Interleukin 4 Inhibits Growth and Induces Apoptosis in Human Breast Cancer Cells

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ABSTRACT

Interleukin-4 (IL-4) is a pleiotropic cytokine produced by mast cells and T lymphocytes that promotes proliferation and immunoglobulin class-switching in B cells. IL-4 receptors (IL-4Rs) are also expressed by nonhematopoietic cells as well as some tumor cells. Unlike its mitogenic effect on B cells, IL-4 inhibits the growth of some cancer cells in vitro. In this study, we show that IL-4R is expressed by breast and ovarian cancer cell lines. Furthermore, anchorage-dependent and -independent growth of breast cancer cell lines MCF-7 and MDA-MB-231 is inhibited by IL-4 treatment, and this effect requires IL-4R. Interestingly, IL-4 only inhibited proliferating breast cancer cells and had no effect on basal, unstimulated growth. We therefore characterized the effect of IL-4 on breast cancer cell growth stimulated by either estradiol or insulin-like growth factor I (IGF-I). In both anchorage-dependent and -independent growth assays, IL-4 inhibited estradiol-stimulated growth. The antiestrogenic effect of IL-4 was not due to IL-4 interference with the estrogen receptor, because IL-4 did not interfere with estrogen receptor-mediated reporter gene transactivation. In contrast, IL-4 had no effect on IGF-I-stimulated proliferation. Because IGF-I is known to inhibit programmed cell death, we examined apoptosis as a possible mechanism of IL-4 action. We established that IL-4 induced apoptosis in breast cancer cells by five independent criteria: (a) morphological indicators including pyknotic nuclei and cytoplasmic condensation; (b) DNA fragmentation; (c) the formation of DNA laddering; (d) the cleavage of poly(ADP-ribose) polymerase; and (e) the presence of cells with sub-G1 DNA content. IL-4 increased the percentage of apoptotic cells in MCF-7 and MDA-MB-231 cells 6.0- and 6.7-fold over that of the control, respectively. Finally, the addition of IGF-1 reversed IL-4-induced apoptosis, suggesting that the mechanism of IL-4-induced growth inhibition in human breast cancer cells is the induction of programmed cell death.

INTRODUCTION

Breast cancer cell growth is controlled by a complex network of steroid hormones, peptide growth factors, and cytokines that induce a myriad of growth effects. The most important proliferative agents include estrogen, IGF-I, and epidermal growth factor (1, 2). In addition, many other molecules contribute to the regulation of breast tumor growth by inducing differentiation as well as growth inhibition. One group of molecules that may be important in the regulation of breast cancer cell growth is the group of cytokines secreted by tumor-infiltrating lymphocytes. IL-4, IL-6, and IL-10 have been reported to be produced by activated T cells found to be associated with breast tumors (3, 4). IL-4 is of particular interest, because it has been reported to be growth inhibitory to breast cancer cells in vitro (5–7).

IL-4 is a pleiotropic cytokine produced by mast cells and T lymphocytes and acts primarily on B cells, in which it induces proliferation and immunoglobulin class-switching (8). IL-4 acts via a multiunit transmembrane receptor, the IL-4R (9, 10), which has also been found on a number of nonhematopoietic cells including some tumor cells (11). In contrast to its mitogenic role in B cells, IL-4 has been reported to be growth inhibitory to some cancer cells in vitro. Indeed, tumor cells engineered to secrete IL-4 have been shown to have decreased growth in vitro and in vivo (12–14). In addition, IL-4 has been shown to have direct antiproliferative effects on some tumor cells including renal carcinomas (15), lung carcinomas (16), gastric carcinomas (17), and breast carcinomas (5–7).

