake. However, cell viability (as determined by trypan blue exclusion) was 100% in both IL-4-treated and untreated groups (data not shown). These data indicate that IL-4 caused cytostatic rather than cytotoxic effects on RCC tumor cells.

To establish that the observed growth inhibitory effects were mediated directly by IL-4, we investigated the effect of a neutralizing antibody directed against human IL-4 on RCC tumor cell proliferation. We found that anti-IL-4 antibody reversed the growth inhibition caused by IL-4 in a concentration-dependent manner (Fig. 3 *B*). At 10 μg/ml, the anti-IL-4 antibody significantly neutralized the antiproliferative effects of IL-4 ($P < 0.03$), however, complete neutralization was not observed. Thus our data corroborate previous findings (18) and further suggest that the antiproliferative effects of IL-4 are mediated through its receptors.

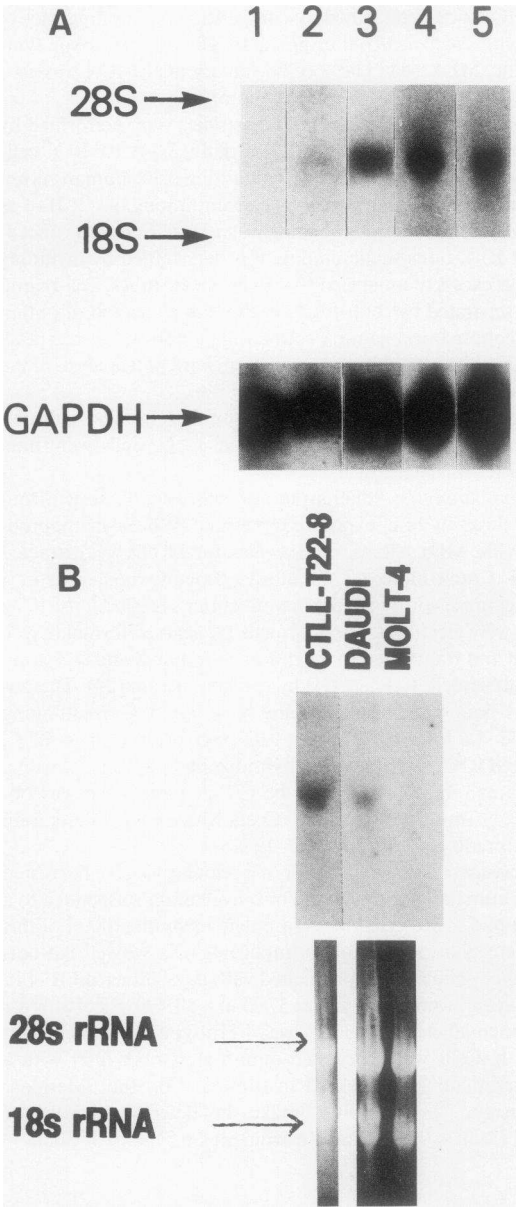

Figure 2. (*A*) Expression of IL-4R mRNA by human RCC cells. Total RNA was isolated from RCC cells, and a 10-μg sample from each was electrophoresed and then transferred to a nylon filter. The immobilized RNA was analyzed with a ^{32}P-labeled cDNA probe for human IL4R. RNA from a mouse T cell line CTLL-2 before and after transfection with the human IL-4R gene (CTLL-T22-8) were included as controls (lanes *1* and *2*, respectively). Lanes *3–5* represent gene expression from different RCC cells (lane *3*, WS-RCC; lane *4*, HL-RCC; lane *5*, PM-RCC). For internal standard the blots were rehybridized with ^{32}P-labeled cDNA probe for GAPDH and shown in the figure (*lower panel*). (*B*) Expression of IL-4R mRNA on Daudi and Molt-4 cells. Total RNA (20 μg) from Daudi, Molt-4, and CTLL-T22-8 was electrophoresed and analyzed for the expression of IL-4R mRNA as described in *A*. Lane *1*, CTLL-T22-8; lane *2*, Daudi; and lane *3*, Molt-4 cell-derived RNA. Lower panel represents ethidium bromide staining of rRNA which indicates that an equivalent amount of RNA was loaded in each lane.

