Fas Signaling in Thyroid Carcinomas Is Diverted from Apoptosis to Proliferation

Constantine S. Mitsiades,1,2 Vassiliki Poulaki,3 Galinos Fanourakis,1,2,4 Elias Sozopoulos,4 Douglas McMillin,1 Zhaoqin Wen,1 Gerassimos Voutsinas,8 Sophia Tseleni-Balafouta,4 and Nicholas Mitsiades1

Abstract

Purpose: The death receptor Fas is present in thyroid carcinomas, yet fails to trigger apoptosis. Interestingly, Fas has been reported to be actually overexpressed in papillary thyroid carcinomas, suggesting that it may confer a survival advantage.

Experimental Design: We investigated the expression and activation status of Fas pathway mediators in thyroid carcinoma cell lines and tumor specimens.

Results: All cell lines tested express Fas-associated death domain, procaspase-8, procaspase-9, and procaspase-3; resistance to Fas-mediated apoptosis could not be attributed to lack of any of these apoptosis mediators. Moreover, Fas death domain mutations were not found in our study. The proteasome inhibitors MG132 and PS-341 (bortezomib, Velcade), which lead to accumulation of the nuclear factor-κB (NF-κB) inhibitor IκB, did not sensitize SW579 cells to Fas-mediated apoptosis, suggesting that resistance to Fas-mediated apoptosis is not due to proteasome or NF-κB activity. Cross-linking of Fas in vitro induced recruitment of Fas-associated death domain—like interleukin-1β-converting enzyme inhibitory protein (FLIP) instead of procaspase-8. Inhibition of FLIP expression with a FLIP antisense oligonucleotide resulted in significant sensitization to Fas-mediated apoptosis. Fas cross-linking promoted BrdUrd incorporation; activated the mitogen-activated protein kinase/extracellular signal-regulated kinase, NF-κB, and activator protein-1 pathways in thyroid carcinoma cells in vitro; and protected cells from tumor necrosis factor—related apoptosis-inducing ligand—induced apoptosis. We also found that good prognosis papillary thyroid carcinoma specimens exhibited higher immunoreactivity for cleaved (activated) caspase-8 than poor prognosis tumors.

Conclusions: In thyroid carcinomas, the proteolytic cleavage and activation of caspase-8 depends on the balance between expression levels for procaspase-8 and FLIP and correlates with favorable clinical prognosis. Fas may actually stimulate proliferation and confer a survival advantage to thyroid cancer cells.

Fas (also known as Apo-1/CD95) is a transmembrane protein of the tumor necrosis factor/nerv growth factor receptor superfamily that transmits an apoptotic signal in susceptible normal and neoplastic cells. On cross-linking and oligomerization by its ligand (FasL), Fas recruits, via a cytoplasmic domain of ~70 amino acid residues (the death domain), an adaptor molecule, Fas-associated death domain (FADD; ref. 1).

FADD, in turn, allows the recruitment of the proenzyme form of caspase-8, also known as FADD-like interleukin-1β-converting enzyme, resulting in proteolytic autoactivation of caspase-8 (induced-proximity model; refs. 2, 3). The complex formed by the cross-linked receptor Fas, FADD, and procaspase-8 has been named the death-inducing signaling complex (DISC). Fas is widely expressed in normal and neoplastic tissues and Fas-mediated apoptosis plays a role in cell-mediated cytotoxicity against virally infected or transformed cells. Fas and Fasl also participate in the inflammatory destruction of target organs in several autoimmune disorders (1, 4, 5).

Thyroid cancer is the most prevalent endocrine neoplasia and is diagnosed annually in ~25,700 new cases in the United States alone (6). Although most thyroid carcinomas are of the well-differentiated papillary histology and respond well to ablation, tumors with more aggressive histology, such as follicular, poorly differentiated, anaplastic, and medullary cancers, lead to almost 1,500 patient deaths annually (6). Several studies have shown that lymphocytic infiltrates, a common finding in papillary thyroid carcinomas, are associated with a more favorable clinical prognosis (7–9), suggesting an
important role for immune-mediated control of thyroid cancer growth. As Fas is a major effector of cell-mediated antitumor cytotoxicity, enhancing Fas-mediated apoptosis may improve outcome in patients with thyroid carcinoma.