Whereas growth inhibition due to IL-4 has been reported, the role of the IL-4R and the mechanism for IL-4-induced growth inhibition have not been well characterized, particularly in breast cancer cells. Therefore, the purpose of this study was to characterize IL-4R expression and utilization by IL-4 in human breast cancer cells, to determine the effect of IL-4 on breast cancer cell growth including E2- and IGF-I-stimulated growth, and to examine apoptosis as a possible mechanism of growth inhibition. We showed by monolayer and anchorage-independent growth assays that IL-4 was growth inhibitory and acted via IL-4R expressed by breast cancer cells. In addition, we showed that IL-4 inhibited the E2-stimulated growth of MCF-7 cells in monolayer and anchorage-independent growth assays but had no effect on IGF-I-stimulated growth. Because IGF-I is known to inhibit programmed cell death, we hypothesized that IL-4 was inducing apoptosis. Accordingly, we showed that IL-4-induced growth inhibition is associated with an increase in apoptosis, and that the induction of apoptosis by IL-4 is inhibited by the addition of IGF-I.

MATERIALS AND METHODS

Materials

MCF-7 cells were provided by C. Kent Osborne (University of Texas Health Science Center, San Antonio, TX) and grown in IMEM (Life Technologies, Inc., Bethesda, MD) with phenol red plus 5% fetal bovine serum (Summit, Fort Collins, CO). MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in IMEM without phenol red plus 5% fetal bovine serum. MD-MB-231 cells were provided by V. Craig Jordan (Northwestern University Medical Center, Chicago, IL). IL-4 was a gift of Satwant Narula (Schering-Plough Research Institute, Kenilworth, NJ). IL-4-neutralizing Ab was provided by Dr. Kathleen dePicha (ImmuneX, Inc., Seattle, WA). IL-4R cDNA was provided by Kevin Moore (DNAX Research Institute, Palo Alto, CA). IGF-I was obtained from GroPep (Adelaide, Australia).

Cell Growth Assays

Monolayer Growth. Growth assays were performed by MTT assay as described previously (18). MCF-7 cells were plated in triplicate at a density of 18,000 cells/well, and MDA-MB-231 cells were plated in triplicate at a density of 12,000 cells/well in 24-well cell culture plates. Cells were allowed to adhere overnight and then washed once in 1× PBS; the culture medium was replaced with SFM overnight. After treatment, 60 μl of MTT (5 mg/ml in PBS) were added to the medium for 4 h. Medium and MTT were then removed, DMSO and 2.5% DMEM were added, and the absorbance was measured at 540 nm.
Anchorage-independent Growth. MCF-7 cells were plated at a density of 1.5 \times 10^4 cells/plate, and MDA-MB-231 cells were plated at 7.5 \times 10^3 cells/plate. Cultures were prepared with a base layer of IMEM supplemented with 20% FCS and containing 0.5% low-melting point agarose (Sea Plaque; FMC Bioproducts, Rockland, ME). MCF-7 and MDA-MB-231 cells were then plated over the base layer in duplicate in IMEM supplemented with 10% FCS and containing 0.5% low melting-point agarose. In addition, cultures were prepared with a base layer of IMEM supplemented with 10% CSS containing 0.5% low-melting point agarose. MCF-7 cells were then plated over the base layer in duplicate in IMEM supplemented with 5% CSS and containing 0.5% low-melting point agarose. IL-4 was added at a concentration of 10 ng/ml, and IL-4R Ab was added as indicated. E2 was added at a final concentration of 10^{-9} M. Cells were allowed to grow for 7-10 days before colonies of at least 20 cells were counted.

RNase Protection Assay

Total RNA from breast and ovarian cancer cell lines cultured in serum-supplemented media was prepared by the guanidinium thiocyanate method (19), measured by spectrophotometry, and checked for integrity by separation on a 1% formaldehyde-agarose gel. RNase protection was performed according to our previously published method (20), and RNA loading was corrected with the ribosomal protein 36B4 (21). Briefly, 20 \mu g of RNA were hybridized with radiolabeled antisense cRNAs transcribed from the IL-4R and 36B4 cDNAs. The RNase protection probe was generated by PCR from an expression vector containing the IL-4R cDNA and a COOH-terminal flag sequence (Eastman Kodak, Rochester, NY). IL-4R fragment PCR was performed using a 5' primer containing a EcoRI restriction site (5'-GGTAGAGGACATGC-CAAAGC) approximately 400 bp upstream of the stop codon and a 3' primer containing an XhoI restriction site followed by the flag sequence. The resulting 400-bp fragment was subcloned into pGEM4Z by a restriction digest. pGEM4Z-IL-4R was linearized with EcoRI, and transcription with T7 RNA polymerase was carried out in the presence of [32P]UTP to produce labeled antisense cRNA. For 36B4, a 145-bp PstI-PstI fragment was cloned into pGEM4Z, linearized with EcoRI, and transcribed with T7 RNA polymerase. After hybridization of RNA with a radiolabeled probe, single-stranded RNA was digested with RNase A, and samples were separated on 8 M urea/6% SDS-PAGE. cRNA was hybridized as a negative control. The gel was dried and was digested with RNase A. and samples were separated on 1% formaldehyde-agarose. IL-4 was added at a concentration of 10 ng/ml, and IL-4R Ab was added as indicated. E2 was added at a final concentration of 10^{-9} M. Cells were allowed to grow for 7-10 days before colonies of at least 20 cells were counted.