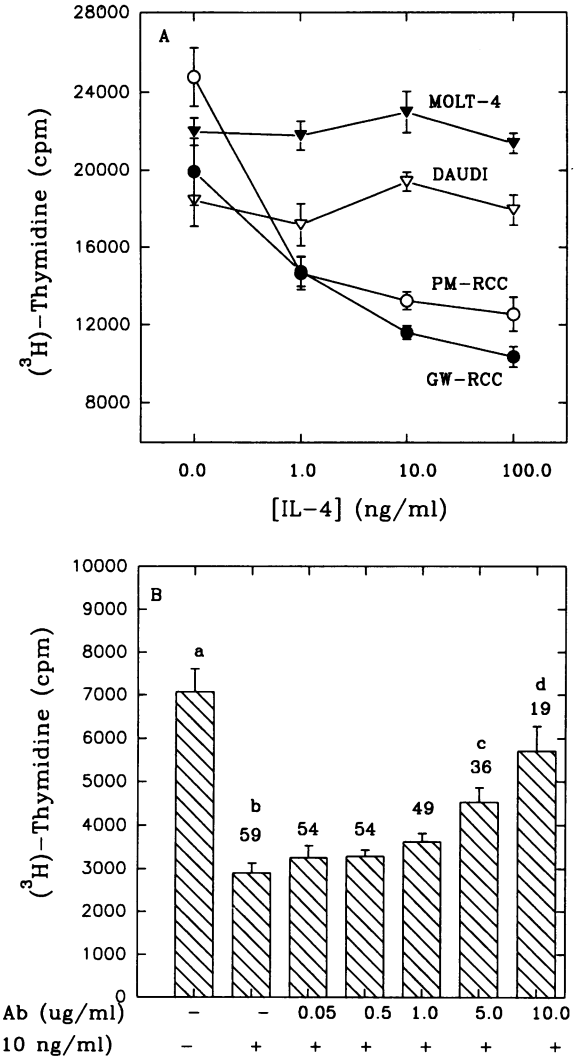

Figure 3. IL-4 inhibits RCC tumor cell growth in vitro. PM-RCC, GW-RCC, Molt-4, or Daudi cells, at a concentration of 5×10^3/well, were cultured with various concentrations of IL-4 for 3–4 d at 37°C and labeled for 18 h with [^{3}H]thymidine as described in Methods (*A*). GW-RCC tumor cells were cultured with or without 10 ng/ml IL-4 in the presence of increasing concentrations of anti-IL-4 antibody for 3 d (*B*). Data are reported as the mean cpm±SD of quadruplicate wells. The numbers shown above the bars in *B* indicate the percent growth inhibition relative to growth in control cultures in which cells were incubated in medium alone. Letters above the bars represent statistical analysis of data by Student's *t* test and the levels of significance are as follows: a vs b, $P < 0.0001$; b vs c, $P < 0.001$; b vs d, $P < 0.001$; a vs c, $P < 0.001$; a vs d, $P < 0.03$.

Effect of IL-4 on normal cell lines. To understand the significance of growth inhibitory effects of IL-4 on RCC tumor cells, we tested the effects of IL-4 on the growth of normal cell lines. Two normal human skin fibroblasts cell lines (969-Sk and 39-Sk) were used in a 4-d proliferation assay and their proliferation was compared to that of RCC tumor cells. IL-4 caused modest stimulation of growth of normal skin fibroblast cells (Fig. 4). At 1 ng/ml, IL-4 caused a modest stimulation of growth of both fibroblast cell lines and this growth stimulation was statistically significant compared to that of control untreated cells ($P < 0.001$). Similarly, the stimulation of normal HUVE cells and human adrenal capillary endothelial (HACE)

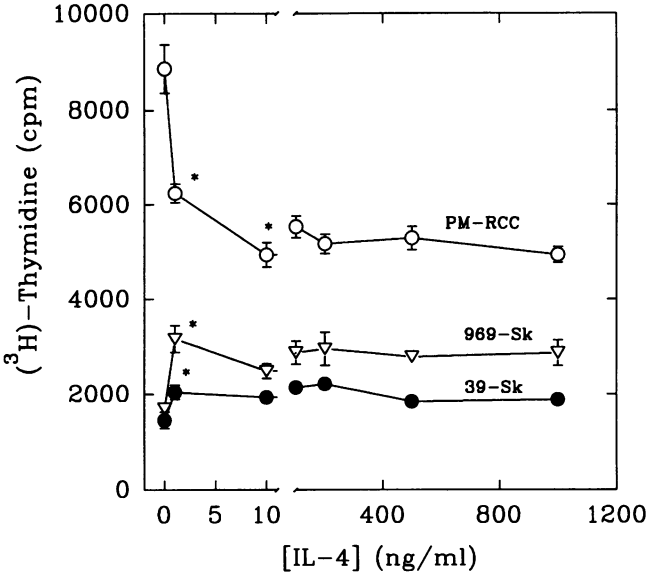

Figure 4. IL-4 stimulates growth of human normal skin fibroblast cell lines in vitro. 5×10^3 fibroblast cell lines (969-Sk and 39 Sk) and RCC tumor cells (PM-RCC) were cultured in the presence of indicated concentrations of IL-4 for 3 d at 37°C and labeled for 18 h with [^{3}H]thymidine as described in Methods. Data are represented as mean cpm±SD of quadruplicate samples. Asterisks represent statistical analyses which indicate that data is highly significant ($P < 0.001$ for PM-RCC, 969-Sk, and $P < 0.01$ for 39-Sk) from respective control (cells cultured in medium alone) groups.

cells have previously been reported (11). In contrast, IL-4 caused the inhibition of PM-RCC tumor cell growth in a dose-dependent manner (Fig. 4). These data further indicate the significance of our observations and confirm cell type–specific effects of IL-4 on growth inhibition.

