Autocrine Production of Interleukin-4 and Interleukin-10 Is Required for Survival and Growth of Thyroid Cancer Cells

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Abstract

Although CD95 and its ligand are expressed in thyroid cancer, the tumor cell mass does not seem to be affected by such expression. We have recently shown that thyroid carcinomas produce interleukin (IL)-4 and IL-10, which promote resistance to chemotherapy through the up-regulation of Bcl-xL. Here, we show that freshly purified thyroid cancer cells were completely refractory to CD95-induced apoptosis despite the consistent expression of Fas-associated death domain and caspase-8. The analysis of potential molecules able to prevent caspase-8 activation in thyroid cancer cells revealed a remarkable up-regulation of cellular $FLIP_L$ ($cFLIP_L$) and PED/PEA-15, two antiapoptotic proteins whose exogenous expression in normal thyrocytes inhibited the death-inducing signaling complex of CD95. Additionally, small interfering RNA FLIP and PED antisense sensitized thyroid cancer cells to CD95-mediated apoptosis. Exposure of normal thyrocytes to IL-4 and IL-10 potently up-regulated cFLIP and PED/PEA-15, suggesting that these cytokines are responsible for thyroid cancer cell resistance to CD95 stimulation. Moreover, treatment with neutralizing antibodies against IL-4 and IL-10 or exogenous expression of suppressor of cytokine signaling-1 of thyroid cancer cells resulted in cFLIP and PED/PEA-15 down-regulation and CD95 sensitization. More importantly, prolonged IL-4 and IL-10 neutralization induced cancer cell growth inhibition and apoptosis, which were prevented by blocking antibodies against CD95 ligand. Altogether, autocrine production of IL-4 and IL-10 neutralizes CD95-generated signals and allows survival and growth of thyroid cancer cells. Thus, IL-4 and IL-10 may represent key targets for the treatment of thyroid cancer. (Cancer Res 2006; 66(3): 1491-9)

Introduction

The balance between cell survival and cell death is controlled by proapoptotic and antiapoptotic factors whose dysregulation contributes to the development of several pathologic conditions, including cancer (1).

CD95 (also known as Fas or Apo-1) is a broadly expressed death receptor involved in many physiologic and pathologic regulatory systems (2). Binding of CD95 ligand to CD95 induces receptor clustering and formation of a death-inducing signaling complex (DISC; ref. 3), which involves the recruitment and activation of caspase-8 through the adaptor molecule Fas-associated death domain (FADD; refs. 4–7). Death receptor signaling can be reduced or neutralized by the presence of high levels of cellular FLIP (cFLIP) or PED/PEA-15, two death receptor inhibitory proteins that prevent caspase-8 activation by interacting with the DED domains of FADD and caspase-8 (2, 8–11).

CD95-mediated apoptosis may play a critical role in tumorigenesis and tumor escape from endogenous growth control mechanisms. Low expression of proapoptotic factors, like CD95 or caspase-8, and high expression of antiapoptotic genes, like *FLIP* or *PED/PEA-15*, have been suggested as selection features acquired by tumor cells to prevent apoptosis induced by death receptors (10–15).

A wide spectrum of malignant tumors originate from thyroid epithelium, such as papillary, follicular, insular, and anaplastic carcinomas. Papillary thyroid carcinoma (PTC) accounts for 85% of differentiated thyroid carcinomas. The remaining epithelial thyroid tumors are predominantly follicular (FTC), whereas the undifferentiated anaplastic carcinomas (UTC) are rare. Thyroidectomy is the primary therapy for all the histologic variants of thyroid epithelial carcinoma, which are resistant to standard chemotherapy (16, 17).

Reportedly, FTC and PTC express both CD95 and CD95L, whereas primary PTC are resistant to CD95-mediated apoptosis (18–20). Although CD95 and its ligand are simultaneously expressed in thyroid cancer cells, such expression does not seem to affect tumor progression, suggesting that a molecular defect in the apoptotic pathway prevents CD95 signaling. The molecular mechanisms responsible for CD95 resistance in thyroid cancer are still unclear.

cFLIP has been shown to be elevated in the human SW579 thyroid cancer cell subline that survived prolonged death receptor stimulation (21). Moreover, in a transgenic mouse model of thyroid follicular cancer, loss of FADD expression impaired CD95 signaling and correlated with tumor development and progression (22). Therefore, refractoriness to CD95-mediated apoptosis may promote thyroid cancer cell survival and contribute to tumor development (20).

A number of cytokines are produced by cancer cells or by the tumor microenvironment. Many studies have addressed the positive or negative effect of different cytokines on the development and progression of cancer (23). It has been reported that interleukin (IL)-4 acts as an autocrine growth factor in pancreatic cancer cells by promoting the activation of AKT-1, signal transducers and activators of transcription (Stat) 3, and mitogen-activated protein kinase (24). In human primary prostate, breast,

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and bladder cancer cells, IL-4 induces up-regulation of cFLIP and Bcl-X_L, which confer resistance to death receptor– and chemotherapeutic drug–induced apoptosis (25).

IL-4 binding to IL-4 receptor α (IL-4Rα) results in tyrosine phosphorylation of several receptor-signal transducers, including Janus family (Jak) tyrosine kinases (Jak1, Jak2, and Jak3), insulin receptor substrate, and Stat6 (26). Jak activity is inhibited by protein-tyrosine phosphatases and by a family of nonenzyme proteins termed suppressor of cytokine signaling (SOCS; ref. 27). Among the prosurvival signals generated by IL-4 are an increased nuclear factor-κB transcriptional activity in prostate cancer cells (28), aberrant activation of Stat3 in glioblastoma multiforme, and Stat6 activation in a variety of cell types. IL-13Rα2, a decoy receptor for IL-13, partially blocks IL-4-mediated activation of Stat6 and promotes phosphorylation of Stat3. These findings indicate that signaling by IL-4 and IL-13 contributes to Stat3 phosphorylation in cancer cells (29).

Another cytokine relevant for thyroid cancer seems to be IL-10. This cytokine is spontaneously secreted by a variety of cancer cells, including melanoma and glioblastoma, where IL-10 has been proposed to promote tumor cell survival, proliferation, and migration (30, 31).

We recently showed that autocrine production of IL-4 and IL-10 in thyroid cancer promotes resistance to cell death induced by chemotherapy through the up-regulation of Bcl-xL and Bcl-2 (23). We speculated that the modulation of apoptosis-related proteins by cytokines was not restricted to chemotherapy resistance but could be involved in the survival and growth of thyroid cancer cells through the blockade of CD95-induced apoptosis. On the basis of our previous results, we therefore investigated the involvement of cFLIP and PED/PEA-15 in the resistance of neoplastic thyrocytes to CD95 stimulation and the effect of IL-4 and IL-10 on cell survival via regulation of apoptosis-related proteins mediated by Jak/Stat signal pathway.

