0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(6):2880–2889

doi: 10.1210/jc.2003-031639

Interleukin-4 Stimulates Papillary Thyroid Cancer Cell Survival: Implications in Patients with Thyroid Cancer and Concomitant Graves' Disease

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IL-4, a pleiotropic cytokine mainly produced by activated helper T lymphocytes type 2 (Th2), is known to protect thyroid cells from autoimmune damage. Acting via its receptors (IL-4Rα), IL-4 has antiproliferative and apoptotic effects in many malignancies. Its effect in thyroid cancer is unknown.

We found that surgical specimens of thyroid carcinomas express both IL-4Rα and IL-4 in the majority of cases. Thyroid glands affected by Graves' disease also express IL-4. We also studied a panel of eight thyroid cancer cell lines from different histotypes and found that thyroid cancer cells express high levels of IL-4Rα although they do not express IL-4.

We then compared the biological effects of IL-4 in TPC-1, a thyroid cancer cell line, and in MCF-7 breast cancer cells. IL-4 very weakly stimulated thyroid cancer cell proliferation, but it was very effective in protecting thyroid cancer cells from apoptosis induced by staurosporin. The protective effect of IL-4 was similar in magnitude to that of IGF-I and was associated with up-regulation of the antiapoptotic molecule Bcl-2 and weak down-regulation of the proapoptotic molecule Bax. Moreover, IL-4 slightly potentiated the survival effect of IGF-I. In contrast, IL-4 reduced growth and induced apoptosis in MCF-7 cells.

Taken together, these findings suggest that thyroid cancer cells receive significant protection from apoptosis by IL-4 produced in the thyroid gland by activated T lymphocytes when concomitant Graves' disease is present. (*J Clin Endocrinol Metab* 89: 2880–2889, 2004)

GRAVES' DISEASE IS an organ-specific autoimmune disorder characterized by the presence of stimulating auto antibodies to the TSH receptor (TSAb) that cause hyperthyroidism (1). We and others have previously reported that thyroid nodules and thyroid cancers are more frequently found in Graves' patients than in euthyroid controls (2). Moreover, differentiated thyroid carcinomas concurrent with Graves' disease are more aggressive at presentation, are more frequently metastatic at distant sites, and have a more adverse outcome compared with similar carcinomas occurring in euthyroid subjects or in patients with nonautoimmune hyperthyroidism (3, 4). One possible mechanism accounting for this increased aggressiveness involves the presence of TSAb. These antibodies, likewise TSH, have been shown to stimulate both the growth and function of well-differentiated thyroid carcinomas (5). We have also hypothesized that cytokines locally produced in the Graves' thyroid gland may concur with TSAb to determine the aggressive features of papillary thyroid cancer in Graves' patients (2, 5). Thyroid glands affected by Graves' disease are infiltrated by activated T lymphocytes predominantly belonging to the Th2 subtype. Accordingly, increased levels of IL-4 and IL-10 have been found both in thyroid-infiltrating lymphocytes (6, 7) and in serum of Graves' patients (8).

IL-4, a pleiotropic cytokine produced by Th2 cells, elicits major regulatory effects in the immune response by inducing Th2 cell differentiation, proliferation, and apoptosis inhibition in both B and T cells. It also induces IgE and IgG1 class switching and major histocompatibility complex class II molecule expression (9). In addition, IL-4 has effects on nonimmune cells, *i.e.* it is mitogenic for fibroblasts and endothelial cells and favors adhesiveness of endothelial cells for T lymphocytes and eosinophils rather than for granulocytes (10).

IL-4 has raised the interest of oncologists because cells from a variety of epithelial malignancies express IL-4 receptors (IL-4Rα). In these malignant cells, including melanoma, colon, renal, and breast cancer cells, IL-4 has antiproliferative and/or apoptotic effects that markedly contrast with its growth-stimulating effects in T cells (11–13). In breast cancer cells, IL-4 antagonizes the antiapoptotic effect of IGF-I (13). These data have suggested that IL-4, or agonist IL-4 analogs, could be used in antitumor therapy.

The data on IL-4 antiproliferative and/or proapoptotic effects in epithelial malignancies may appear in contrast with the observation that thyroid carcinomas concurrent with Graves' disease are usually aggressive.

To investigate this issue, we studied the presence of IL-4Rα

Abbreviations: CDK, Cyclin-dependent kinase; cFLIP, cellular FLICE-inhibitory protein; FBS, fetal bovine serum; IL-4R, IL-4 receptors; MTT, methyl thiazolyl tetrazolium; SDS, sodium dodecyl sulfate; TSAb, antibodies to the TSH receptor.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

and the biological effects of IL-4 in a panel of thyroid cancer cells. By comparison, we studied MCF-7 breast cancer cells, where IL-4 is known to be antimitogenic and proapoptotic (13).

We found that thyroid cancer specimens express both IL-4R and IL-4 in the majority of cases, whereas thyroid cancer cell lines express variable levels of IL-4Rα but not IL-4. IL-4 protected thyroid cancer cells from apoptosis induced by exposure to staurosporin with a potency similar to that of IGF-I. In contrast, IL-4 induced apoptosis and antagonized the survival effect of IGF-I in breast cancer MCF-7 cells.

The survival effect of IL-4 in thyroid cancer cells was associated with up-regulation of the antiapoptotic molecule Bcl-2 and down-regulation of the proapoptotic molecule Bax. These data suggest, therefore, that IL-4 produced by Th2 lymphocytes may contribute to thyroid cancer growth and aggressiveness in Graves' patients and that antagonists to these cytokines or to their receptors may be helpful in the treatment of aggressive thyroid papillary carcinomas.

Materials and Methods

Cells and human tissue specimens

Human thyroid cancer cell lines BC-PAP (papillary), FRO (follicular), WRO (follicular), ARO (anaplastic), and TPC-1 (papillary) provided by Dr. A. Fusco and M. Santoro (Naples, Italy); FF-1 (anaplastic) established in our laboratory; and 8505-C (papillary) purchased from DMSZ (Braunschweig, Germany) were grown in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and streptomycin. The human thyroid papillary cancer cell line PAP was provided by Prof. Edgar Selzer (Vienna, Austria) and grown in RPMI 1640 (Sigma) and DMEM (Sigma) (1:1) supplemented with 2 mM glutamine, 10% FBS, 100 μg/ml penicillin, and streptomycin. Normal thyroid cells in primary culture were obtained from surgical specimens after treatment with 1 mg/ml collagenase IV (Sigma). The human MCF-7 cell line was cultured in MEM (Sigma) plus 10% FBS, 100 μg/ml penicillin, and streptomycin. The human acute T cell leukemia cell line (JURKAT) was cultured in RPMI 1640 (Sigma) supplemented with 10% FBS, 2 mM glutamine, 100 μg/ml penicillin, and streptomycin. In all cell cultures, medium was routinely changed every 2 d.

