Thyroid Cancer Resistance to Chemotherapeutic Drugs via Autocrine Production of Interleukin-4 and Interleukin-10

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ABSTRACT

We investigated the mechanisms responsible for the widespread refractoriness to chemotherapeutic drugs observed in thyroid cancers. We show that malignant epithelial cells from papillary, follicular, and anaplastic thyroid carcinomas express high levels of Bcl-2 and Bcl-xL. Exogenous expression of either Bcl-2 or Bcl-xL in normal thyrocytes was sufficient to prevent chemotherapeutic drug-induced cytotoxicity. All of the histological thyroid cancer variants examined produced interleukin-4 (IL-4) and interleukin-10 (IL-10), which increased Bcl-2 and Bcl-xL levels and protected thyroid cells from chemotherapeutic agents. Exposure to neutralizing antibodies against IL-4 and IL-10 resulted in down-modulation of Bcl-2 and Bcl-xL, death of a considerable percentage of thyroid cancer cells, and sensitization of the residual tumor population to cytotoxic drug-induced apoptosis. In conclusion, autocrine production of IL-4 and IL-10 promotes thyroid cancer cell progression and resistance to chemotherapy through the up-regulation of antiapoptotic proteins. Thus, IL-4 and IL-10 may represent new therapeutic targets for the treatment of thyroid cancer.

INTRODUCTION

Thyroid cancer is the most common endocrine malignancy and is responsible for about 60% of deaths secondary to endocrine cancer (1, 2). Three major types of malignant tumors originate from the thyroid epithelium. The more differentiated PTCs and FTCs account for most of the malignant tumors, whereas the UTCs are extremely rare (3). Clinical trials with chemotherapeutic drugs have produced rare responses in thyroid cancer (4–6). However, most patients with thyroid cancer will eventually experience relapse despite initial therapy (10, 11). They activate the intrinsic apoptotic pathway of the multidrug resistance gene is altered in a small subset of thyroid carcinomas (7–9), but the molecular bases implicated in the intrinsic apoptotic pathway are not fully understood. Antineoplastic drugs are a class of cytotoxic compounds that selectively target proliferating cells by interfering with DNA replication or transcription (10, 11). They activate the intrinsic apoptotic pathway that involves the release from mitochondria of death factors such as cytochrome c and Apaf-1, resulting in the formation of a holoenzyme complex (called the apoptosome) that triggers activation of caspase 9 and executioner caspases responsible for completion of the death program (12, 13). A network of cellular proteins influences the ultimate outcome of drug-induced damage (14). The relative levels and competing dimerization between Bcl-2 family members regulate cytochrome c release from mitochondria, thus determining cell susceptibility to apoptotic signals (15). High expression of the antiapototic members of the Bcl-2 family is commonly found in human cancers and contributes to both neoplastic cell expansion and resistance to the therapeutic action of cytotoxic drugs (16, 17). Accordingly, several studies have shown that functional blockade of Bcl-2 or Bcl-xL can either restore the apoptotic process in tumor cells or sensitize these tumors to chemotherapies and radiotherapies (18–21). Thus, up-regulation of antiapoptotic Bcl-2 family members promotes cancer growth and impairs the effectiveness of therapeutic procedures. As suggested by experimental evidences on cell lines and primary cells (22), the considerable resistance of thyroid tumors to chemotherapeutic drugs may result from altered expression of antiapoptotic proteins.

Cytokines promote the expression of a variety of genes involved in the survival or death of different target cells (23–26). Three functionally distinct subsets of T-helper cells have been characterized on the basis of cytokine production (27). Th1 cells secrete IFN-γ and other cytokines associated with inflammation and cell-mediated immune responses. In contrast, Th2 cells promote the humoral response and produce IL-4, IL-5, and IL-10, whereas Th3 cells inhibit the immune response through the release of IL-10 and transforming growth factor β-1 (27–29). We recently demonstrated that cytokines released by T-helper lymphocytes can contribute to thyroid destruction through the modulation of proapoptotic and antiapoptotic proteins in target cells. IFN-γ contributes to autoimmune thyrocyte destruction through the up-regulation of CD95, caspase-3, and caspase-8, whereas IL-4 and IL-10 promote thyrocyte survival during nondestructive autoimmune thyroid destruction through the up-regulation of cellular FLICE inhibitory protein (cFLIP) and Bcl-xL (30, 31).