Luciferase Assay

MCF-7 cells that had been stably transfected with ERE-luciferase reporter construct were a kind gift of Dr. Michel Pons (INSERM U-58, Montpellier, France; Ref. 22). Cells were treated with E2 (10^{-9} M), IL-4 (10 ng/ml), or IGF-I (5 nM) as indicated for 48 h. Cell lysates were collected by three cycles of freeze-thawing in 1X reporter lysis buffer (Promega, Madison, WI). Total protein was measured by Bio-Rad (Hercules, CA) assay, and luciferase activity was determined using the luciferase assay system from Promega.

Apoptosis Assays

For all apoptosis assays, MDA-MB-231 cells were treated in SFM alone, whereas MCF-7 cells were treated in SFM supplemented with 0.25% FCS.

H&E and TUNEL Staining. Cells were plated in 8-well chamber slides (NUNC, Inc., Naperville, IL) at a density of 7.5 \times 10^3 cells and allowed to adhere overnight. SFM was placed on the cells overnight, and cells were then treated with IL-4 or SFM alone. IL-4 was added at 50 ng/ml for 48 h. Chambers were then removed, and slides were fixed in 70% ethanol. A TUNEL assay was performed following the manufacturer's instructions using the TACS 2 dUTP kit from Trevigen, Inc. (Gaithersburg, MD).

DNA Laddering. Cells were plated in 10-cm dishes and allowed to grow to 70% confluence. Medium was removed, cells were washed once with 1X PBS, and SFM was added before treatment with 10 ng/ml IL-4 or 1 \mu g/ml paclitaxel. After 7 days, the cells were harvested in trypsin-EDTA, washed with 1X PBS, and then lysed in 0.15 M NaCl, 10 mM Tris-HCl (pH 7.8), 2 mM MgCl_2, 1 mM DTT, and 0.5% NP40 on ice for 40 min. Nuclei were collected by centrifugation at 1,500 rpm for 10 min and lysed in 0.35 M NaCl, 10 mM Tris-HCl, 1 mM MgCl_2, and 1 mM DTT on ice for 20 min. Nuclear lysates were extracted once with phenol-saturated chloroform, and fragmented DNA was precipitated with 0.01 M MgCl_2 and 2.5 volumes of ethanol at -20°C overnight. DNA was collected by centrifugation at 16,000 rpm for 30 min. DNA was resuspended in 50 \mu l of Tris-EDTA with 0.1 mg/ml RNase A for 1 h at 37°C followed by a 1-h digestion with 1 mg/ml protease K. DNA was then electrophoresed in 1.5% agarose gels containing ethidium bromide.

PARP Cleavage. Cells were treated with 10 ng/ml IL-4 or 1 \mu g/ml paclitaxel for 0, 2, and 4 days and harvested with trypsin-EDTA, pelleted, and washed with 1X PBS. Protein from MDA-MB-231 cells was extracted using a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1% NP40, 100 mM NaCl, 100 mM sodium orthovanadate, 100 \mu g/ml leupeptin, 20 \mu g/ml aprotinin, and 10^{-7} M phenylmethylsulfonyl fluoride. Protein (50 \mu g) was analyzed by 7.5% SDS-PAGE. After transfer of the proteins to nitrocellulose, the membrane was incubated in 5% milk Tris-buffered saline-Tween and then immunoblotted with a 1:1000 dilution of the anti-PARP Ab (Boehringer Mannheim, Indianapolis, IN). Horseradish peroxidase-conjugated goat-anti-rabbit secondary Ab was added at a 1:2000 dilution, and proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