Human thyroid cancer specimens were obtained at surgery and stored in liquid nitrogen until processing. Informed consent was obtained from patients.

Immunohistochemistry

Immunohistochemical staining was performed on 7-μm-thick sections of unfixed tissue specimens. Sections were cut with a cryostat at −30 C, fixed with acetone at −20 C for 10 min, and hydrated with PBS at room temperature for 45 min. After blocking in 2% normal serum for 20 min, sections were incubated overnight with the anti-IL-4 or anti-IL-4R polyclonal antibodies (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA). Specific labeling was detected with biotin-conjugated anti-mouse/antirabbit IgG and avidin-biotin peroxidase complex. Sections were counterstained with either hematoxylin QS or Nuclear Fast Red (Bio-Optica, Milano, Italy), examined, and photographed using an Olympus BH-2 microscope (Olympus, Orangeburg, NY). In every experiment, sections were incubated with secondary antibody alone to further verify the specificity of the reaction. Both the intensity and the proportion of stained cells were determined by examining the entire slide and scored as: + (low staining intensity in less than 10% cells in the section); ++ (moderate staining intensity in 10–40% cells in the section); or +++ (high and diffuse staining intensity in more than 50% cells in the section).

Immunoblot analysis

Cell lysates were prepared in RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail (Roche Biochemical Inc, Basel, Switzerland). Samples were resuspended in loading buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% milk-Tris-buffered saline with Tween 20 and then immunoblotted with primary antibodies (1 μg/ml). Appropriate horseradish peroxidase-conjugated secondary antibodies were added at 1:2000 (Amersham Biosciences, Little Chalfont, UK), and proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

The following antibodies were used for Western blotting: anti-IL-4Rα polyclonal antibody (Santa Cruz Biotechnology); anti-p27 monoclonal antibody (Exalpha Biologicals, Inc., Boston, MA); anti-p21 polyclonal antibody (Santa Cruz Biotechnology); anti-Bax monoclonal antibody (Trevigen Inc., Gaithersburg, MA); anti-Bcl-2 monoclonal antibody and anti-Bcl-X_L monoclonal antibody (Santa Cruz Biotechnology); anti-FLIP polyclonal antibody (Calbiochem, La Jolla, CA); and anti-β-actin monoclonal antibody (Sigma).

IL-4Rα and IL-4 measurement

IL-4Rα and IL-4 transcripts were measured by RT-PCR. Total RNA (1 μg) was reverse transcribed with Superscript II (Invitrogen, Paisley, UK) and Random Examers. Two microliters of the synthesized cDNA were then combined in a PCR using primers 5′-AAC-CAG-AGT-GAG-TAT-GAG-GAT-3′ (forward) and 5′-CCG-TTC-CAG-AGC-GAA-GTG-CTT-3′ (reverse) specific for IL-4Rα (fragment size, 309 bp). The IL-4 transcript was detected using primers 5′-TCA-CCT-CCC-AAC-TGC-TTC-CC-′ (forward) and 5′-TGT-CGA-GCC-GTT-TCA-GGA-AT-3′ (reverse) (fragment size, 327 bp) for first PCR and primers 5′-CAC-GGA-CAC-AAG-TGC-GAT-AT-3′ (forward) and 5′-GCA-GCG-AGT-GTC-CTT-CTC-AT-3′ (reverse) for nested PCR. ELE-1 amplification (ubiquitous gene) was performed using the following primers: 5′-ATT-GAA-GAA-ATT-GCA-GGC-TC-3′ (forward) and 5′-TGG-AGA-AGA-GGA-GCT-GTA-TCT-3′ (reverse) (fragment size, 280 bp).

Viability assay

Cell viability was measured by the methyl thiazolyl tetrazolium (MTT) test (MTT, Amersham Biosciences), which measures the ability of viable mitochondria to reduce soluble tetrazolium salts to insoluble formazan (14). MTT dye conversion is, therefore, proportional to the number of viable cells. Cells (1×10^3) were seeded in 96-well plates. After 24 h, medium was removed and replaced with BSA (0.1%). After an additional 24 h, 10 nM IGF-I or 50 ng/ml IL-4 was added in fresh medium. MTT conversion was measured 1, 3, 5, and 7 d later by dissolving formazan in dimethylsulfoxide and reading the absorbance at 405 nm. For dose response experiments, increasing IL-4 concentrations were used (10, 25, 50, 70, and 100 ng/ml). Cell viability was measured 4 d later, as described above.

Cell growth studies

Cell growth was measured by [^{3}H]thymidine incorporation: cells (3×10^4/well) were plated in 24-well tissue culture plates and grown in their regular medium for 48 h. Medium was then replaced with fresh medium containing 0.1% BSA. Twenty-four hours later, either 50 ng/ml IL-4 or 10 nM IGF-I was added to each well. After 24 h, 18.5 kBq/well of [^{3}H]thymidine was added for 4 h. Cell monolayers were then washed twice with cold buffer, incubated with cold 10% TCA solution for 30 min, solubilized with 0.1 N NaOH, and counted by liquid scintillation.

Invasion assay

Cell ability to invade collagen-coated filters was measured with the Boyden's chamber technique. Approximately 10^5 cells, resuspended in 200 μl medium, were placed on 6.5-mm diameter polycarbonate filters (8-μm pore size, Corning Costar Corporation, Camdridge, MA) coated at the upper and lower side with 1.0 mg/ml collagen (invasion assay). Either IL-4 (50 ng/ml) or IGF-I (10 nM) in 1 ml medium was added to the lower compartment. The plates were incubated at 37 C with 5% CO_2 overnight, and then the collagen at the upper side of the filter was

removed with a cotton swab. Cells that had migrated to the lower side of the filter were fixed with 11% glutheraldeyde for 15 min at room temperature and stained with 0.1% crystal violet in 20% methanol for 20 min. After three washes with water and complete drying, the crystal violet was solubilized by immersion of the filters in 10% acetic acid. The concentration of the solubilized crystal violet was evaluated as absorbance at 590 nm.

Apoptosis assay

Apoptosis was determined with Cell Death Detection ELISA PLUS kit (Roche Diagnostic, Mannheim, Germany), a photometric enzyme immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments after induced cell death.

Briefly, cells (2×10^4/well) were plated in 12-well tissue culture plates and grown in their regular medium for 24 h. Medium was then replaced with fresh medium containing IL-4 (50 ng/ml) or IGF-I (10 nM) in the presence or absence of staurosporin (Sigma) (2 nM for TPC-1 cell line and 40 nM for MCF-7 cells) for 24 h. At the end of incubation, the absorbance was read at 405 nm.