Regulation of IL-4R by IL-4. To understand the mechanism of inhibition of tumor cell growth by IL-4, we investigated the regulation of IL-4R by IL-4. PM-RCC cells were cultured with or without IL-4 (20 ng/ml) for a 4–72-h period. Cells were then harvested using versene and the expression of IL-4R and its affinity were determined by equilibrium binding and Scatchard plot analysis. IL-4 significantly downregulated its receptors after 4 h of incubation (data not shown) and this down regulation was optimal after 72 h of incubation. ^{125}IL-4 specifically bound to RCC tumor cells in a concentration-dependent manner but this binding was significantly decreased after 72-h treatment with IL-4 (Fig. 5 *A*). Scatchard plot analysis of the binding data revealed that the density of IL-4 receptors was decreased from 2,957±703 (n = 2) IL-4R molecules/cell on untreated cells to 1,298±645 (n = 2) IL-4R molecules/cell on treated cells (Fig. 5 *B*). However, the dissociation constant in IL-4 treated cells (98±19 pM) was similar to that of untreated cells (94±14 pM).

Discussion

In this study, we demonstrate that RCC tumor cells express high affinity IL-4R. The expression of high affinity IL-4R on human RCC tumor cells has not been previously reported. Our results demonstrate the expression of IL-4R at the protein and gene level. The IL-4R appear to be functional as IL-4 was able

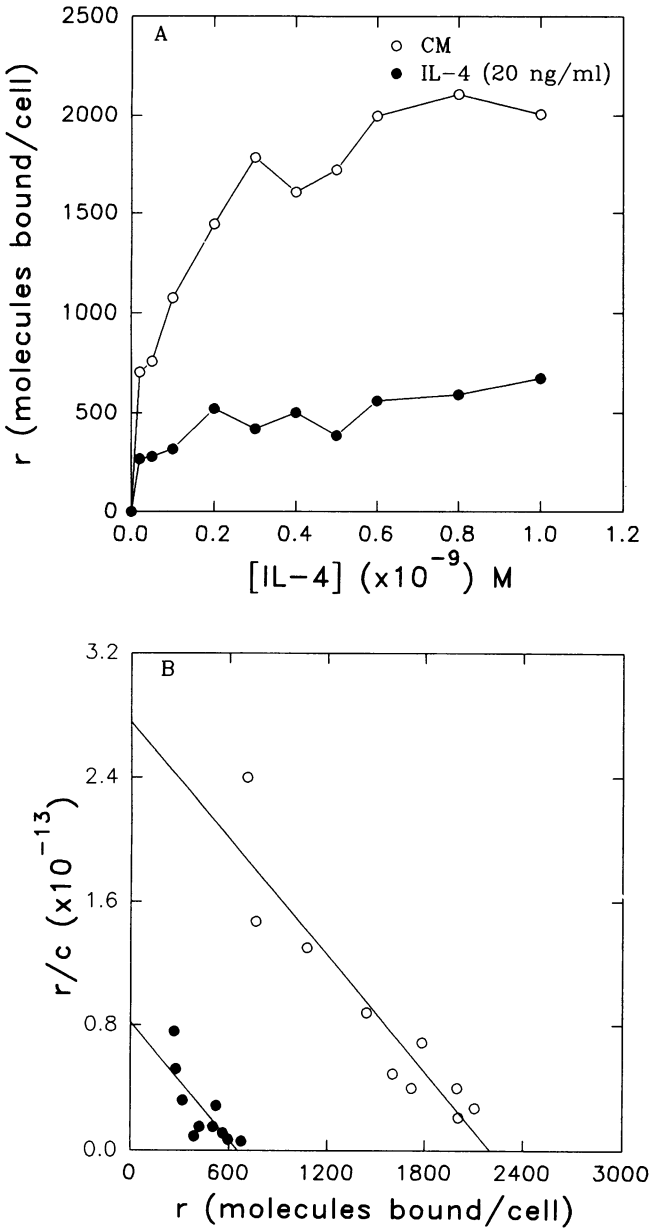

Figure 5. IL-4 inhibits IL-4 R on human RCC tumor cells. Tumor cells (PM-RCC) were cultured with or without IL-4 (20 ng/ml) for 72 h. Cells were then harvested, washed, and stripped to remove excess IL-4, and further incubated with 50 pM–1 nM ^{125}I-IL-4 for 2 h at 4°C. In duplicate tubes, 100–200-fold molar excess unlabeled IL-4 was included to determine nonspecific binding. The number of specifically bound ^{125}I-IL-4 molecules/cell was determined and used to plot the binding curve shown in *A* and the Scatchard plot shown in *B*. Data are represented as a single experiment performed in duplicate and representative of two separate experiments.

to inhibit RCC tumor cell growth in vitro. A neutralizing antibody to IL-4 was able to abrogate the growth inhibitory effects of IL-4.