Materials and Methods

Human tissues. Thyroid tissues affected by PTC ($n = 8$; age 45 ± 5), FTC ($n = 8$; age 48 ± 3), and UTC ($n = 4$; age 67 ± 4) were obtained at the time of thyroidectomy, whereas normal thyroid tissue was obtained from the contralateral lobe of thyroid glands surgically removed for a euthyroid nodule ($n = 14$; age 49 ± 8) in accordance with the ethical standards of the institutional committee responsible for human experimentation. Histologic diagnosis was based on the behavioral characteristics of carcinoma cells and nuclear atypia.

Thyroid cell purification. Thyroid tissues from normal, PTC, FTC, and UTC were digested for 2 hours with collagenase (1.5 mg/mL; Life Technologies, Grand Island, NY) and hyaluronidase (20 μg/mL; Sigma Chemical Co., St. Louis, MO) in DMEM as previously described (23). After 12 additional hours of culture, thyroid cells were allowed to grow in monolayer for the immunocytochemistry or detached with trypsin + EDTA following exposure to cytokines for functional and protein expression analyses. Thyrocytes were cultured in the presence or absence of human recombinant IL-4 (20 ng/mL), IL-10 (40 ng/mL), IFN-γ (1,000 IU/mL; Euroclone, Paignton, United Kingdom), anti-human IL-4 (10 μg/mL), and antihuman IL-10 (R&D Systems, Minneapolis, MN) for 48 hours. Anti-CD95 [monoclonal antibody (mAb) CH-11, IgM; Upstate Biotechnology, Inc., Lake Placid, NY] or control IgM (Sigma Chemical) were used to determine sensitivity to CD95-induced apoptosis in thyrocytes. CD95L mAb (Nok-1, mouse IgG1, PharMingen, San Diego, CA) was used to block apoptosis induced by CD95.

Survival and death assays. Apoptotic events among neoplastic thyrocytes were evaluated by DNA staining and flow cytometry analysis. The percentage of hypodiploid nuclei was evaluated as previously described (32). Additionally, DNA staining was evaluated by immunofluorescence analysis of green fluorescent protein (GFP)–positive cells stained with ethidium bromide. Alternatively, freshly purified thyrocytes were plated in 96-well plates in triplicate at 15,000 per well and cultured. The number of viable cells was detected by CellTiter Aqueous Assay kit (Promega Corporation, Madison, WI) following the instructions of the manufacturer. HuT78 cells were plated at 2×10^5/mL and treated with CD95-activating antibody, CH-11 (200 ng/mL), and were used as a positive control for cell death measurement.

Immunohistochemistry. Immunohistochemical stainings were done on 5-μm-thick paraffin-embedded thyroid sections. Before immunostaining, dewaxed sections were treated for 10 minutes in microwave oven in 0.1 mol/L citrate buffer. Deparaffinized sections were incubated for 10 minutes with TBS containing 3% bovine serum albumin (BSA) to block unspecific staining. Following elimination of excess serum, specimens were exposed for 1 hour to specific antibodies against CD95 (apo-1, mouse IgG1, kindly provided by P. Krammer, Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center, Heidelberg, Germany), IL-4 (B-S4 mouse IgG1, Caltag Laboratories, Burlingame, CA), IL-10 (B-N10 mouse $IgG2_a$, Caltag Laboratories), IFN-γ (B27, mouse IgG1, Caltag Laboratories), IL-4Rα (C-20 rabbit IgG, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), IL-10R (C-20 rabbit IgG Santa Cruz Biotechnology), PED/PEA-15 (rabbit IgG, kindly provided by G. Condorelli), or isotype-matched controls at appropriate dilutions. cFLIP expression was done on freshly purified thyrocytes fixed in 2% paraformaldehyde in PBS as follows. After two washes in PBS, cells were permeabilized in PBS containing 0.1% Triton X-100. Cells were then exposed for 1 hour at 37°C to anti-cFLIP (rabbit polyclonal IgG, Upstate Biotechnology) opportunely diluted in 3% BSA and 0.05% Tween 20 in PBS. Following exposure to primary antibody, cells were treated with biotinylated anti-rabbit or anti-mouse immunoglobulins, washed in PBS, and then incubated with streptavidin peroxidase (Dako LSAB 2 kit, Dako Corporation Carpinteria, CA). Staining was detected using 3-amino-9-ethylcarbazole (AEC) as a colorimetric substrate. Counterstaining of cells was done using aqueous hematoxylin. Caspase-8 (8CSP03, mouse IgG1, Lab Vision Corporation, Westinghouse, CA) and FADD (N-18, goat polyclonal IgG, Santa Cruz Biotechnology) expression was detected by immunofluorescence in purified thyroid cells. Cells were fixed and permeabilized as described above. Following exposure to primary antibody, cells were treated with fluorescein-conjugated anti-mouse or anti-goat antibodies (Molecular Probes, Inc., Eugene, OR). Counterstaining of cells was done using Hoechst.

Terminal deoxynucleotidyl transferase–mediated nick end labeling. Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) reaction was done on paraffin-embedded CD95L-labeled thyroid sections (6 μm). Specimens were postfixed with paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. Apoptosis was determined by *In Situ* Cell Death Detection, AP kit (Boehringer Mannheim, Indianapolis, IN). DNA strand breaks were detected by 5-bromo-4-chloro-3-indolyl-phosphate (Dako) substrate. Positive control was done by pretreating specimens with DNase I (1 μg/mL) to introduce nonspecific strand breaks, whereas the negative control was represented by CD95L-positive specimens subjected to the same staining for TUNEL without terminal deoxynucleotidyl transferase.

Western blotting. Cell pellets were resuspended in ice-cold NP40 lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EGTA, and 1% NP40] containing protease inhibitors as previously described (33). Immunoblotting of antibodies specific for actin (Ab-1, mouse IgM, Calbiochem, Darmstadt, Germany), CD95L (G247-4, mouse IgG1, PharMingen), CD95 (C-20, Santa Cruz Biotechnology), cFLIP (NF6 mouse IgG1, Alexis Biochemicals, Lausen, Switzerland), PED/PEA-15 (rabbit IgG kindly provided by G. Condorelli), IL-4R (MAB304, mouse IgG1, R&D Systems), IL-10R (MAB217, mouse IgG2B, R&D Systems, Inc.), FADD (1, mouse IgG1, PharMingen), caspase-8 (5F7, mouse monoclonal IgG2b, Upstate Biotechnology), phospho-Jak1 ($Tyr^{1022/1023}$, rabbit polyclonal, Cell Signaling Technology, Inc., Beverly, MA), phospho-Stat6 (Tyr^{641}, rabbit polyclonal, Cell Signaling Technology), and SOCS-1 (C-20, goat polyclonal, Santa Cruz Biotechnology) was detected by

horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies (Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, United Kingdom) and visualized with the chemiluminescence detection system (SuperSignal West Dura Extended duration Substrate, Pierce Biotechnology, Inc., Rockford, IL).