Statistical analysis

Differences between means were evaluated using the Student's paired *t* test, as indicated in *Results*. The statistical program Statview 5.01 for Macintosh (SAS Institute, Inc., Cary, NC) was used.

Results

IL-4 and IL-4R expression in thyroid cancer cells and surgical specimens

IL-4 and IL-4Rα expression was first examined in a panel of eight thyroid cancer cell lines (four papillary, two follicular, and two anaplastic) and in one primary cell culture from normal thyroid by RT-PCR. MCF-7 human breast cancer cells were also examined. Phytoemoagglutinin-stimulated peripheral blood leukocytes were used as positive control for IL-4 expression; JURKAT cells were used as positive controls for IL-4Rα.

IL-4 transcripts were detected only in phytoemoagglutinin-stimulated peripheral blood leukocytes but neither in thyroid cells nor in MCF-7 cells (data not shown). In contrast, IL-4Rα transcripts were expressed in all thyroid cancer cell lines as well as in normal thyroid cells (Fig. 1A). IL-4Rα protein expression was evaluated by Western blot and results expressed in all cells studied, with highest values in follicular and anaplastic cancer cells (Fig. 1B).

To investigate the *in vivo* relevance of IL-4 in thyroid cancer, the expression of IL-4 and IL-4Rα was evaluated in surgical specimens by both RT-PCR and immunohistochemistry. We obtained specimens from: 1) normal thyroids (n = 8); 2) thyroids affected by Graves' disease (n = 10); and 3) thyroid carcinomas of different histotypes (n = 24). No IL-4 or IL-4Rα were found in normal thyroid specimens. In Graves' glands, IL-4Rα mRNA was found in all cases, although IL-4Rα protein was undetectable by immunohistochemistry; IL-4 mRNA was found in 80% of cases and IL-4 protein in 50% (data not shown). In cancer specimens, IL-4Rα mRNA was found in all cases studied and IL-4Rα protein in approximately 70% of cases. IL-4 was detected in 14 of 16 cases by RT-PCR and in 17 of 24 cases by immunohistochemistry. IL-4 immunostaining was localized at the level of the malignant thyrocytes in 11 cases, whereas it was localized at both the thyrocytes and the stromal cells in six cases (Table 1 and Fig. 2). In one case of papillary cancer concurrent with

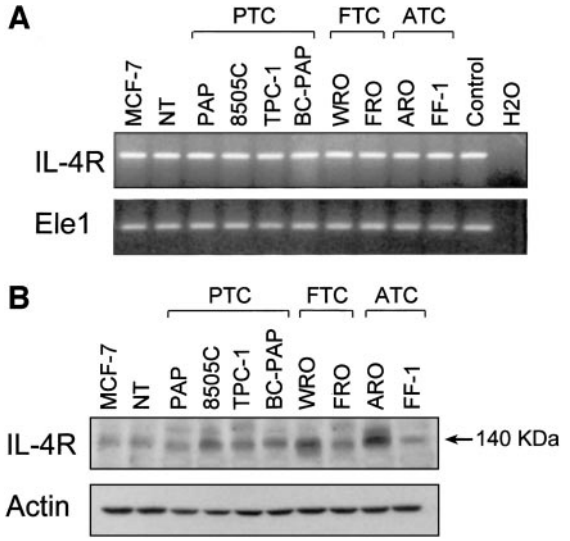

FIG. 1. IL-4Rα mRNA and protein expression in thyroid cells and in MCF-7 breast cancer cells. A, RT-PCR analysis of IL-4Rα expression in MCF-7 cells, primary cultures from normal thyroid (NT), and papillary (PTC), follicular (FTC), or anaplastic (ATC) thyroid cancer cell lines. Products of PCR amplification were resolved by electrophoresis on 2% agarose gels and stained with etidium bromide. The Ele-1 gene shows proper normalization of the RNA extracted from each sample. B, Western blot analysis of IL-4Rα expression in MCF-7 cells, primary cultures from normal thyroid, and cell lines derived from either papillary, follicular, or anaplastic thyroid cancer cell lines. Cell lysates were subjected to SDS-PAGE and blotted with an anti-IL-4Rα antibody. Filters were then stripped and reprobed with an anti-β-actin antibody to control protein loading. A representative experiment of three is shown.

Graves' disease, both IL-4 and IL-4Rα were found in both the neoplastic and the nonneoplastic tissues (Table 1). In papillary carcinomas, there was an association between lymphocyte infiltration and IL-4 positivity: in seven cases, both features were present; in three cases, only IL-4 was found; and in two cases, only lymphocyte infiltration was found. No apparent association between lymphocyte infiltration and IL-4 was present in follicular or anaplastic carcinomas (Table 1).

IL-4 increases viability in thyroid cancer cells

We then evaluated the effect of IL-4 on cell viability in thyroid cancer cells maintained in serum-free medium in monolayer culture. IGF-I (10 nM), previously described as a relevant mitogenic and antiapoptotic factor for cultured thyroid cancer cells, was used as a control growth factor. Human breast cancer cells MCF-7, known to be growth-inhibited by IL-4 and growth-stimulated by IGF-I (13), were studied in parallel experiments. IL-4 was used at 50 ng/ml, a dose able to elicit maximal effects in most model systems, and cell viability was evaluated by MTT staining 4 d later.

IL-4 increased cell viability (up to 171% of unstimulated) in all papillary and anaplastic thyroid cell lines; although in one papillary cell line (8505C), this increase was very modest and not significant. IL-4 did not increase cell viability in follicular cancer cell lines, rather decreased it in one cell line (WRO) (Table 2). As expected, IL-4 reduced cell viability in MCF-7 cells (77% of unstimulated). IGF-I increased cell vi-

TABLE 1. IL-4 and IL-4R expression in human thyroid cancer specimens by immunohistochemistry (IHC) and RT-PCR; lymphocyte infiltration is also shown

	IL-4			IL-4Rα		Lymphocyte infiltration
	IHC/T[a]	IHC/S[b]	PCR	IHC	PCR	
Papillary cancer						
050CA	++	−	+	−	+	−
106CA	++	−	+	+	+	+
114CA	++	−	n.e.	−	n.e.	+
160CA	+	−	n.e.	+	n.e.	+
252CA	−	−	n.e.	+	n.e.	+
290CA	+++	−	n.e.	+	n.e.	+
321CA	++	++	+	+++	+	+
340CA	++	+++	+	++	+	−
350CA	+	++	+/−	+	+	+
360CA	−	−	−	+	+	+
370CA	−	−	n.e.	−	n.e.	−
380CA	+	++	−	−	+	−
Papillary cancer in Graves' disease						
161 Graves' tissue	+	+	+	+	+	+
161 Cancer tissue	+	+	+	++	+	+
Follicular cancer						
097CA	+	−	+	+++	+	−
267CA	+	+	+	+	+	−
283CA	+	−	+	+	+	−
371CA	−	−	n.e.	+	n.e.	−
Anaplastic cancer						
017CA	−	−	+	−	+	−
096CA	++	−	+	++	+	−
103CA	−	−	+	++	+	+
238CA	+/−	−	n.e.	−	n.e.	−
241CA	+	−	+	++	+	+
261CA	−	+/−	n.e.	−	n.e.	−
333CA	+	−	+	+	+	−

n.e., Not evaluated.
[a] T, Thyrocytes.
[b] S, Stromal cells.

ability in all thyroid cancer cell lines; although in one of them (8505C), this increase was not significant. MCF-7 cell viability increased markedly in response to IGF-I (Table 2).