Flow Cytometry. Cells were treated with 10 ng/ml IL-4 or 1 \mu g/ml paclitaxel as indicated for 48 h. Cells were harvested with trypsin-EDTA and washed with 1X PBS. Protein from MDA-MB-231 cells was extracted using a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1% NP40, 100 mM NaCl, 100 mM sodium orthovanadate, 100 \mu g/ml leupeptin, 20 \mu g/ml aprotinin, and 10^{-7} M phenylmethylsulfonyl fluoride. Protein (50 \mu g) was analyzed by 7.5% SDS-PAGE. After transfer of the proteins to nitrocellulose, the membrane was incubated in 5% milk Tris-buffered saline-Tween and then immunoblotted with a 1:1000 dilution of the anti-PARP Ab (Boehringer Mannheim, Indianapolis, IN). Horseradish peroxidase-conjugated goat-anti-rabbit secondary Ab was added at a 1:2000 dilution, and proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

Fig. 1. RNase protection assay of IL-4R mRNA expression in breast cancer and ovarian cancer cell lines. Samples (20 \mu g) of RNA from eight breast cancer and three ovarian cancer cell lines were examined for IL-4R mRNA by RNase protection assay. MDA-MB-10A is a derivative of the MDA-MB-231 cell line. iRNA was included as a negative control, and 36B4 was included as a loading control.

Fig. 2. Monolayer growth and dose response of MCF-7 and MDA-MB-231 cells to IL-4. MCF-7 and MDA-MB-231 cells were plated in triplicate in 24-well plates and then treated with increasing concentrations of IL-4. Growth was determined by the MTT assay after 3 days. MCF-7 cells; O, MDA-MB-231 cells. MCF-7 cells were cultured in SFM plus 0.25% serum, whereas MDA-MB-231 cells were cultured in SFM alone.

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Statistics
Growth in soft agar was analyzed using Student's t test, and Ps were determined for a two-tailed test with 2 degrees of freedom. The results of the flow cytometric quantitation of apoptosis were also analyzed using Student's t test, and Ps were determined for a two-tailed test with 4 degrees of freedom.

RESULTS

IL-4 Acts via the IL-4R and Induces Growth Inhibition in Breast Cancer Cells. IL-4 is known to act at a multiunit transmembrane receptor, IL-4R. IL-4R is composed of two subunits: (a) the ligand-binding chain, IL-4Ra; and (b) the γ-common chain (23, 24). It has been reported that IL-4Ro is necessary for IL-4 activity but may also act independently of the γ-common chain (25, 26). Therefore, to determine whether IL-4 may act via the IL-4R, we examined the expression of IL-4Ra mRNA in breast cancer cell lines using the RNase protection assay. Fig. 1 shows that IL-4Ra mRNA was expressed by six of seven breast cancer cell lines (the MDA-10A cell line is a derivative of MDA-MB-231) and two of three ovarian cancer cell lines.

IL-4 has been reported to be growth inhibitory to some cancer cells in vitro (5-7, 15-17). To determine the effect of IL-4 on breast cancer cell growth, we treated MCF-7 and MDA-MB-231 cells with IL-4 in SFM over a course of 4 days. We found that IL-4 had no effect on MCF-7 cell growth when cells were cultured in SFM alone (data not shown). However, MCF-7 cells are hormone- and growth factor-dependent cells, and no basal growth was observed in SFM under our experimental conditions. The addition of 0.25% FCS resulted in proliferation. Under these conditions, IL-4 inhibited MCF-7 cell growth over a range of concentrations (Fig. 2). MDA-MB-231 cells are hormone- and growth factor-independent breast cancer cells, and proliferation was consistently observed in SFM. In these cells, IL-4 also inhibited growth over a range of concentrations (Fig. 2). Approximately 10 ng/ml IL-4 resulted in optimal inhibition in both cell lines. In addition, we examined the growth of MDA-MB-435A cells after IL-4 treatment and found that the cell line was unaffected by IL-4 (data not shown). This is consistent with the observation that MDA-MB-435A cells did not express detectable IL-4R mRNA.