Production of retroviral particles and infection of thyrocytes. $cFLIP_L$, PED/PEA-15 cDNAs were cloned in PINCO vector (23, 33). The amphotropic packaging cell line Phoenix was transfected by standard calcium phosphate/chloroquine method, and culture supernatants containing retroviral particles were collected 48 hours after transfection. Infection was done by culturing thrice 5×10^5 thyrocytes in 1 mL of 0.45 mmol/L filtered supernatant containing viral particles. Gene transfer efficiency was evaluated by flow cytometry analysis based on the expression of the GFP reporter. The levels of cFLIP, PED/PEA-15 expression were evaluated by immunoblot analysis using lysates of primary thyroid cancer cells for comparison.

SOCS1 and PED/PEA-15 antisense were cloned in a new lentiviral vector (Tween) generated by engineering pRRLsin.cPPT.hCMV.GFP.Wpre. In this vector, the hCMV.GFP cassette was substituted with the hCMV.hPGK.GFP (34). PED/PEA-15 antisense (34) and SOCS-1 (kindly provided by Dr. A. Yoshimura, Department of Infectious Diseases, University of Miyazaki, Japan; ref. 35) were subcloned in the *Xba*I and in the *Eco*RV sites of the Tween vector, respectively. Lentiviral supernatants were produced by calcium phosphate transient trasfection in the packaging human embryonic kidney cell line 293T (34). Viral supernatants were collected and exposed to cells as described above for the retroviral procedure.

FLIP small interfering RNA (siRNA) is a target-specific 20 to 25 nucleotides along with its corresponding sense RNA oligonucleotides, whereas scrambled is a control siRNA nontargeting 20 to 25 nucleotides (Santa Cruz Biotechnology). These RNAs were dissolved in dilution buffer according to the instructions of the manufacturer. Thyroid cancer cells were transfected by LipofectAMINE 2000 (Invitrogen, Life Technologies, Rockville, MD). FLIP siRNA (40 nmol/L) or scrambled oligonucleotide was complexed in serum-free media with LipofectAMINE. After 20 minutes, the complexes were added to thyroid cancer cells or PED antisense-transfected cells and cultured for 48 hours. Transfection was monitored by immunoblot analysis.

Results

Refractoriness of thyroid carcinoma cells to CD95-mediated apoptosis. Primary PTC cells are resistant to CD95-induced apoptosis (18). To determine the sensitivity of less-differentiated tumors, we investigated CD95 sensitivity in FTC and UTC cells. Primary neoplastic cells from all histologic variants of thyroid carcinomas showed similar resistance to CD95-mediated apoptosis (Fig. 1*A*). To investigate whether such a general resistance to CD95-mediated apoptosis paralleled with the expression of the ligand in all the different variants, we analyzed CD95 and CD95L expression in thyroid cancer cells. Immunohistochemical and immunoblot analyses showed that CD95 was expressed in all the histologic variants of thyroid cancinomas (Fig. 1*B-C*), whereas immunoblot analysis of freshly purified neoplastic thyroid cancer cells revealed that CD95L was highly expressed in both differentiated and undifferentiated forms, as shown by the comparison with transgenic cardiomyocytes overexpressing CD95L (Fig. 1*D*). We then

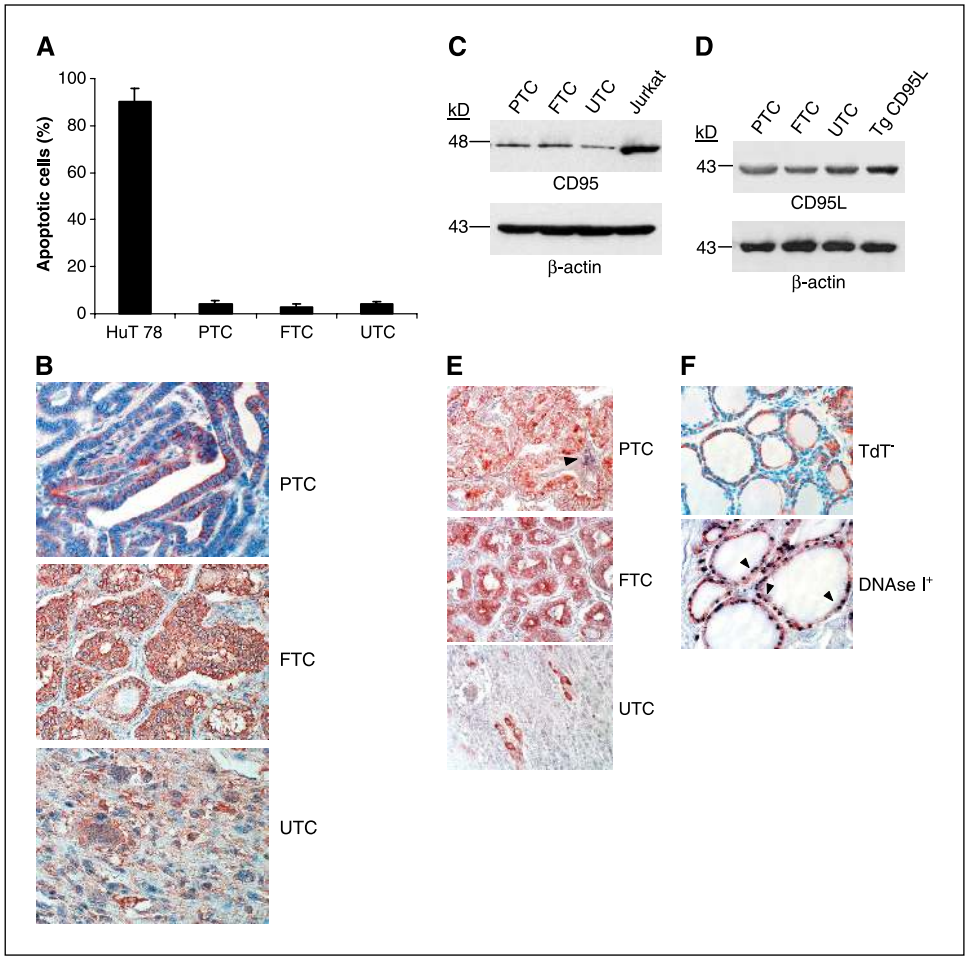

Figure 1. Resistance to CD95-induced apoptosis and CD95/CD95L expression in thyroid carcinoma cells. *A,* percentage of apoptotic cells in freshly purified thyroid epithelial cells from PTC, FTC, and UTC exposed for 24 hours to CD95 (CH11; 200 ng/mL). The HuT78 lymphoma cell line was used as positive control. *Columns,* mean of three independent experiments; *bars,* SD. *B,* immunohistochemical analysis of CD95 on paraffin sections of PTC, FTC, and UTC thyroid glands revealed by AEC (*red staining*). *C* and *D,* immunoblot analyses of CD95 and CD95L in freshly purified thyroid cell lysates from PTC, FTC, and UTC. Jurkat cells and CD95L transgenic heart (*Tg CD95L*) were used as positive control. Loading controls were done by detecting β-actin in the same membrane blots. *E, in situ* TUNEL detection in CD95L-labeled paraffin-embedded sections from PTC, FTC, and UTC thyroids. Apoptotic nuclei are stained in dark blue. *F,* negative and positive control for the detection of DNA breaks by TUNEL experiment (*dark blue*) done on normal thyroid paraffin-embedded sections. *Black arrowheads,* TUNEL-positive cells. One representative experiment of four done with cells from different donors.

measured the rate of apoptosis *in vivo* in thyroid cancer. Double-labeled immunohistochemistry for CD95L staining and TUNEL reaction revealed the absence of apoptotic thyrocytes in thyroid carcinomas (Fig. 1*E*). Negative and positive controls are shown in Fig. 1*F*. Thus, although both CD95 and CD95L are expressed in thyroid carcinoma cells, CD95 does not seem to be active *in vitro* or *in vivo*.