TPC-1 cells showed the highest increase in cell viability in response to IL-4 (141 ± 7% of unstimulated) among thyroid papillary cells. A similar response (140 ± 6%) was observed after IGF-I stimulation. TPC-1 cells, therefore, were chosen to further study the effect of IL-4 and were compared with MCF-7 cells. Time-course and dose-response studies confirmed that IL-4 and IGF-I have similar effects in TPC-1 cells but diverging effects in MCF-7 (Fig. 3). Maximal IL-4 effect was observed after 5–7 d and with a dose of 50–100 ng/ml.

IL-4 does not affect thyroid cancer cell proliferation

To evaluate whether IL-4 increased cell viability in TPC-1 cells by either increasing cell proliferation or decreasing the apoptosis rate, [^{3}H]-thymidine incorporation was carried out after cell exposure to either IL-4 or IGF-I. Parallel experiments were carried out in MCF-7 cells. Exposure to IL-4 slightly increased [^{3}H]-thymidine incorporation in TPC-1 cells, although not significantly. In contrast, IL-4 significantly reduced growth in MCF-7 cells ($P = 0.03$) (Fig. 4). IGF-I significantly increased [^{3}H]-thymidine incorporation in both cell lines ($P = 0.007$ and $P = 0.004$ in TPC-1 and MCF-7, respectively) (Fig. 4).

We also investigated whether IL-4 affects the expression of cyclin-dependent kinase (CDK) inhibitors, such as $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, that negatively regulate cyclin/CDK complexes and cell cycle progression. In particular, $p27^{Kip1}$ has a key role in mediating G1 arrest by contact inhibition or serum deprivation and mainly targets cyclin D/CDK4. We found that IL-4 exposure reduces both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ in TPC-1 cells. In particular $p21^{Cip1/Waf1}$ levels reached 51% of unstimulated cells at 24 h, whereas $p27^{Kip1}$ levels were reduced to 34% and 58% of unstimulated cells after 12 h and 24 h, respectively (Fig. 5). In contrast, IL-4 increased $p21^{Cip1/Waf1}$ levels at 24 h in MCF-7 cells (150% of unstimulated cells) and reduced $p27^{Kip1}$ levels to 88% of unstimulated after 24 h (Fig. 5).

IL-4 stimulates chemoinvasion in thyroid cancer cells

We evaluated the ability of IL-4 to induce chemoinvasion in TPC-1 cells. Chemoinvasion was measured as the cell ability to cross a collagen barrier in Boyden chambers. Both IL-4 and IGF-I similarly stimulated chemoinvasion in TPC-1 thyroid cancer cells. This stimulation was weak for both ligands but consistent: 123 ± 6% for IL-4 ($P = 0.0013$) and 123 ± 4.7% for IGF-I ($P = 0.0012$). In MCF-7 cells, only IGF-I (129 ± 8%, $P = 0.028$) but not IL-4 (110 ± 12%, $P = 0.45$) stimulated chemoinvasion.

IL-4 protects thyroid cancer cells from apoptosis

Because IL-4 did not elicit a consistent proliferative effect in TPC-1, we interpreted the increased cell viability as mostly

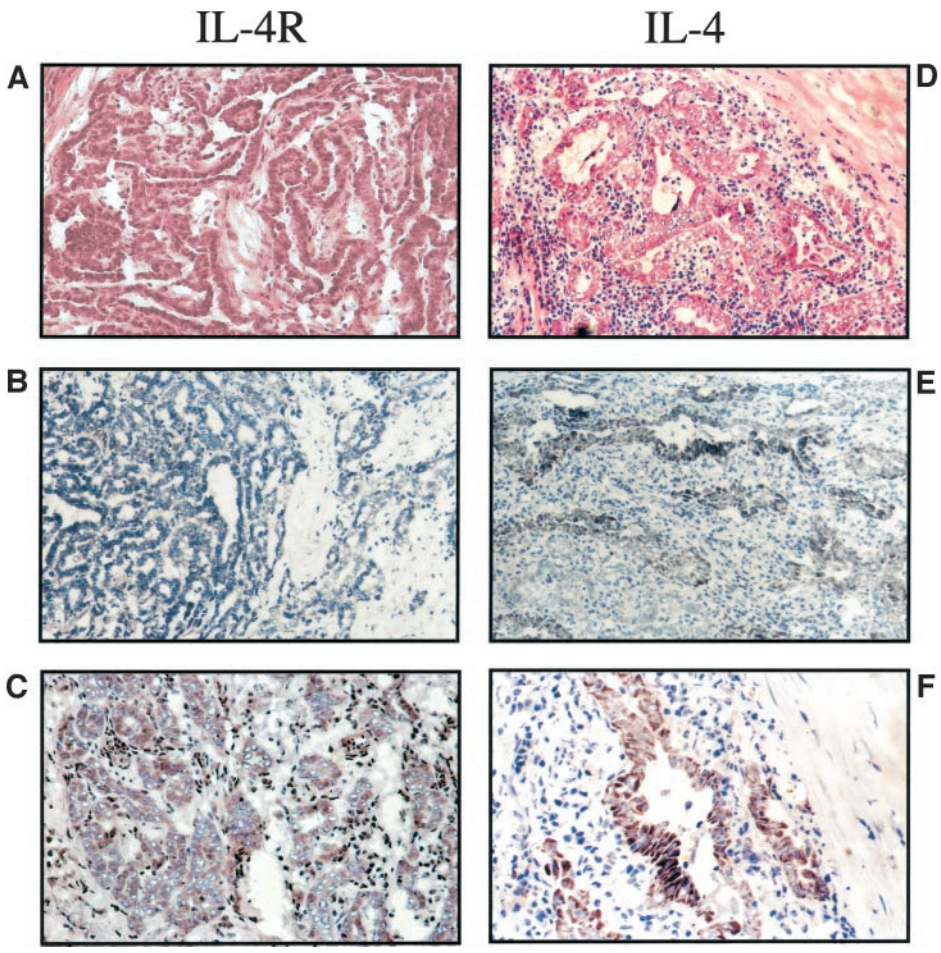

FIG. 2. Micrographs of papillary cancer specimens showing immunohistochemical staining for IL-4Rα and IL-4. Papillary cancer (340CA): A, Hematoxylin and eosin staining; B and C, immunohistochemistry (IHC) staining for IL-4Rα, original magnification $\times$10 and $\times$20, respectively. Papillary cancer (321CA): D, Hematoxylin and eosin staining; E and F, IHC staining for IL-4, original magnification $\times$10 and $\times$20, respectively.