We speculated that the modulation of apoptosis-related proteins by T-helper cytokines was not restricted to normal thyrocytes but could be involved in the survival of thyroid cancer cells. We, therefore, investigated the presence of IL-4 and IL-10 in the thyroid cancer microenvironment and whether they were responsible for refractoriness to chemotherapy.

MATERIALS AND METHODS

Specimens. Thyroid tissues from patients affected by PTC (n = 8; age, 45 ± 5 years), FTC (n = 8; age, 48 ± 3 years), and UTC (n = 4; age, 67 ± 4.5 years) were obtained at thyroidectomy, in accordance with the ethical standards of the Institutional Responsible Committee on human experimentation. Control thyroid specimens were obtained from the uninvolved, controlateral capsules (n = 8; age, 48 ± 3 years, and on nuclear atypia (shape and chromatin pattern). Transgenic mouse hearts expressing human Bcl-xL, provided by G. Condorelli (Thomas Jefferson University, Philadelphia, PA), and HL-60 cells were used as positive controls.

Thyroid Cell Purification, Culture, and Cell Death Quantitation. Tissues from normal, PTC, FTC, and UTC thyroids were digested for 2 h with collagenase (1.5 mg/ml; Life Technologies, Inc., Grand Island, NY) and hyaluronidase (20 µg/ml; Sigma Chemical Co., St. Louis, MO) in DMEM (30). Thyrocytes were purified from the digested tissues by hematopoietic cell...
depletion with anti-CD45-coupled beads (Dynal, Wirral Merseyside, United Kingdom) and 12 h of flask adherence, which allowed removal of other cells. After an additional 12 h of culture, thyroid cells were allowed to grow in monolayer for immunocytochemistry or were detached with trypsin/EDTA after exposure to cytokines or chemotherapeutic agents for functional and protein analyses. Thyrocytes were cultured in standard DMEM with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, United Kingdom) in the presence or absence of human recombinant IL-4 (20 ng/ml), IL-10 (40 ng/ml), or IFN-γ (1000 IU/ml; Euroclone, Paignton, United Kingdom) with or without cisplatinum (300 ng/ml), doxorubicin (5 μM), or Taxol (5 μM; Sigma Chemical Co.). Autocrine release of IL-4 and IL-10 was neutralized, exposing thyrocytes for 48 h with 10 μg/ml Abs specific to IL-4 (3007, mouse IgG1; R&D Systems, Inc., Minneapolis, MN) or IL-10 (23738, mouse IgG2b; R&D Systems).

Death of normal and neoplastic thyrocytes was evaluated by DNA staining, followed by flow cytometry analysis as described previously (30). Alternatively, freshly purified thyrocytes were plated in flat-bottomed 96-well plates in triplicate at 20,000 cells/well and cultured. The number of viable cells was detected using CellTiter Aqueous Assay kit (Promega Corp., Madison, WI).

Immunostaining Procedure. Immunohistochemical stainings were performed on 5-μm-thick paraffin-embedded sections or in thyrocyte monolayers. Slides were incubated for 10 min in TBS containing 3% BSA to block unspecific staining. After elimination of excess serum, specimens were exposed for 1 h to specific Abs against Bcl-xL (H-5, mouse IgG1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Bcl-2 (124, mouse IgG1; DAKO Corp., Carpinteria CA), IL-4 (B-S4 mouse IgG1; Caltag Laboratories, Burlingame, CA), IL-10 (B-N10 mouse IgG2a; Caltag Laboratories), IFN-γ (B27, mouse IgG1; Caltag Laboratories), human cytokeratin (AE1/AE3, mouse IgG1; DAKO Corp.), or isotype-matched controls at appropriate dilutions. After two washes in TBS, sections were treated with biotinylated antirabbit or antimouse immunoglobulins, washed in TBS, and incubated in streptavidin peroxidase (DAKO LSAB 2 kit; DAKO Corp.). Staining was detected using 3-amino-9-ethylcarbazole as a colorimetric substrate. Counterstaining of cells and tissue sections was performed using aqueous hematoxylin.

Fig. 1. Resistance to apoptotic cell death induced by chemotherapeutic drugs in thyroid cancer cells. Percentage of cell death in freshly purified thyroid epithelial cells from control, PTC, FTC, and UTC glands exposed for 6, 12, and 24 h to cisplatinum (300 ng/ml), doxorubicin (5 μM), and taxol (5 μM). Data are mean ± SD of four independent experiments, each performed with cells from different donors.