Fas is expressed in most thyroid carcinomas, yet its cross-linking fails to induce apoptosis in thyroid carcinoma cell lines in vitro (10). In fact, many thyroid carcinoma cells simultaneously express their own FasL, which may confer them a protective immunomodulatory effect (11) and clearly has no negative effect on their survival. The protein synthesis inhibitor cycloheximide sensitizes thyroid carcinoma cells to Fas-mediated apoptosis, suggesting the presence of a short-lived inhibitor of the Fas pathway in these cells (10, 12). Cross-linking of Fas failed to induce recruitment and activation of caspase-8 whereas transfection of a constitutively active caspase-8 construct effectively killed the SW579 papillary carcinoma cell line, arguing that the action of the putative inhibitor occurs upstream of caspase-8 activation (10). Such an inhibitory effect on Fas-induced caspase-8 activation and apoptosis has been reported in many other models for FADD-like interleukin-1β–converting enzyme inhibitory protein (FLIP; ref. 13). In thyroid carcinomas, FLIP has been shown to suppress apoptosis triggered by another member of the tumor necrosis factor family, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)/Apo2L (14). The role of FLIP in Fas signaling in thyroid carcinomas has not been studied. Moreover, Fas has been reported to be actually overexpressed in papillary thyroid carcinomas compared with paired normal thyroid tissue (15), which raises the possibility that Fas expression may actually confer a survival advantage to thyroid cancer cells.

In the present study, we investigated the role of FLIP in caspase-8 activation and Fas-mediated apoptosis in thyroid carcinoma. We found that FLIP interferes with caspase-8 activation in vitro and that higher immunoreactivity for cleaved (activated) caspase-8 correlates with better prognosis in papillary thyroid carcinoma specimens. Fas cross-linking activated the mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase (MEK)/ERK, nuclear factor–κB (NF–κB), and activator protein 1 (AP-1) pathways and stimulated proliferation in thyroid carcinoma cells in vitro. Our study shows that thyroid carcinoma cells can divert Fas signaling from apoptosis to proliferation and may actually exploit Fas expression to their advantage.

Materials and Methods

Human tissues

Archival formalin-fixed and paraffin-embedded thyroid specimens from 72 patients (21 male, 51 female) with thyroid carcinomas, ages 14 to 78 years (average ± SD, 45.5 ± 14.5 years), were retrieved retrospectively from the files of the Pathology Department, University of Athens, Greece. They represented 52 papillary, 2 follicular, 1 anaplastic, and 17 medullary carcinomas. A subgroup of papillary carcinomas that exhibited a series of good prognostic features [i.e., small size (larger diameter <4 cm)] confined to the thyroid gland and the classic well-differentiated histologic picture without vessel invasion were labeled group A (good prognosis, n = 10). Another subgroup showed extensive areas of moderate/low differentiation or squamous differentiation, large clinical size, or even disseminated (size >4 cm and/or infiltration of the thyroid capsule or represented recurrence after initial surgery) and were labeled group B (poor prognosis, n = 22). Fresh normal thyroid tissue was obtained from the contralateral lobe of thyroid glands removed surgically for a nodule from spontaneously euthyroid patients. All thyroid specimens were removed from patients followed at the Endocrine Unit of the Evgenidion Hospital, Athens, Greece. All studies on patient material were conducted in accordance with the Declaration of Helsinki principles and Institutional Review Board policies.

Cell lines

Thirteen previously described thyroid carcinoma cell lines were used in this study. The papillary thyroid carcinoma cell lines BHP-2, BHP-5, BHP-7, BHP-10, BHP-14, BHP-17, and BHP-18, and BHP-19 were generous gifts of Dr. Jerome M. Hershman (West Los Angeles Veterans Affairs Medical Center, Los Angeles, CA; refs. 16, 17). The SW579 cell line, derived from a poorly differentiated human thyroid adenocarcinoma (poorly differentiated carcinoma with nuclear features of papillary carcinoma and squamous differentiation), and the TT cell line, derived from a medullary thyroid carcinoma, were purchased from American Type Culture Collection (Manassas, VA). The anaplastic carcinoma cell lines FRO and ARO and the follicular carcinoma cell line WRO were a generous gift of Dr. James A. Fagin (University of Cincinnati School of Medicine, Cincinnati, OH; ref. 18). The neuroectodermal cell line SK-N-MC, which is exquisitely sensitive to Fas-mediated apoptosis (19, 20), was also purchased from American Type Culture Collection and served as a control.