Thyroid cancer cells express cFLIP$_L$ and PED/PEA-15. To explore the mechanism responsible for CD95 refractoriness, we investigated whether an altered expression of key factors of the proximal CD95 pathway could be involved in impaired transmission of the apoptotic signal. Therefore, we evaluated the presence and measured the expression levels of FADD, caspase-8, cFLIP, and PED/PEA-15 in normal and neoplastic thyroid cells. Whereas caspase-8 and FADD expression levels were comparable in both normal and cancer thyrocytes (Fig. 2*A-C*), cFLIP$_L$ and PED/PEA-15 levels were ~3-fold higher in freshly purified thyroid neoplastic cells compared with normal thyrocytes (Fig. 2*D-G*). In contrast, cFLIP$_S$ was undetectable in both freshly purified normal and thyroid neoplastic cells (Fig. 2*E*). Behcet's disease resting T lymphocytes (36) and MCF-7 cells were used as positive controls. Thus, cFLIP$_L$ and PED/PEA-15 up-regulation in thyroid cancer cells may confer resistance to CD95-induced apoptosis.

Exogenous cFLIP or PED/PEA-15 expression confers protection from CD95-induced apoptosis in normal thyrocytes. To investigate the ability of cFLIP or PED/PEA-15 up-regulation to

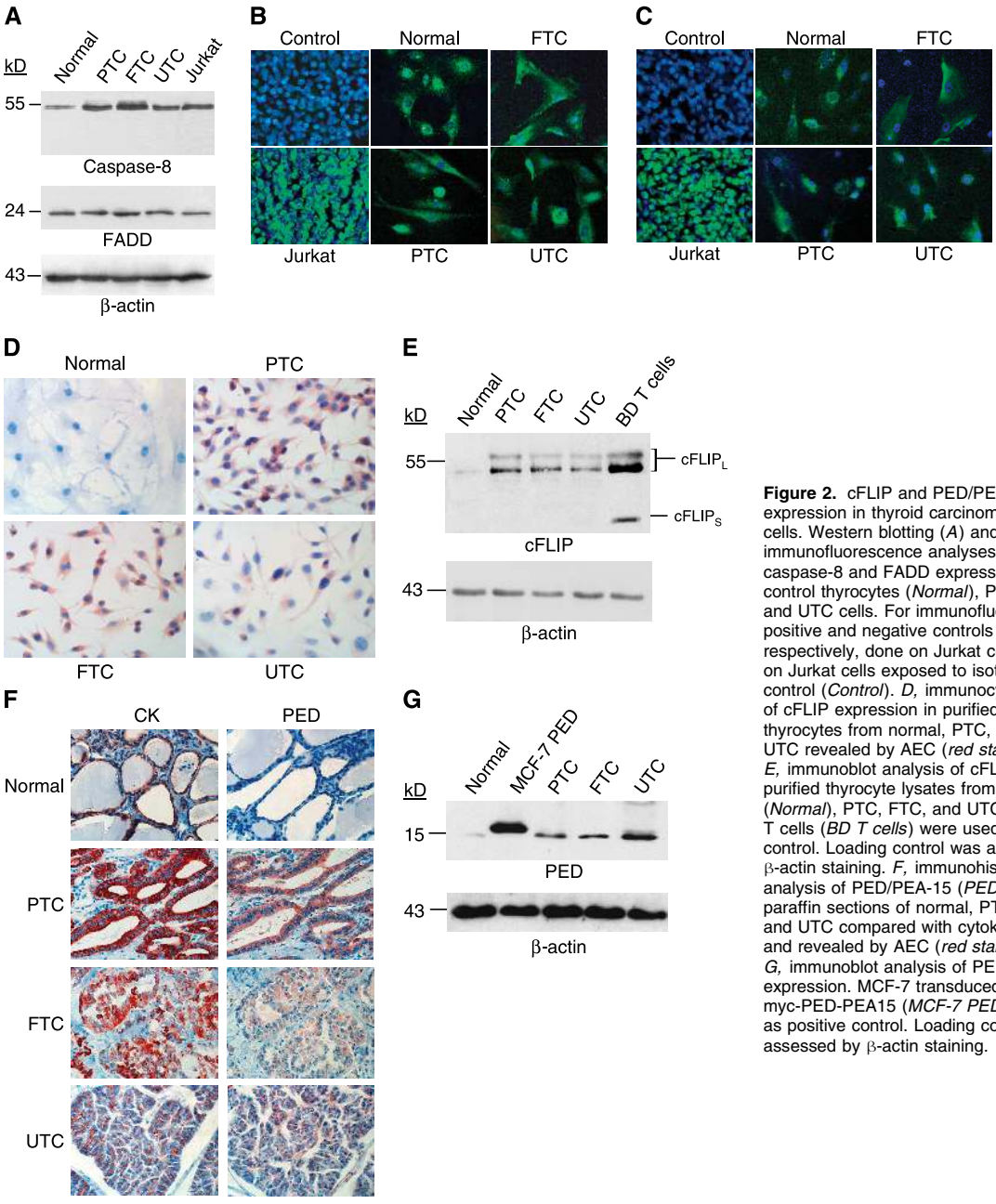

Figure 2. cFLIP and PED/PEA-15 expression in thyroid carcinoma cells. Western blotting (*A*) and immunofluorescence analyses (*B* and *C*) of caspase-8 and FADD expression in primary control thyrocytes (*Normal*), PTC, FTC, and UTC cells. For immunofluorescence, positive and negative controls were, respectively, done on Jurkat cells and on Jurkat cells exposed to isotype-matched control (*Control*). *D,* immunocytochemistry of cFLIP expression in purified thyrocytes from normal, PTC, FTC, and UTC revealed by AEC (*red staining*). *E,* immunoblot analysis of cFLIP in freshly purified thyrocyte lysates from control (*Normal*), PTC, FTC, and UTC. BD resting T cells (*BD T cells*) were used as positive control. Loading control was assessed by β-actin staining. *F,* immunohistochemical analysis of PED/PEA-15 (*PED*) on serial paraffin sections of normal, PTC, FTC, and UTC compared with cytokeratin (*CK*) and revealed by AEC (*red staining*). *G,* immunoblot analysis of PED/PEA-15 expression. MCF-7 transduced with myc-PED-PEA15 (*MCF-7 PED*) were used as positive control. Loading control was assessed by β-actin staining.