TABLE 2. Changes in MCF-7 and in thyroid cancer cell viability after 4-d exposure to either 50 ng/ml IL-4 or 10 nM IGF-I

Cells	IL-4	IGF-I
MCF-7	77 ± 10[a]	158 ± 7[a]
TPC-1	141 ± 7[a]	140 ± 6[a]
PAP	126 ± 5[b]	110 ± 1[b]
8505C	118 ± 11[c]	128 ± 12[c]
BC-PAP	127 ± 5[b]	144 ± 3[a]
WRO	76 ± 5[b]	146 ± 11[b]
FRO	82 ± 8[c]	124 ± 8[b]
ARO	133 ± 10[b]	125 ± 9[b]
FF-1	171 ± 5[a]	277 ± 6[a]

Cell viability was measured by MTT and expressed as percent of unstimulated (unstimulated = 100). Values indicate mean ± SE of three separate experiments.

Difference in respect to basal cell viability:

[a] $P < 0.01$.

[b] $P < 0.05$.

[c] Not significant.

due to protection from apoptosis. To better study this effect, we then evaluated the possible protective effect of IL-4 on apoptosis induced by exposure to staurosporin, a potent apoptotic agent. TPC-1 or MCF-7 cells were plated in monolayers in the presence or the absence of 2 nM staurosporin, and protection from staurosporin-induced apoptosis was then evaluated by adding either IL-4 (50 ng/ml) or IGF-I (10 nM) or both compounds. Apoptosis was evaluated by measuring histone-associated-DNA-fragments (mono- and oligonucleosomes), as described in *Materials and Methods*. In TPC-1 cells, neither IL-4 nor IGF-I affected basal apoptosis whereas both of them effectively reduced staurosporin-induced apoptosis in a similar manner. The simultaneous treatment with the two compounds had an additive effect on apoptosis reduction (Fig. 6A). In MCF-7 cells, IL-4 did not significantly affect staurosporin-induced apoptosis, whereas IGF-I was highly protective and almost completely inhibited the effect of staurosporin. The combined effect of the two compounds was similar to that of IGF-I alone (Fig. 6A).

To assess the protective effect of IL-4 over time, cells were plated at high density and maintained in the presence or the absence of staurosporin for 6 d, with or without the addition of either IL-4 or IGF-I or both ligands. Cell survival was then assessed by MTT staining.

Staurosporin reduced cell survival by approximately 30–40% in both cell lines. In agreement with results obtained measuring histone-associated-DNA-fragments, both IL-4 and IGF-I were equipotent in protecting TPC-1 cells from the effect of staurosporin. The simultaneous exposure to both ligands completely blocked the effect of staurosporin (Fig. 6B). Different results were obtained in MCF-7 cells. IL-4 slightly potentiated the effect of staurosporin, whereas IGF-I was protective. The simultaneous addition of IL-4 reduced IGF-I protection (Fig. 6B).

IL-10, another Th2 cytokine present in Graves' disease,

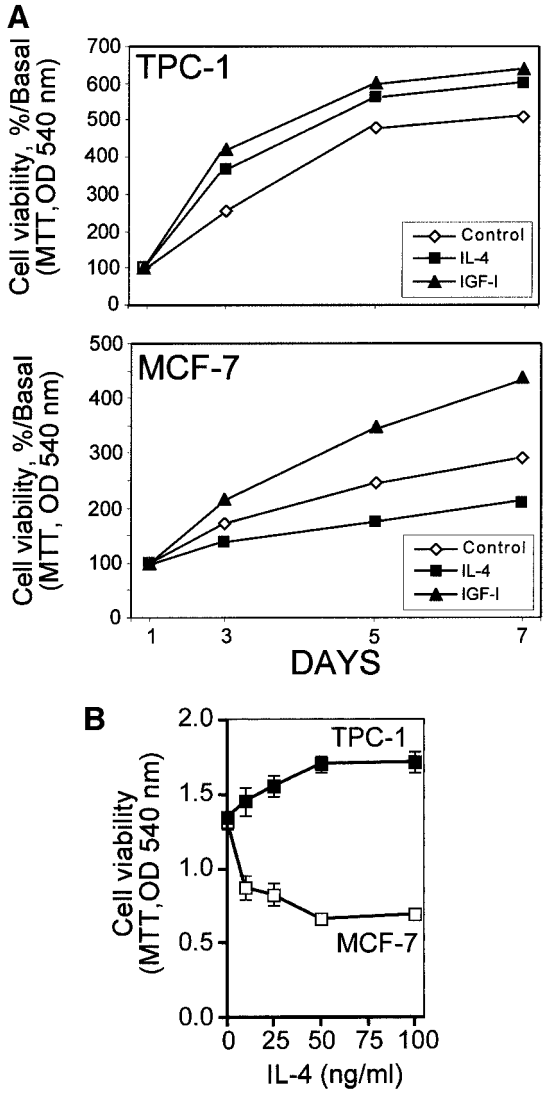

FIG. 3. A, Time-course of IL-4 or IGF-I effect on cell viability. Subconfluent cells were serum-starved and cultured for 3, 5, and 7 d in the presence or the absence of either IL-4 (50 ng/ml) or IGF-I (10 nM). Cell viability was measured by MTT staining. In TPC-1 cells, both IL-4 and IGF-I increased cell viability, with a maximal effect at 5–7 d. In MCF-7 cells, cell viability was reduced by IL-4 but increased by IGF-I. B, Dose-response effect of IL-4 on cell viability. Serum-starved TPC-1 or MCF-7 cells were cultured in the presence or the absence of increasing doses of IL-4 for 4 d. Cell viability was then measured by MTT staining. Cell viability was increased in TPC-1 cells and decreased in MCF-7 cells in a dose-dependent manner, with a maximal effect at 50 ng/ml IL-4. Each figure represents the mean ± SD of three separate experiments.

was also tested in parallel experiments and produced results very similar to those of IL-4: it protected thyroid cancer cells from staurosporin-induced apoptosis with a potency similar to IL-4, whereas it potentiated the effect of staurosporin in MCF-7 cells (data not shown).