Fig. 2. Expression of antiapoptotic molecules in thyroid cancer. a and b, immunohistochemical analysis of Bcl-xL and Bcl-2 on paraffin sections of control thyroid gland, PTC, FTC, and UTC revealed by 3-aminio-9-ethylcarbazole (red staining). Black arrows indicate Bcl-2 expression on less differentiated areas of UTC. c and d, immunoblot analysis of Bcl-xL and Bcl-2 in freshly purified thyroid cell lysates from control, PTC, FTC, and UTC thyroids. Bcl-xL transgenic hearts and HL-60 cells were used as positive controls. Loading controls were performed by detecting β-actin in the same membrane blot. One representative experiment of four performed with cells from different donors is shown.
**Protein Isolation and Western Blotting.** Cell pellets were resuspended in ice-cold NP40 lysis buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 1% NP40] containing 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (1 μg/ml). Lysates (30 μg/lane) were fractionated on SDS-polyacrylamide gels and blotted to nitrocellulose. Membranes were blocked for 1 h with nonfat dry milk in TBS containing 0.05% Tween 20 and successively incubated for 3 h with Abs specific to actin (Ab-1, mouse IgM; Calbiochem, Darmstadt, Germany), Bcl-2 (124, mouse IgG1; Upstate Biotechnology Inc.), Bcl-xL (H-5, mouse IgG1; Santa Cruz Biotechnology), IL-4 (R107, mouse IgG1; R&D Systems), IL-10 (23738, mouse IgG2b; R&D Systems), and IFN-γ (25723, mouse IgG2b; R&D Systems). After washing, the blots were incubated for 1 h with horseradish peroxidase-conjugated antimouse Ab (Amersham) and visualized using an enhanced chemiluminescence detection system (SuperSignal West Dura Extended Duration Substrate; Pierce Chemical Co., Rockford, IL). Recombinant IL-4, IL-10, and IFN-γ (Euroclone) were used as positive controls.

**Production of Retroviral Particles and Infection of Thyrocytes.** Bcl-2 and Bcl-xL cDNAs were cloned into the PINCO vector (32). The amphotrophic Phoenix packaging cell line was transiently transfected with PINCO using the calcium-phosphate/chloroquine method. Infection was performed by culturing 5 × 10⁵ thyrocytes in 1 ml of 0.45 mM filtered supernatant containing viral particles. Then, cells were centrifuged for 45 min at 1800 rpm and placed back in the CO₂ incubator for 2 h. Three infection cycles were performed before the thyrocytes were placed back in supplemented medium. Sorted and enriched positive cells were plated and exposed to cisplatinum, doxorubicin, and taxol for evaluation of cell death.

**RESULTS**

**Thyroid Cancer Cells Are Resistant to Chemotherapy-induced Cell Death.** To investigate the sensitivity of thyroid carcinomas to conventional chemotherapeutic drugs, we measured the viability of freshly purified thyroid cells exposed to cisplatinum (300 ng/ml), doxorubicin (5 μM), and taxol (5 μM) using dosages equivalent to those reached during cancer treatment. In line with the modest efficacy reported in clinical trials, primary neoplastic cells from all histological variants of thyroid carcinomas showed a scarce sensitivity to the different drugs used (Fig. 1). Thus, the inefficacy of chemotherapy likely results from direct resistance of thyroid cancer cells to cytotoxic drugs.

**Thyroid Cancer Cells Express Bcl-2 and Bcl-xL.** Refractoriness to chemotherapy may result from the activity of antiapoptotic genes. Therefore, we evaluated the expression of a number of relevant antiapoptotic proteins. We found by immunohistochemistry that thyroid carcinoma cells were strongly immunoreactive to Bcl-2 and Bcl-xL (Fig. 2, a and b). To determine more accurately the difference between normal and malignant thyroid cells, freshly purified control and neoplastic cells were lysed and analyzed by immunoblot. We found that Bcl-xL was weakly expressed in control cells but 4- to 5-fold up-regulated in all of the histological cancer variants, whereas Bcl-2 was found ~3-fold higher in FTC cells and 2-fold higher in PTC and UTC cells (Fig. 2, c and d). The levels of Bcl-2 in UTC were not homogeneous, because the more differentiated areas expressed higher amounts of this antiapoptotic protein. The ability of Bcl-xL and Bcl-2 overexpression to protect some cell types against the cytotoxic effect of chemotherapeutic drugs suggests a potential role of these proteins in thyroid cancer resistance to drug-induced cytotoxicity.