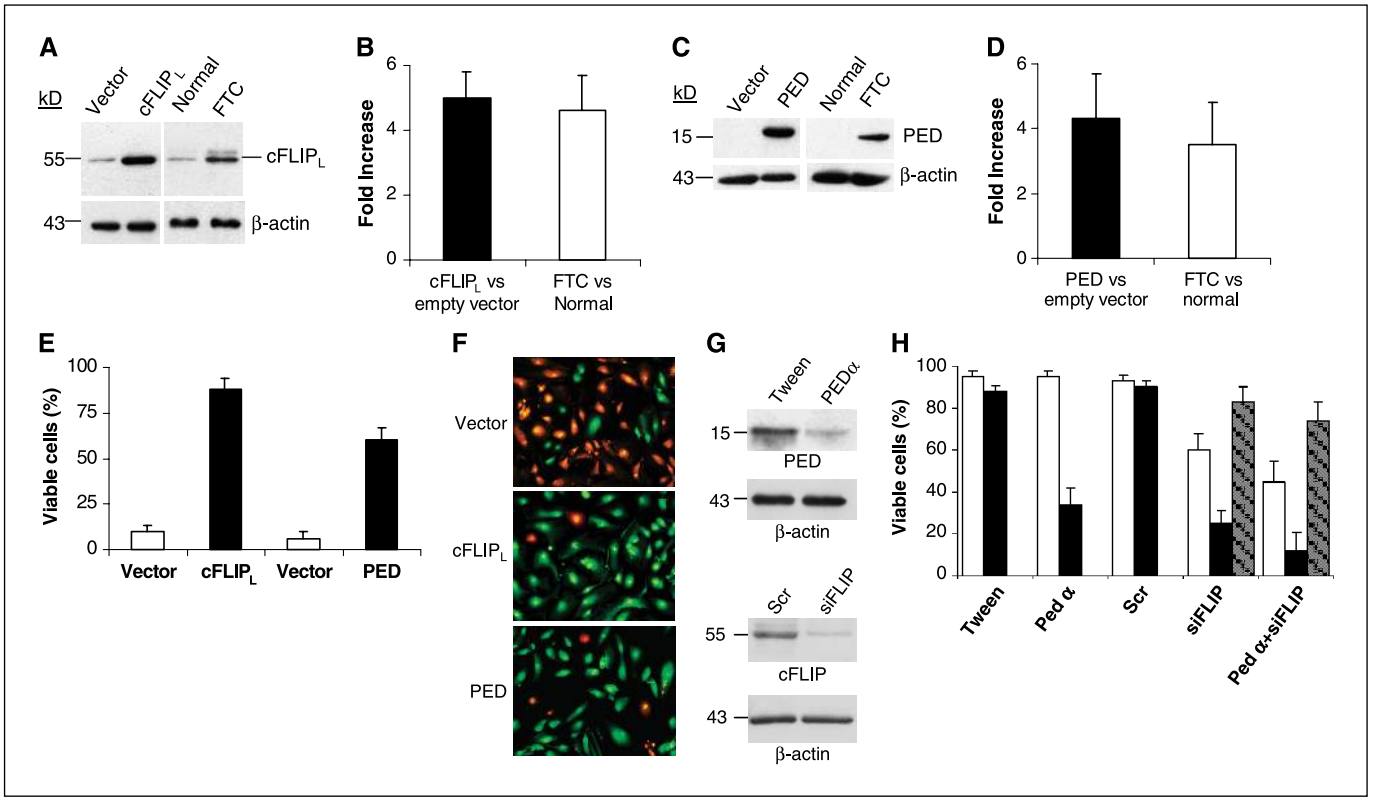

Figure 3. Exogenous cFLIP$_L$ and PED/PEA-15 expression prevents CD95-induced apoptosis in thyroid cells. *A* and *C,* immunoblot analysis of cFLIP and PED/PEA-15 expression in normal thyrocytes transduced with empty PINCO vector (*Vector*), cFLIP, or PED compared with normal cells and FTC-nontransduced thyrocytes. Loading controls were done on the same membrane blot detected for β-actin. *B* and *D,* comparison of cFLIP$_L$ or PED expression levels in normal versus FTC thyrocytes transduced as in (*A* and *C*). Data show fold increase of cFLIP$_L$ or PED in normal versus *FTC* gene–transduced thyrocytes compared with empty vector–transduced thyrocytes (*black columns*) or in FTC versus normal thyrocytes (*white columns*). *E,* percentage of viable cells in normal thyrocytes transduced with empty vector or cFLIP$_L$ or PED and exposed for 24 hours to CD95 agonistic mAb (CH11; 200 ng/mL) after 48 hours of IFN-γ (1,000 IU/mL) pretreatment. Viability was analyzed on propidium iodide–stained cells using flow cytometry and by CellTiter Aqueous Assay kit. *F,* immunofluorescence analysis of GFP-positive monolayer cells treated as in (*E*) and stained with ethidium bromide. *G,* immunoblot analysis of cFLIP and PED on scrambled (*Scr*) or empty vector (*Tween*) and PED antisense cDNA (*PEDα*) or siRNA FLIP (*siFLIP*) transfected FTC cells. Loading control was done by β-actin staining. *H,* percentage of cell death in FTC thyrocytes transduced with empty vector, PED antisense cDNA, scrambled oligos, or siRNA FLIP, and exposed for 24 hours to CD95 agonistic mAb as in (*E*) or to neutralizing antibodies against CD95L (*Nok-1*). Representative of three independent experiments. □, w/o; ■, anti-CD95; ▨, NOK-1.

promote the resistance of neoplastic thyrocytes to CD95-induced apoptosis, normal thyrocytes were transduced with cFLIP or PED/PEA-15 using a retroviral vector carrying the *GFP* as a reporter gene. Retroviral infections were monitored by immunoblot analysis to confirm the efficiency of gene delivery (Fig. 3*A* and *C*). *FLIP* and *PED/PEA-15* gene expression levels in transduced thyrocytes were comparable with those observed in primary FTC thyrocytes (Fig. 3*B* and *D*). Despite the presence of IFN-γ, which sensitizes thyrocytes to CD95-induced apoptosis, control thyrocytes transduced with cFLIP or PED/PEA-15 acquired a considerable resistance to CD95 stimulation (Fig. 3*E-F*), indicating that these DISC inhibitory proteins are functionally active in thyroid cells.