We then examined the ability of IL-4 to inhibit colony formation of MCF-7 and MDA-MB-231 cells in an anchorage-independent growth assay. MCF-7 and MDA-MB-231 cells were plated in soft agar containing FCS with or without 10 ng/ml IL-4. Colony formation in both cell lines was significantly (P < 0.02 and P < 0.001 for MCF-7 and MDA-MB-231 cells, respectively) reduced by IL-4 (Fig. 3A). In addition to fewer colonies, IL-4 treatment also reduced the size and changed the appearance of colonies (Fig. 3B).

To determine whether the interaction of IL-4 and IL-4R was required for growth inhibition, we used a neutralizing Ab to the ligand-binding chain of the receptor (27, 28). MCF-7 and MDA-MB-231 cells were grown in monolayer culture and preincubated with Ab before IL-4 treatment. We found that IL-4-mediated growth inhibition was prevented by blocking the IL-4 ligand-binding chain with neutralizing Ab (Fig. 4). As controls, an equal concentration of a non-specific monoclonal Ab did not prevent IL-4-induced growth inhibition, and incubation with IL-4R Ab alone was not toxic to the cells.
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A.

B.

C.

Fig. 5. Effect of IL-4 on E2-stimulated growth and ER-mediated gene transcription. A, MCF-7 cells were plated in SFM and treated with increasing concentrations of E2 alone or with IL-4 (10 ng/ml), and growth was measured after 3 days by the MTT assay (•, E2; O, E2 plus IL-4). Data shown are representative of two separate experiments. Error bars, mean ± SE of quadruplicate samples. B, MCF-7 cells were plated in a final concentration of 5% CSS alone or with E2 (10^-'' M) and treated with 10 ng/ml IL-4. IL-4R-neutralizing Ab was added at 200 ng/ml, and colony formation was determined after 10 days. Data shown are representative of four separate trials. C, MCF-7 cells that had been stably transfected with an ERE-luciferase construct were treated with E2, IGF-I, IGF-I plus E2, IL-4, or IL-4 plus E2 for 24 h and measured luciferase activity. The result shown is representative of three separate trials. Error bars, mean ± SE of triplicate samples.

(data not shown). In the anchorage-independent growth assay, we again confirmed that IL-4 was acting via the IL-4R by adding neutralizing Ab to IL-4-treated cells before plating in soft agar. The addition of 200 ng/ml Ab to MCF-7 cells completely reversed the growth inhibition observed after IL-4 treatment alone, whereas 500 ng/ml Ab partially reversed the IL-4 inhibition of colony formation in MDA-MB-231 cells (Fig. 3A). Therefore, the growth-inhibitory effects of IL-4 seem to be mediated specifically through the IL-4R.

IL-4 Inhibits E2-stimulated Growth but Does Not Affect ER-mediated Gene Transcription. MCF-7 cells require estrogen or peptide growth factors such as IGF-I for optimal monolayer growth. Fig. 2 shows that the growth of MCF-7 cells was inhibited by IL-4 when proliferation was induced by the addition of serum. We were therefore interested in finding out whether proliferation induced by either E2 or IGF-I was sensitive to growth inhibition by IL-4.

MCF-7 cells in monolayer culture were treated with increasing concentrations of E2 alone or E2 plus IL-4 for 3 days. IL-4 inhibited E2-stimulated growth under these conditions (Fig. 5A). We then used an anchorage-independent growth assay to confirm the IL-4-mediated inhibition of E2-stimulated growth. MCF-7 cells were plated in soft agar containing either CSS alone or CSS plus E2. As in our monolayer growth experiments, IL-4 had no effect on basal, unstimulated colony formation in CSS alone. However, in the presence of E2, IL-4 treatment significantly (P < 0.01) reduced the colony size and number (Fig. 5B). Again, this effect was prevented by the addition of IL-4R-neutralizing Ab.