IL-4 differentially affects apoptosis regulatory molecules in MCF-7 and TPC-1 cells

To gain insight into the mechanisms underlying the differential effect of IL-4 on cell survival, we analyzed the effect

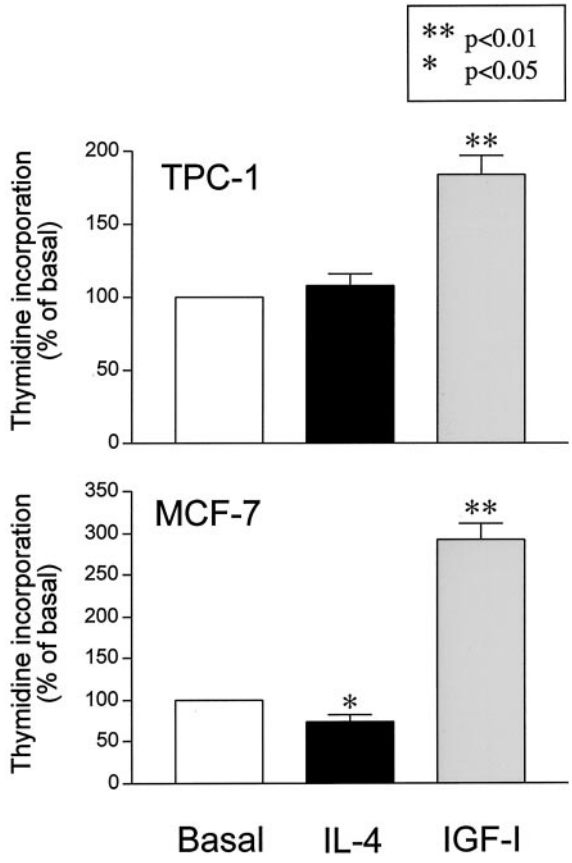

FIG. 4. Proliferative effect of IL-4 and IGF-I. Serum-starved TPC-1 or MCF-7 cells were cultured in the presence or the absence of IL-4 (50 ng/ml) or IGF-I (10 nM) for 2 d and then pulsed with [^{3}H]-thymidine. In TPC-1 cells, [^{3}H]-thymidine incorporation was very weakly stimulated by IL-4 and consistently stimulated by IGF-I. In MCF-7 cells, [^{3}H]-thymidine incorporation was reduced by IL-4 and stimulated by IGF-I. Figures represent the mean ± SD of five separate experiments.

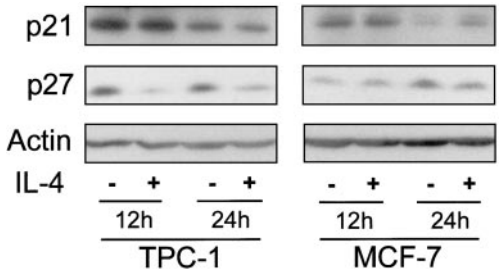

FIG. 5. Effect of IL-4 on p21$^{Cip1/Waf1}$ and p27^{Kip1} expression. Monolayer cultures were incubated with either IL-4 (50 ng/ml) or IGF-I (10 nM) in serum-free medium for either 12 or 24 h. Cell lysates were subjected to SDS-PAGE and blotted with anti-p21$^{Cip1/Waf1}$ or p27^{Kip1} antibodies. Filters were then stripped and reblotted with an anti-β-actin antibody as control (*lower panel*). A representative of three experiments is shown.

of IL-4 on a panel of molecules involved in apoptosis regulation.

Bcl-2, Bcl-X_L, and Bax belong to the same family and are major regulators of the mitochondrial arm of apoptosis pathway; Bcl-2 and Bcl-X_L are antiapoptotic, whereas Bax is proapoptotic.

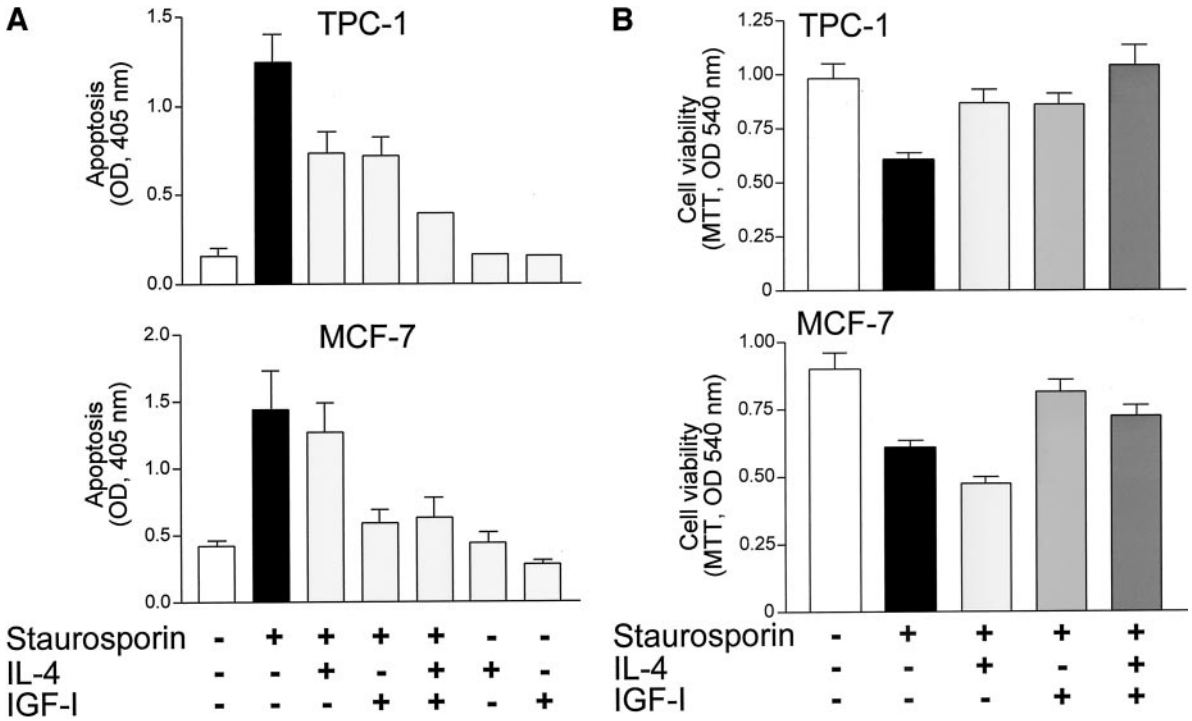

FIG. 6. Protection by either IL-4 or IGF-I on staurosporin-induced apoptosis. A, Serum-starved cells were incubated with either IL-4 (50 ng/ml) or IGF-I (10 nM) or both ligands in the presence or absence of staurosporin for 24 h. Apoptosis was detected by measuring cytoplasmic histone-associated DNA fragments by ELISA. B, Cells were plated at high density and maintained with either IL-4 (50 ng/ml) or IGF-I (10 nM) or both ligands in the presence or absence of staurosporin for 6 d. Cell survival was then assessed by MTT staining. Each figure represents the mean ± SD of five separate experiments.