Accordingly, the down-regulation of either cFLIP or PED in FTC cells, obtained with FLIP siRNA or PED/PEA-15 antisense (Fig. 3*G*), resulted in considerable CD95 sensitization (Fig. 3*H*). Importantly, the simultaneous targeting of both cFLIP or PED resulted in FTC cell apoptosis that was significantly prevented by CD95L neutralization (Fig. 3*H*), indicating that cFLIP and PED/PEA-15 up-regulation protects thyroid cancer cells from constitutive triggering of CD95.

Autocrine production of IL-4 and IL-10 protects neoplastic thyrocytes from CD95-induced apoptosis. We previously showed that thyroid cancer cells from all the histologic variants produce considerable amounts of IL-4 and IL-10 (23). Before analyzing the functional effects of these cytokines on thyroid cancer, we evaluated the expression of cytokine receptors in normal and neoplastic thyroid samples. Immunohistochemistry on paraffin-embedded sections and immunoblot analysis on freshly purified cells showed that IL-4 and IL-10 receptors were expressed in thyroid cells from both normal and cancer tissues (Fig. 4*A-B*). To investigate the possible involvement of IL-4 and IL-10 on CD95 resistance in thyroid cancer, normal thyrocytes were exposed to IL-4 or IL-10 and analyzed for expression of those DISC inhibitory proteins that were up-regulated in thyroid cancer. Both IL-4 and IL-10 considerably increased cFLIP and PED/PEA-15 (Fig. 4*C*) in thyrocytes, suggesting that autocrine/paracrine IL-4 and IL-10 production may protect thyroid cancer cells from death receptor stimulation through the up-regulation of cFLIP and PED/PEA-15.

SOCS-1 down-regulates cFLIP$_L$ and PED/PEA-15 expression levels and sensitizes thyroid cancer cells to CD95-induced apoptosis. To determine whether IL-4 and IL-10 protect thyroid cancer cells through the activation of the Jak/Stat pathway, we measured the activation of Jak1 and Stat6 in FTC cells. Interestingly, the levels of phosphorylated Jak1 and Stat6 were found to be 4-fold higher in purified FTC cells compared with normal

thyrocytes (Fig. 5*A-B*), indicating that the Jak/Stat pathway is constitutively activated in thyroid cancer cells.

Cytokine-mediated Jak-Stat signaling is negatively regulated at multiple levels by a number of molecules, including SOCS family members. To determine if the constitutive activation of Jak1/Stat6 is involved in up-regulation of cFLIP and PED/PEA-15 in thyroid cancer cells, we evaluated the functional effect of SOCS-1 expression in FTC cells. SOCS-1 lentiviral infection was monitored by immunoblot analysis to validate the efficiency of gene transfer (Fig. 5*C*). Exogenous expression of SOCS-1 significantly reduced the expression of both cFLIP and PED/PEA-15 (Fig. 5*C*) in FTC cells, which became sensitive to CD95-induced apoptosis (Fig. 5*D-E*). These findings suggest that the constitutive activation of the Jak/Stat pathway is responsible for cFLIP and PED/PEA-15 up-regulation and resistance to CD95 stimulation in thyroid cancer.

IL-4 and IL-10 neutralization promotes CD95-mediated apoptosis in thyroid cancer cells. To directly show that autocrine/paracrine production of IL-4 and IL-10 protects thyroid cancer cells from the deadly interaction of CD95 and CD95 ligand, we investigated the effects of IL-4 and IL-10 neutralization.

The combined treatment of primary FTC cells with neutralizing antibodies against IL-4 and IL-10 for 2 days dramatically reduced the expression of cFLIP or PED/PEA-15, whereas the single antibodies showed a modest effect (Fig. 6*A-B*), indicating that both cytokines promote the up-regulation of these antiapoptotic proteins in thyroid cancer.

To determine the direct contribution of IL-4- and IL-10-induced cFLIP and PED/PEA-15 up-regulation in thyroid cancer cell resistance to CD95 stimulation, thyroid cancer cells were pretreated for 2 days with neutralizing antibodies against IL-4 and IL-10 and stimulated with anti-CD95 for 24 hours. In line with the ability to down-regulate cFLIP and PED/PEA-15, the combined neutralization of IL-4 and IL-10 sensitized thyroid cancer cells to CD95-induced apoptosis (Fig. 6*C*), confirming the antiapoptotic role of these cytokines in thyroid cancer. These data suggest that the simultaneous expression of CD95 and CD95 ligand in thyroid cancer cells is counterbalanced by cytokine-mediated neutralization of the DISC. Accordingly, the rate of apoptosis induced by IL-4 and IL-10 neutralization in thyroid cancer was massively reduced by the treatment with an antibody that prevented the binding of CD95 ligand to CD95 (Fig. 6*D*). Furthermore, a prolonged analysis of tumor cell growth indicated that IL-4 and IL-10 neutralization extinguishes the tumor cells in 2 weeks through the interaction between CD95 and CD95 ligand as indicated by the ability of CD95 ligand blockade to considerably restore tumor cell growth (Fig. 6*E*). These data indicate that autocrine/paracrine production of IL-4 and IL-10 is required for survival and growth of thyroid cancer cells, which in the absence of these cytokines are committed to die through the interaction of CD95 and CD95 ligand.

Discussion

Tumorigenesis is a pathologic developmental process characterized by the prevalence of oncogenic events over physiologic tumor suppression mechanisms. Alterations in the apoptotic machinery in cells prone to neoplastic transformation contribute to tumor formation and development (37, 38).