Because E2 exerts its effects through dimerization of ERs, DNA binding, and transactivation at EREs (29), we investigated the possibility that IL-4 inhibits E2-stimulated growth by disrupting ER transactivation. We treated MCF-7 cells that had been stably transfected with an ERE-luciferase reporter construct with E2, IGF-I, IGF-I plus E2, IL-4, or IL-4 plus E2 for 24 h and measured luciferase activity. We have shown previously that IGF-I alone stimulates transcription, and IGF-I plus E2 further enhances the transcriptional activity of the ER to levels above that of either E2 or IGF-I alone (30). Therefore, IGF-I was included in this experiment as a positive control for ER transactivation. IL-4 treatment of MCF-7 cells had no effect on the E2-stimulated induction of luciferase, because there was no statistically significant difference between control and IL-4 alone or E2 and IL-4 plus E2 (Fig. 5C). Thus, IL-4 does not seem to exert its effects by...
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Disruption of the ER transactivation of EREs. It is therefore likely that IL-4 inhibits estrogen-stimulated growth via an interaction with events distal to ER activation.

Previous work has shown that E2 and IGF-I stimulate MCF-7 monolayer cell growth with approximately equal effects (31, 32). Whereas IL-4 inhibited estrogen-stimulated growth, IL-4 had no effect on IGF-1-stimulated growth in monolayer culture (Fig. 6). One possible explanation for this difference is that in addition to stimulating proliferation, IGF-I has been described to induce antiapoptotic mechanisms in cancer cells (33, 34). Therefore, we considered the possibility that IL-4-induced growth inhibition was due to the induction of apoptosis.

IL-4 Induces Apoptosis. To determine whether IL-4 increases the rate at which the breast cancer cells undergo apoptosis, we first examined IL-4-treated MCF-7 cells and untreated controls with H&E and performed a TUNEL assay. H&E-stained IL-4-treated breast cancer cells showed morphological indicators of apoptosis including reduction of the cytoplasm and chromatin condensation. Furthermore, a greater fraction of IL-4-treated cells was positive for fragmented DNA by the TUNEL assay (Fig. 7A). IL-4-treated MDA-MB-231 cells also exhibited morphological indicators of apoptosis as well as positive staining for DNA fragmentation by the TUNEL assay (data not shown). We then confirmed that fragmented DNA detected by the TUNEL assay resulted from apoptosis. We treated MDA-MB-231 cells with IL-4, SFM, or paclitaxel for 4 days and then collected fragmented DNA from each sample. DNA laddering was observed in the presence of paclitaxel and IL-4 but not in SFM controls (Fig. 7B). Similar results were obtained for MCF-7 cells cultured with SFM, IL-4, or paclitaxel supplemented with 0.25% serum (data not shown). Paclitaxel was included as a positive control for apoptosis (35). In addition, we determined the cleavage of the IL-1β-converting enzyme substrate, PARP, by Western blotting (36, 37). Immunoblotting with an Ab against the COOH-terminal portion of PARP demonstrated a Mr 116,000 intact PARP protein as well as a Mr 85,000 apoptosis-specific cleavage fragment in paclitaxel and IL-4-treated MDA-MB-231 cell lysates (Fig. 7C).

We then analyzed propidium iodide-stained MCF-7 and MDA-MB-231 cells by flow cytometry. This is a convenient method for quantifying apoptotic cells, because each cell can be scored for DNA content, and the percentage of apoptotic cells corresponds to the percentage of cells with a DNA content of less than 2 n. MDA-MB-231 cells were treated with SFM alone, IL-4, or paclitaxel, whereas MCF-7 cells were treated with SFM, IL-4, or paclitaxel with the addition of 0.25% serum. Paclitaxel was again included in the experiment as a positive control for apoptosis. IL-4 resulted in a 6.0- and 6.7-fold increase in apoptosis over that of untreated controls in MCF-7 and MDA-MB-231 cells, respectively (Fig. 8A).