After 12–24 h exposure to IL-4, Bcl-2 expression increased by approximately 4-fold in TPC-1, whereas it remained unchanged in MCF-7 cells (Fig. 7A). Bcl-X_L expression was increased in TPC-1 cells by 30% after 12 h and reduced by 20% after 24 h. In MCF-7 cells, Bcl-X_L expression was reduced by 24% and 40%, after 12 and 24 h exposure to IL-4, respectively. Proapoptotic protein Bax expression was slightly affected by IL-4 treatment, changing in an opposite way in the two cell types (−18% and −16% in TPC-1 cells *vs.* +12% and +12% in MCF-7 cells after 12 h and 24 h, respectively) (Fig. 7A).

Exposure to staurosporin reduced both Bcl-2 and Bcl-X_L expression in the two cell lines studied. Bcl-2 was reduced by 40% and 30% in TPC-1 and MCF-7 cells, respectively. Treatment with IL-4 was able to make TPC-1 cells almost completely recover basal Bcl-2 levels, whereas it was not able to block Bcl-2 reduction in MCF-7 cells. Bcl-X_L was reduced by 32% and 46% in TPC-1 and in MCF-7, respectively. Treatment with IL-4 caused a slight further reduction in TPC-1 and a more marked reduction in MCF-7 cells (Fig. 7B).

In addition to reducing antiapoptotic molecules Bcl-2 and Bcl-X_L, staurosporin increased the levels of proapoptotic molecule Bax (to 140% and 280% of untreated cells in TPC-1 and in MCF-7, respectively). The addition of IL-4 not only blocked the staurosporin-mediated Bax increase in TPC-1 cells but also reduced Bax levels to approximately 60% of the level of untreated cells. By contrast, in MCF-7 cells, IL-4 did not affect staurosporin-mediated Bax increase.

To evaluate the potential role of IL-4 on the caspase arm of apoptosis, caspase 3 activation was assessed by Western blot by an antibody that detects both procaspase and the cleaved caspase fragment. We also measured cellular FLICE-inhibitory protein (cFLIP), an antagonist of the effector caspases, by Western blot. No significant effect of IL-4 on caspase-3 processing or cFLIP expression was found in both TPC-1 and MCF-7 cells (data not shown). Exposure to staurosporin reduced cFLIP levels by approximately 30% in both cell lines. Neither IL-4 nor IGF-I significantly affected these changes (data not shown).

Discussion

Herein we show that IL-4 elicits, in most thyroid cancer cells, multiple biological effects, which may favor tumor progression and spreading. These effects are peculiar to thyroid cancer cells, because IL-4 has previously been shown to elicit antiproliferative and/or proapoptotic effects in a variety of solid tumors, including melanoma, colon, renal, and breast cancer (15). To our knowledge, the only other epithelial malignancy in which IL-4 was found to be weakly mitogenic is neck squamous cell carcinoma (16).

First we observed that human thyroid cells in culture, both normal and malignant, express IL-4R mRNA and protein. When thyroid surgical specimens were examined by immunohistochemistry, IL-4R was found in most malignant specimens but not in normal thyroid specimens. These observations indicate that most thyroid carcinomas express high IL-4Rα levels, independently of the histotype. The expression of the ligand, IL-4, was not found in established thyroid cancer cell lines but was found in approximately 70% of fresh

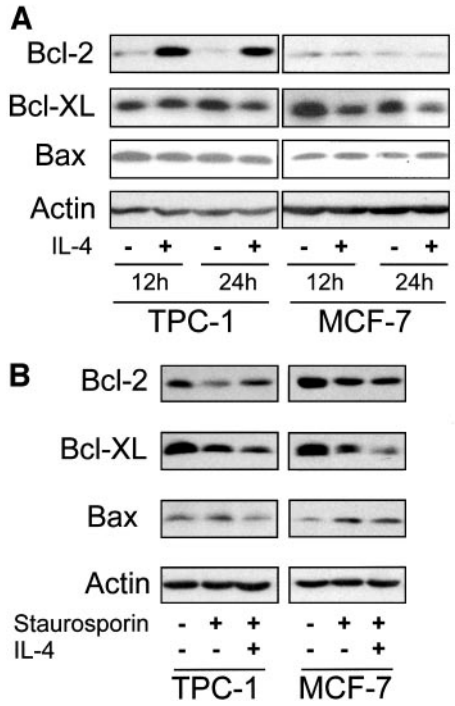

FIG. 7. Changes in the expression of mitochondrial proteins Bcl-2, BcL-XL, and Bax after cell exposure to IL-4. Cells cultured in the absence (A) or in the presence (B) of staurosporin were exposed to either IL-4 (50 ng/ml) or IGF-I (10 nM). Cell lysates were then subjected to SDS-PAGE and blotted with antibodies (either anti-Bax or anti-Bcl-2 or anti-Bcl-X_L). Filters were then stripped and reprobed with an anti-β-actin antibody to control protein loading. A representative of three experiments is shown.

specimens from thyroid carcinomas. In 17 of 24 cases, IL-4 immunoreactivity appears to be localized in the malignant thyrocytes. Moreover, IL-4 was present in over 80% of specimens from thyroid glands affected by Graves' disease. In one case of papillary thyroid cancer concomitant with Graves' disease, IL-4 was found both in the malignant and in the nonmalignant tissue. IL-4 was not present in normal thyroid specimens. These observations are in agreement with previous data indicating that IL-4, together with other Th2 cytokines (*e.g.* IL-10), is expressed by lymphocytes infiltrating thyroid glands affected by Graves' disease (7, 17). While this paper was being reviewed, Stassi *et al.* (18) reported that primary cultures from thyroid carcinomas from different histotypes, and apparently free of lymphocyte infiltration, express IL-4. They concluded that malignant thyrocytes may express IL-4 in an autocrine manner (18). Taken together, these findings indicate that thyroid cancer cells overexpress IL-4Rα and are exposed to IL-4, locally produced either by thyrocytes themselves or by infiltrating lymphocytes. IL-4 production may be especially important in tumors concomitant with Graves' disease.