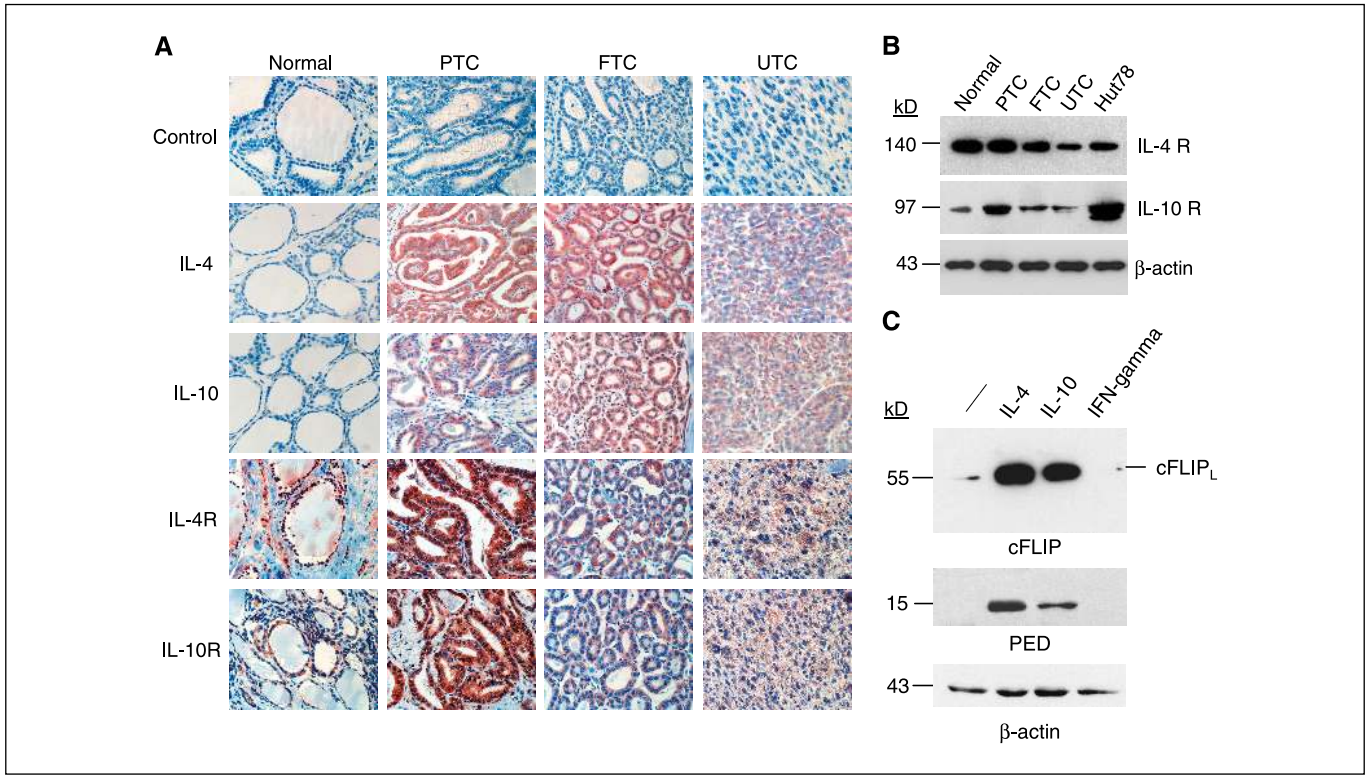

Figure 4. IL-4 and IL-10 up-regulate cFLIP$_L$ and PED/PEA-15 expression levels in thyroid cells. *A,* immunohistochemical analysis of IL-4, IL-10, IL-4R, and IL-10R on paraffin-embedded sections from normal, PTC, FTC, and UTC thyroids (*red staining*). *B,* Western blotting analysis of IL-4R and IL-10R in freshly purified thyroid cells from control (*normal*), PTC, FTC, and UTC thyroid glands. HuT78 cells were used as positive control. Loading control was done by detecting β-actin. *C,* immunoblot analysis of FLIP or PED in normal thyrocytes treated with 20 ng/mL IL-4, 40 ng/mL IL-10, or 1,000 IU/mL IFN-γ for 48 hours. β-actin detection was used as loading control. One representative of three independent experiments.

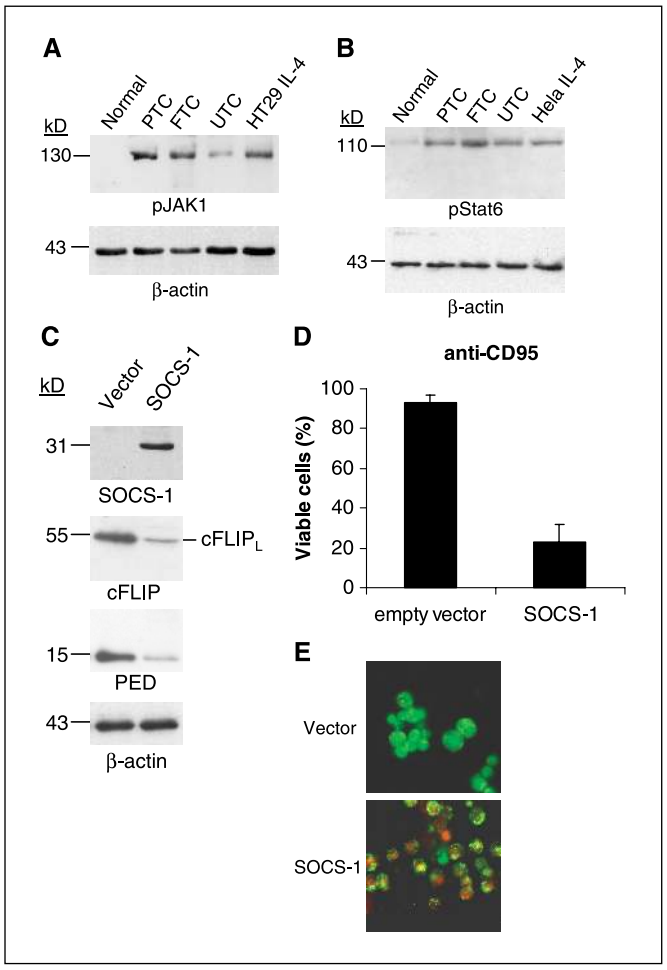

Figure 5. Exogenous expression of SOCS-1 sensitizes thyroid carcinoma cells to CD95-mediated apoptosis. *A* and *B,* immunoblot analysis of pJak1 and pStat6 in freshly purified thyrocyte lysates from control (*Normal*), PTC, FTC, and UTC. HT29 and HeLa cells treated with 20 ng/mL of IL-4 for 10 minutes (HT29 IL-4; HeLa IL-4) were used as positive control. Loading control was assessed by β-actin staining. *C,* Western blotting analysis of SOCS-1, cFLIP, and PED in FTC cells transduced with empty vector (*Vector*) or SOCS-1. Loading control was done by β-actin staining. *D,* percentage of viable cells in FTC cells transduced with empty vector or SOCS-1 and exposed for 24 hours to CH11. *E,* immunofluorescence analysis of GFP-positive suspended cells treated as in (*D*) and stained with ethidium bromide.

Several molecular alterations may be responsible for the neoplastic transformation of thyroid follicular cells. Death receptor signaling plays a key role in nonneoplastic thyroid diseases. The interaction between CD95 and its ligand promotes thyrocyte destruction in the presence of proinflammatory cytokines, such as IFN-γ, IL-1β, and tumor necrosis factor-α. In contrast, IL-4 and IL-10 antagonize death receptor signaling through the up-regulation of antiapoptotic proteins acting at the DISC and at the mitochondrial level along the apoptotic pathway. As a consequence, thyrocytes are killed by autocrine production of CD95 and CD95L during the inflammatory response in Hashimoto's thyroiditis, whereas they are protected by the presence of IL-4 and IL-10 during the early phases of Graves'disease (33, 39, 40).

This complex network of cytokines and receptors seems particularly relevant in thyroid cancer. Our data show that all the histologic variants of thyroid carcinomas express CD95 and CD95L, and are resistant to CD95-induced apoptosis. Although loss of FADD expression correlated with tumor development and progression in a transgenic mouse model of thyroid follicular cancer, we did not observe an altered expression of the proapoptotic components required for CD95 signaling. Instead, our data clearly indicate that refractoriness to CD95-induced apoptosis in thyroid cancer cells results from high expression of cFLIP and PED/PEA-15, which inhibit the "extrinsic" apoptotic pathway by preventing the interaction between the adaptor molecule FADD and procaspase-8. Thus, as in other tumors, cFLIP and PED/PEA-15 represent two major candidates for cancer cell resistance to death receptor stimulation.