The role of IL-4R on human RCC tumor cells is not known. IL-4R may have some regulatory role in tumor cell growth in vivo. Previous studies have suggested that IL-4R may be associated with an oncogene or may itself be a novel oncogene (26). However, these possibilities are unlikely because our studies and those of others (17, 18) show that IL-4 has antiproliferative effects on tumor cells.

Recently, IL-4 has also been shown to inhibit the growth of human hematopoietic cell derived tumors such as multiple myeloma, lymphoma (27), and chronic myelomonocytic leukemia cells (28). Moreover, IL-4 increased the antiproliferative effects mediated by tumor necrosis factor on various human tumor lines including breast carcinoma, epidermoid carcinoma, and histiocytic lymphoma cells (29). Thus our data agree with published reports on growth inhibitory function of IL-4.

Interestingly, in contrast to the inhibition of RCC tumor cell growth, IL-4 did not inhibit the growth of control Molt-4 and Daudi lymphoblastoid cell lines (Fig. 3). Molt-4 cells did not express IL-4R at the mRNA level or on the cell surface consistent with the lack of IL-4 effect on these cells. However, Daudi cells did express IL-4R mRNA but expressed a lower number of IL-4R on cell surface (< 100 molecules bound/cell) (Table I) compared to RCC tumor cells (Fig. 1). Daudi cell lines have been shown to express varying number of IL-4R, however, receptor numbers observed in our studies were lower than those observed previously (19, 25). Difference in cell lines may explain this difference. Thus lower numbers of IL-4R present on Daudi cells might explain the lack of IL-4 effect on these cells.

The mechanism for IL-4 unresponsiveness on Daudi and Molt-4 cells based on receptor number/cell cannot be explained in the light of data obtained with normal cells. Normal fibroblast and endothelial cells expressed a higher number of IL-4 receptors than Daudi cells, however, in contrast to data observed on RCC tumor cells, IL-4 caused a modest growth stimulation of normal cells. These data are in agreement with a published report in which IL-4 has been shown to be a mitogen for normal HUVE and human adrenal capillary endothelial cell lines (11). IL-4 has also been shown to be a growth factor for murine normal fibroblast cell lines (30), however, its effects on the growth of normal human fibroblast cells has not been reported. We observed a modest but significant growth-stimulatory effect of low concentration of IL-4 on two human normal fibroblast cell lines (Fig. 4).

We considered that the difference in receptor affinities may account for differences in IL-4 response in various cell types. However, we have found that the K_d was not different on normal, lymphoblastoid, and RCC tumor cells. These different cell types might differ in the mechanisms of receptor internalization, processing, and/or signal transduction triggered by IL-4 binding to its receptors. Irrespective of the mechanism of action, our data suggest that IL-4 may be a useful agent for the regulation of RCC tumor growth in vivo without mounting inhibitory effects on normal cells.

Our data also demonstrate that IL-4 can downregulate its own receptors. Transient downregulation of IL-4R by IL-4 on human Jijoye cell line has been previously reported (31). However, on resting T and B lymphocytes IL-4 has been shown to upregulate its receptors (32). The significance of downregulation of IL-4R by IL-4 on human RCC tumor cells remains to be determined. It is possible that the downregulation of IL-4R may be involved in the mechanism of inhibition of tumor growth by IL-4.

IL-4 has also been shown to upregulate classes I and II HLA antigens on some tumor cells including RCC cells (17, 18). In murine studies perilymphatic administration of low doses of IL-4 has been shown to cause the rejection of established tumors by host immune cells (14). Furthermore, murine tumor

cells transduced with IL-4 gene can abrogate the growth of tumor cells in vivo (15) or cause the regression of established tumor at a distant site due to enhanced tumor immunogenicity and enhancement of CTL response (16). These findings, particularly, in a murine experimental model for RCC using IL-4 transduced Renca tumor cells (16), reinforce the importance of our data demonstrating the expression of functional IL-4R on human RCC tumor cells. Taken together, these observations can help develop strategies for the treatment of human RCC in which IL-4R may be used as a target for IL-4 itself, IL-4 toxin therapy, or, alternatively, in gene therapy (16).

Acknowledgments

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