Second, we found that IL-4 exerted a potent antiapoptotic effect and protected cells from apoptosis induced by staurosporin. This effect was comparable with that of IGF-I, a major survival factor in thyroid cancer (19). In contrast with observations in thyroid cancer cells and in agreement with previous observations (13), we found that IL-4 induced apoptosis in MCF-7 breast cancer cells and did not protect those cells from staurosporin-induced apoptosis.

IL-4 also elicited opposite effects on cell growth of TPC-1 *vs.* MCF-7 cells: it weakly stimulated thymidine incorporation in thyroid cancer cells, whereas it inhibited growth in MCF-7 cells. This IL-4 effect on MCF-7 growth inhibition is small probably as a consequence of our procedure (cells are cultured in the absence of serum), because IL-4 mainly inhibits MCF-7 cell growth induced by FCS and estrogen (13).

Finally, IL-4 had a stimulatory effect on TPC-1 cell chemoinvasion, a property related to *in vivo* cancer invasion and metastatic potential. The degree of chemo-invasion stimulated by IL-4 was rather weak, but it was comparable with that observed in response to IGF-I, a well-known stimulator of cell motogenesis (20). The observed dissociation between the proliferative and the chemoinvasion effect in response to IL-4 in malignant thyrocytes is not surprising. A similar dissociation has been observed in SKUT-1 leiomiosarcoma cells in response to insulin (21) and in the estrogen receptor-negative breast cancer cells MDA-MB231 in response to IGF-I (22). It appears, therefore, that mitogenic and motogenic pathways stimulated by IL-4 in thyroid cancer cells may be dissociated; likewise, it may happen in response to other growth factors. These non-growth-related motogenic effects, nevertheless, may be relevant to the metastatic process.

To gain insight into the molecular mechanisms underlying the different effects on apoptosis and proliferation in TPC-1 and in MCF-7 cells, we studied the effects of IL-4 on a panel of apoptosis regulatory molecules. In particular, members of the Bcl-2 family have a key role in regulating cytochrome c efflux from the mitochondria into the cytosol and consequent activation of IL-1β-converting enzyme family of proapoptotic cysteine proteases (23). Because these mitochondrial proteins may heterodimerize, the net effect on apoptosis protection results from the balance between antiapoptotic (Bcl-2, Bcl-X_L) and apoptotic proteins (Bax, Bad) (24). We found that IL-4 markedly increases Bcl-2 expression and slightly decreases Bax in TPC-1 cells. In contrast, IL-4 decreased Bcl-X_L and slightly increased Bax in MCF-7 cells. These findings may help explain the antiapoptotic effect in TPC-1 and the proapoptotic effect in MCF-7 cells. These data confirm previous findings indicating that Bax is less modulated by cytokines than Bcl-2 or Bcl-X_L (25).

Cell exposure to staurosporin induced apoptosis and reduced Bcl-2 and Bcl-X_L expression both in TPC-1 and MCF-7 cells. IL-4 antagonized, and almost completely blocked, Bcl-2 reduction in TPC-1 but not in MCF-7 cells. Furthermore, IL-4 slightly affected Bcl-X_L levels in TPC-1, whereas it caused a more marked reduction in MCF-7 cells. IL-4 also blocked the staurosporin-mediated Bax increase in TPC-1 cells, whereas it did not affect staurosporin-mediated Bax increase in MCF-7 cells.

No clear effect of IL-4 was observed on caspase-3 cleavage and on the expression of cFLIP, a effectore caspase inhibitor (26).

In partial accordance with our present data, Stassi *et al.* (27) recently reported that both IL-4 and IL-10 protect Graves' thyrocytes from CD95-mediated apoptosis. However, at variance with our data in thyroid cancer cells, these authors found that IL-4 up-regulates Bcl-X_L and cFLIP in primary

cultures from normal thyroid. However, in their last paper, the same authors reported that IL-4 protects malignant thyrocytes from chemotherapeutic drugs by up-regulating both Bcl-2 and Bcl-X_L (18).

We found that IL-4 was weakly mitogenic in thyroid cancer cells, as assessed by thymidine incorporation. The progression in the cell cycle requires cyclin/CDK complexes that result in phosphorylation of the retinoblastoma protein. Upon phosphorylation, retinoblastoma releases the transcription factor E2F that activates transcription of genes involved in cell cycle progression (28). Cyclin/CDK complexes are negatively regulated by CDK inhibitors, such as $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and others (29, 30). In particular, $p27^{Kip1}$ has a key role in mediating G1 arrest by contact inhibition or serum deprivation and mainly targets cyclin D/CDK4 but also other cyclin/CDK complexes (31). We found that IL-4 elicits different effects on the expression of CDK inhibitors in the two cell lines. IL-4 down-regulates both $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ in TPC-1 cells. The effect on $p27^{Kip1}$ is, however, predominant. In contrast, IL-4 up-regulates $p21^{Cip1/Waf1}$ and does not affect $p27^{Kip1}$ in MCF-7 cells. These data indicate a positive effect of IL-4 on cell cycle progression in thyroid cancer cells but confirm a negative effect on cell cycle progression in MCF-7 cells.

Taken together, these findings suggest that, in patients with Graves' disease and a concomitant thyroid papillary cancer, IL-4 and IL-10 produced by infiltrating lymphocytes may affect thyroid cancer biology and may potentiate the role of other antiapoptotic factors such as IGFs (19, 32). An impaired ability of the cell to undergo apoptosis in response to genotoxic insults may play a role in the accumulation of mutations that eventually leads to malignant transformation. Accordingly, others and we (2, 33) have reported that Graves' patients have an increased rate of thyroid cancer. Moreover, the combined effect of increased survival, growth, and invasion in thyroid cells exposed to IL-4 may stimulate thyroid cancer aggressiveness and hematogenous spread. Interestingly, although the majority of Graves'-associated carcinomas are well differentiated and belong to the papillary histotype that is usually scarcely angioinvasive, these tumors may behave aggressively and show a higher rate of distant metastases and of persisting/relapsing disease than similar tumors occurring in euthyroid patients (3, 4).

These data suggest, therefore, that cytokines produced by Th2 lymphocytes may contribute to thyroid cancer aggressiveness in Graves' patients and that antagonists to these cytokines or to their receptors may be helpful in the treatment of these aggressive thyroid papillary carcinomas.

Acknowledgments

Received September 23, 2003. Accepted March 7, 2004.

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This work was supported, in part, by the Associazione Italiana per la Ricerca sul Cancro (to A.B. and R.V.) and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (Cofin 2003 to A.B.).

V.V. is the recipient of a fellowship from Fondazione Italiana per la Ricerca sul Cancro. F.F. is the recipient of a fellowship from the American Italian Cancer Foundation.

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