We previously showed that thyroid cancer cells produce high levels of IL-4 and IL-10. Thyroid cell production of IL-4 and IL-10 is acquired during neoplastic transformation; in normal thyroid tissue, we detect only IL 4 and IL-10 receptors but not the two

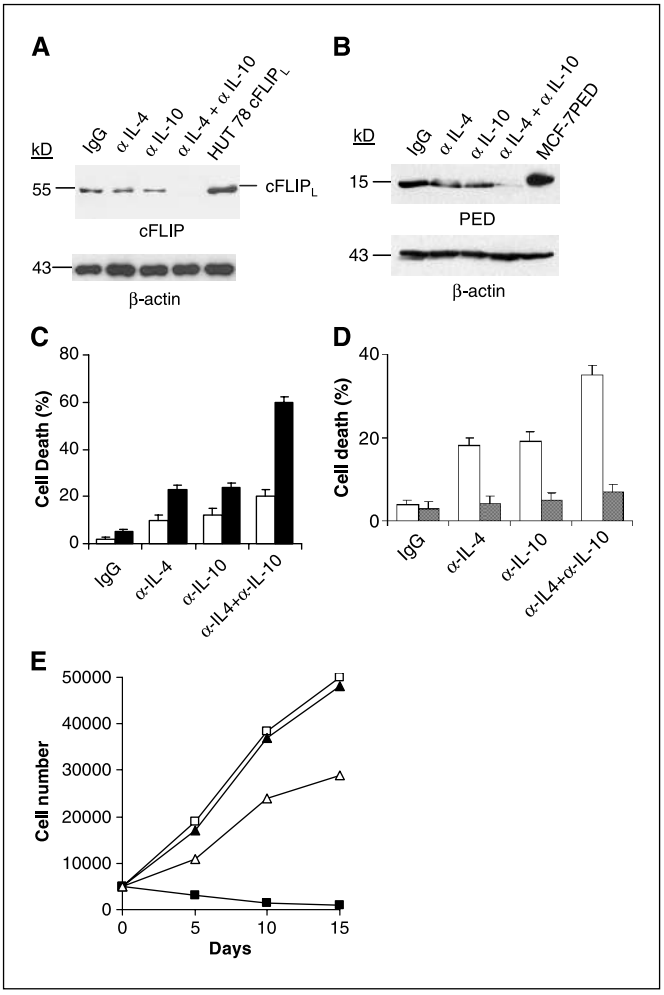

Figure 6. Synergistic effect of neutralizing antibodies against IL-4 and IL-10 on CD95-mediated apoptosis in thyroid carcinoma cells. *A* and *B,* immunoblot analysis of cFLIP or PED on FTC cells treated with 10 μg/mL of control IgG (*IgG*), anti-IL-4, anti-IL-10, or a combination of anti-IL-4 and anti IL-10 for 48 hours. HuT78 FLIP and MCF-7 PED cells were used as positive controls. Loading controls were done by detecting β-actin on the same membrane. *C,* cell death percentage of purified of FTC cells pretreated as in (*A*) and then exposed for 24 additional hours to CD95 mAb (CH11). □, Control; ■, anti-CD95. *D,* cell death percentage of cells treated for 96 hours with neutralizing IL-4 and IL-10 antibodies in the absence (*Control*) or presence of 2 μg/mL blocking CD95L mAb (*Nok-1*). *Columns,* mean of four independent experiments; *bars,* SD. □, Control;▨, NOK-1. *E,* effects of neutralizing antibodies against CD95L (Nok-1), IL-4, and IL-10 on FTC cell growth. Representative of three independent experiments. —□—, Control; —▲—, NOK-1; —△—, NOK-1 + anti-IL-4 + anti-IL10; —■—, anti-IL-4 + anti-IL10.

cytokines. The ability of IL-4 and IL-10 to up-regulate cFLIP and PED/PEA-15 in normal thyrocytes, together with the dramatic down-regulation of both proteins observed in thyroid cancer cells after exposure to IL-4 and IL-10 neutralizing antibodies, clearly indicates that high expression of cFLIP and PED/PEA-15 in thyroid cancer results from autocrine production of IL-4 and IL-10.

Three functionally distinct subsets of T-helper cells have been characterized on the basis of cytokine production (41). T_H1 cells produce IL-2, IFN-γ, and other cytokines, which are critical to cell-mediated immunity, whereas T_H2 cells produce IL-4, IL-5, and IL-10, promoting antibody production and the humoral immunity. In contrast, T_H3 cells release IL-10 and transforming growth factor β-1, which inhibit the immune response (42–44).

Increased IL-4 production as part of a general Th2 cytokine profile has been reported to occur during progressive tumor growth in murine models of renal cell carcinoma and adenocarcinoma, suggesting that a shift from Th1 to Th2 response could be responsible for cancer development and spreading. Accordingly, we have shown that IL-4 and IL-10 can promote cancer cell survival in response to therapeutic drugs and immune response through the up-regulation of antiapoptotic proteins (23).

In B-cell chronic lymphocytic leukemia, increased IL-4 production prevents spontaneous and hydrocortisone-induced apoptosis of leukemia cells by increasing Bcl-2 expression (45). IL-4 has raised the interest of oncologists because cells from a variety of epithelial malignancies express IL-4Rα. It has been additionally shown that both Stat6 and IRS-2 are required for the mitogenic response of T lymphocytes to IL-4. Interestingly, in the absence of both IRS-2 and Stat6, IL-4 is still a potent antiapoptotic agent even in the absence of IL-4-induced Bcl-2 expression and Akt activation (46, 47).

Here, we showed that autocrine IL-4 and IL-10 production in thyroid cancer results in constitutive activation of the Jak/Stat pathway that promotes the up-regulation of $cFLIP_L$ and PED and refractoriness to CD95 stimulation. As a result, IL-4 and IL-10 secretion neutralizes the interaction of CD95L with CD95 that occurs in thyroid cancer as a possible attempt to counterbalance tumor transformation. Thus, the presence of IL-4 and IL-10 in the thyroid cancer microenvironment contribute to thyroid cancer cell survival and proliferation. These findings may have important implications for both the pathogenesis and the therapy of thyroid cancer.

A variety of chemotherapeutic drugs have been investigated for the treatment of thyroid cancer. However, they have shown a very limited efficacy because of the high chemoresistance of thyroid cancer cells that remains a critical obstacle for the treatment of less-differentiated tumors unable to uptake radio-iodine. We showed that IL-4 and IL-10 neutralization is able to extinguish thyroid cancer cells within 2 weeks of culture. The possibility to antagonize these cytokines or their receptors could be exploited for therapeutic purposes to induce tumor regression or sensitization to death receptor stimulation or chemotherapeutic drugs.

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