**Exogenous Bcl-2 and Bcl-xL Protect Thyrocytes from Cell Death Induced by Chemotherapeutic Agents.** To prove that Bcl-xL and Bcl-2 up-regulation protects thyrocytes from apoptosis induced by chemotherapeutic drugs and may be responsible for cancer cell survival, control thyrocytes were transduced with a retroviral vector (PINCO) carrying the green fluorescent protein as a gene reporter. After infection, thyrocytes transduced with empty vector, Bcl-xL, and Bcl-2 were sorted by flow cytometry and exposed to cisplatinum, doxorubicin, and taxol to evaluate the extent of chemotherapy-induced apoptosis. The infections were monitored by immunoblot analysis to confirm the efficiency of gene delivery (Fig. 3a). Thyrocytes transduced with either Bcl-xL or Bcl-2 were almost completely protected from the cytotoxic effects of the chemotherapeutic agents (Fig. 3b and c), indicating that overexpression of either of the two genes is sufficient to prevent thyroid cell death. Thus, Bcl-xL and Bcl-2 represent likely candidates for mediating refractoriness of thyroid cancer cells to chemotherapy.

**Autocrine Production of IL-4 and IL-10 in Thyroid Cancer Cells.** To investigate whether the tumor microenvironment influences thyroid cancer cell phenotype and function, we evaluated the presence of cytokines previously found to modulate thyrocyte susceptibility to...
apoptosis. All of the histological variants expressed increased immunoreactivity to IL-4 and IL-10, whereas IFN-γ was not detectable (Fig. 4a). Interestingly, the reactivity against T H 2 cytokines localized to thyroid follicles, suggesting that neoplastic thyroid cells were the source of production for both IL-4 and IL-10 (Fig. 4a). To rule out the possibility that the reactivity observed in tumor cells was exclusively caused by the release of T H 2 cytokines by infiltrating T cells, freshly purified thyroid cancer cells were analyzed by immunocytochemistry and immunoblot. As observed with immunohistochemistry, purified thyroid cancer cells were intensely reactive to both IL-4 and IL-10, whereas IFN-γ was not present (Fig. 4, b and c), demonstrating that malignant thyroid cells produce considerable amounts of antiapoptotic T H 2 cytokines.

IL-4 and IL-10 Protect Thyrocytes from Cell Death Induced by Chemotherapeutic Agents. We next investigated whether IL-4 and IL-10 modulate sensitivity to chemotherapy-induced apoptosis and expression of antiapoptotic proteins in thyroid cells. Interestingly, both IL-4 and IL-10 prevented death of normal thyrocytes exposed to cisplatinum, doxorubicin, and taxol (Fig. 5a), suggesting that autocrine production of these cytokines in thyroid cancer cells is responsible for refractoriness to chemotherapy. Furthermore, both IL-4 and IL-10 up-regulated Bcl-xL and Bcl-2 after 48 h of culture (Fig. 5b), whereas IFN-γ was not effective, indicating that autocrine release of IL-4 and IL-10 results in up-regulation of antiapoptotic proteins and subsequent protection of thyroid cancer cells from chemotherapy.

Neutralization of IL-4 and IL-10 Promotes Thyroid Cancer Cells Apoptosis. To formally prove that the production of IL-4 and IL-10 inside the tumor is responsible for up-regulation of antiapoptotic proteins in thyroid cancer cells, we treated follicular carcinoma cells for 2 days with IL-4- and IL-10-neutralizing Abs and then measured Bcl-xL and Bcl-2 expression. The levels of these proteins decreased significantly in thyroid tumor cells exposed to both anti-IL-4 and anti-IL-10 Abs, whereas the blockade of a single cytokine had a lower effect (Fig. 6a). Importantly, a substantial number of thyroid cancer cells underwent apoptosis on neutralization of IL-4 and IL-10, indicating that these cytokines act as survival factors for thyroid cancer (Fig. 6b). After IL-4 and IL-10 blockade, thyroid cancer cell apoptosis was dramatically increased by the exposure to...
chemotherapeutic drugs (Fig. 6c), confirming the hypothesis that cytokine-mediated increase of Bcl-xL and Bcl-2 was responsible for tumor cell resistance to chemotherapy. Similar data were obtained with cells from PTC (data not shown). Although we could not analyze the effect of neutralizing anti-IL-4 and anti-IL-10 Abs on apoptosis sensitization of UTC, down-regulation of Bcl-xL and Bcl-2 in PTC and FTC, through the inhibition of T-helper cytokines, results in spontaneous death of a considerable percentage of thyroid cancer cells and sensitization of the survival tumor population to chemotherapy-induced cytotoxicity.