We hypothesized that if IL-4-induced growth inhibition was in fact due to apoptosis, the addition of IGF-I would rescue the cells. Specifically, we anticipated that IGF-I would rescue IL-4-induced apop-
tosis in the IGF-I-responsive cell line, MCF-7, but not in the IGF-I-nonresponsive cell line, MDA-MB-231 (38). As a result, we treated MCF-7 and MDA-MB-231 cells with IL-4 plus IGF-I and determined the percentage of apoptotic cells by flow cytometry. We found that the addition of IGF-I to MCF-7 cells partially protected the cells from apoptosis, resulting in a reduction of apoptotic cells from 8.6 to 3.1%, a statistically significant difference \( (P < 0.05; \text{Fig. 8B}) \). As expected, the addition of IGF-I had no effect on IL-4-induced apoptosis in MDA-MB-231 cells.

**DISCUSSION**

Whereas IL-4 has been considered as an antitumor therapy, the focus has been mainly on the ability of IL-4 to increase the host antitumor response. This is believed to occur primarily through the up-regulation of MHC class I and II antigens by IL-4 (39, 40). Recently, however, researchers have begun to examine the role of IL-4 as a direct mediator of growth inhibition. This was demonstrated in breast cancer cells by Toi et al. (5) in 1992. Using human breast and colon carcinoma cell lines, Toi et al. demonstrated that cells bind iodinated IL-4, and that IL-4 reduces the growth of several cancer cell lines in vitro. Blais et al. (6) showed that IL-4 could inhibit the E2-stimulated growth of MCF-7 cells.

Our results confirm these observations and show that IL-4R seems to be required for this growth inhibition. Moreover, IL-4 induces programmed cell death in breast cancer cells. We showed IL-4 induction of apoptosis in breast cancer cells through cellular morphology, TUNEL assay, DNA laddering, and PARP cleavage, and the level of apoptosis induced by IL-4 was quantitated by flow cytometry. Moreover, the effects of IL-4 are only apparent in cells that appear to be actively proliferating, suggesting that entry into the cell cycle may be required for the IL-4 induction of apoptosis.

Our data also suggest that IL-4 initiates an active response in breast cancer cells that results in cell death. We have previously shown that IGF-I, a mitogen for breast cancer cells, and IL-4 share a common signaling pathway via the insulin receptor substrate 1 molecule (41). It is curious that a cell death pathway and a cell growth pathway can both activate the same intracellular adaptor protein. Whereas the mechanism of differential biological effects through a common signaling molecule has not yet been elucidated, we find that IGF-I can protect breast cancer cells from IL-4, suggesting that the IGF-I-stimulated antiapoptotic pathway is dominant over the IL-4 apoptotic signaling pathway. There are several potential explanations for this observation: (a) IL-4 could be deficient in activating intracellular signaling pathways compared with IGF-I, and the resultant effect could be the induction of apoptosis; (b) IL-4 and IGF-I could stimulate insulin receptor substrate 1 in a temporally different pattern than IGF-I that would lead to a different time course of activation of downstream signaling molecules; and (c) a completely different set of signaling molecules could be activated by IL-4 that is responsible for its biological effects. Because Janus-activated kinase/signal transducers and activators of transcription activation by IL-4 is known to occur in lymphocytes (10), and signal transducers and activators of transcription activation has been associated with cell growth inhibition (42), this could be a potential mechanism of IL-4-induced cell growth inhibition. It is also noteworthy that E2 cannot protect the cells from IL-4-mediated cell death, suggesting that the mitogenic pathways activated by IGF-I and E2 are distinct.

In conclusion, our data suggest that IL-4 not only inhibits the growth of breast cancer cell lines but may represent a potential pathway to affect cell death in breast cancer cells. The high frequency of IL-4R expression suggests a broad application for potential therapies, and the ability of IL-4 to inhibit growth and induce apoptosis suggests a potential mechanism for such antitumor therapies. Finally, the possible connection between IL-4 signaling and IGF-I signaling in breast cancer cells suggests another avenue for further study into how breast cancer cells grow and die.

**ACKNOWLEDGMENTS**

We thank Dr. Kathleen dePicha (Immunex, Inc., Seattle, WA) for M(57) IL-4R Ab and Dr. Kevin Moore (DNAX Research Institute, Palo Alto, CA) for the IL-4R cDNA. We also thank Dr. Craig Allred and Nancy Ransom for technical assistance. We thank Dr. Susan Hilsenbeck for statistical analysis and Dr. Gary Chamness for critical review of the manuscript.
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