DISCUSSION

Cancer cell resistance to chemotherapy is a major concern in clinical oncology, resulting in increased tumor growth and decreased patient survival. The ability of cancer cells to resist the cytotoxic effects of chemotherapy follows genetic alterations of the mechanisms that control cell cycle checkpoints and apoptosis (33, 34). Because cytotoxic drugs act by altering the balance between proapoptotic and antiapoptotic signals, inappropriate activation of antiapoptotic genes may result in the development of drug resistance (16).

We found that all of the histological variants of thyroid epithelial cancer are weakly sensitive to chemotherapy and express high levels of Bcl-2 and Bcl-xL. Moreover, exogenous expression of either Bcl-2 or Bcl-xL was sufficient to protect normal thyrocytes from chemotherapy, whereas targeting the increased expression of antiapoptotic proteins sensitized thyroid cancer cells to chemotherapy.

Bcl-2 and Bcl-xL are two antiapoptotic proto-oncogenes localized to the outer mitochondrial membrane, which counteract the propagation of the apoptotic signals generated by a wide array of stimuli,
including chemotherapeutic agents (12, 15–17). Analysis of a considerable number of cancer cell lines has shown a strong negative correlation between basal Bcl-XL levels and sensitivity to chemotherapeutic drugs (35). Furthermore, treatment of certain malignant cells with antisense oligonucleotides or with plasmid constructs overexpressing antisense Bcl-2 or Bcl-XL decreased the rates of proliferation and resistance to cytotoxic chemotherapeutic agents (18–21). Thus, understanding the mechanisms underlying Bcl-2 and Bcl-XL overexpression may allow the development of effective therapeutic strategies.

Whereas in follicular lymphomas Bcl-2 overexpression results from 14;18 translocation, in most cases increased Bcl-2 and Bcl-XL expression in cancer is an indirect event (36). Several cytokines are known to increase Bcl-2 or Bcl-XL expression. IL-3 increases Bcl-XL levels and promotes survival and expansion of myeloid and pro-B cells (37). Similarly, IL-5 and IL-15 activate Bcl-XL transcription and protect eosinophils and mast cells from apoptosis (38, 39), respectively, whereas IL-10 up-regulates Bcl-2 expression and promotes growth and survival of germinal center B cells (40). Moreover, the increased TNP1 profile of T cells in chronic lymphocytic leukemia results in overproduction of IL-4, which in turn induces Bcl-2 overexpression and increased survival of malignant B cells (26). Thus, certain cytokines promote cancer cell survival and expansion by stimulating the expression of antiapoptotic genes.

Here, we have demonstrated that thyroid carcinoma cells produce two antiapoptotic T-helper cytokines that alter sensitivity to cytotoxic drugs. Expression of Bcl-2 and Bcl-XL is decreased after treatment with neutralizing Abs against IL-4 and IL-10, which results in the spontaneous death of a considerable percentage of cancer cells, indicating that these cytokines act as survival factors for thyroid tumor cells and may contribute to the progression of thyroid cancer. Moreover, neutralization of both IL-4 and IL-10 dramatically increased the effectiveness of antineoplastic drugs on thyroid cancer cells. Although we could not test the ability of blocking Abs against IL-4 and IL-10 to promote thyroid cancer cell death, we have shown that while affecting TNP1 antitumor activity by inducing immune deviation or suppressing the immune response, TNP2 and TNP3 cytokines can directly act on target cancer cells by increasing their growth and allowing malignant neoplasia to counteract cytotoxic therapies. This may be significant in devising new therapeutic tools for sensitizing thyroid cancer cells to chemotherapy.

The poor cytotoxic effect of chemotherapy on thyroid cancer has directed most of the nonsurgical therapeutic efforts on radioiodine therapy, which exploits the iodide uptake potential of thyroid follicular cells to deliver iodine-131 to well-differentiated thyroid cancer cells (2, 41). However, a significant number of patients do not respond to radioiodine and have a very poor prognosis (42). The sensitization of thyroid cancer cells to chemotherapy may dramatically improve the survival rate of these patients, whereas targeting the ability of thyroid cancer cells to exploit IL-4 and IL-10 as survival factors may provide a powerful support to alternative treatments.

In conclusion, we provide evidence that IL-4 and IL-10 can directly act on cancer cells by increasing the expression of antiapoptotic proteins, thus promoting tumor cell progression and resistance to chemotherapy. This is clearly observed in thyroid tumors in which IL-4 and IL-10 are produced by epithelial cancer cells. These cytokines likely represent key therapeutic targets for thyroid cancer treatment.

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