All cells were grown in DMEM (BioWhittaker, Walkersville, MD) with 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% FCS (Life Technologies, Inc., Gaithersburg, MD) unless stated otherwise.

Materials

The Vectastain Elite ABC kit for immunohistochemistry was obtained from Vector Laboratories, Inc. (Burlingame, CA); the anti-Fas CH11 antibody from Panvera (Madison, WI); 3,3′-diaminobenzidine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma (St. Louis, MO); Complete-TM mixture of proteinase inhibitors from Roche Applied Science (Indianapolis, IN); and enhanced chemiluminescence kit, which includes the peroxidase-labeled antimouse and antirabbit secondary antibodies, from Amer- sham (Arlington Heights, IL). The proteasome inhibitor MG132 was from Calbiochem (La Jolla, CA); the proteasome inhibitor bortezomib (PS-341, Velcade) from Millennium Pharmaceuticals (Cambridge, MA); and recombinant TRAIL-LZ from Immunex Corporation (Seattle, WA).

Immunohistochemistry

Immunohistochemistry was done and evaluated as previously described (11). Intensity of positive staining was evaluated on a scale of 0 to 3 (0 and 3 corresponded to the absence and highest degree of staining, respectively) with a 25× lens by an expert thyroid pathologist (S.T-B.). The primary antibodies used for immunohistochemistry were mouse monoclonal antibody for procaspase-8 (Upstate Biotechnologies, Lake Placid, NY); rabbit polyclonal antiserum against FADD and cleaved caspase-8 (Cell Signaling, Beverly, MA); and polyclonal antibody for FLIP (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoblotting analysis

Immunoblotting analysis was done as previously described (21). The proteins were visualized with the enhanced chemiluminescence technique. The primary antibodies used for immunoblotting were mouse monoclonal antibody for tubulin and glyceraldehyde-3-phosphate dehydrogenase and rabbit polyclonal antibodies for caspase-3 and caspase-9 (Santa Cruz Biotechnology); monoclonal antibody for caspase-8 and rabbit polyclonal antibody for FLIP (Upstate Biotechnologies); and rabbit polyclonal antiserum against FADD, phospho-MEK, and phospho-ERK (Cell Signaling).

Fas DD sequencing

Genomic DNA from our thyroid carcinoma cell lines was extracted and the Fas exon 9, encoding the Fas DD, was PCR
amplified using the following primers: forward, 5'-GGTGTTCACAAT-TGGGATTTTCA-3', and reverse, 5'-CTGAACTTGTGGTGTTCTCCT-3'. Cycle sequencing of the purified PCR products was carried out with one of the PCR primers using the Big-dye terminator sequencing kit (Applied Biosystems, Foster City, CA). The Sephadex G-50-purified cycle sequencing products were analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

**Reverse transcription-PCR.** Total RNA from our thyroid carcinoma cell lines was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA with oligo(dT) primer using Superscript II reverse transcriptase (Invitrogen). The cDNA products were amplified for 35 cycles using Taq DNA polymerase (Invitrogen) using the exon 3 primer FasF3' (5'-GGCTGAACTGAC-CTGCCGT-3') and the exon 7 primer FasR7' (5'-CTGCAATGTTCTCTACTCC-3'), both of which are common to transmembrane Fas and the alternatively spliced, shorter, soluble Fas transcript. The primer sequences for caspase-8 were CaspF8', 5'-GCATTAGGGACAGGAAT- GGA-3', and CaspR8', 5'-TTATTCACAGTGGCCATCCC-3', and CaspF8R', 5'-GACTTCAGCAGACATCCTAC-3' as described above, and visualized by enhanced chemiluminescence.

**DISC immunoprecipitation.** Immunoprecipitation of the Fas DISC was carried out as previously described (22). Briefly, 10^7 cells were either first stimulated with biotin-labeled anti-Fas APO-1 antibody (Kamiya Biomedical Company, Seattle, WA, 2 μg/ml) for 10 minutes and then lysed in a lysing buffer [20 mmol/L Tris-HCL (pH 7.4), 1% Triton X-100, 10% glycerol, and 150 mmol/L NaCl] supplemented with protease inhibitors (Complete-TM; stimulated cells) or first lysed and then incubated overnight with the biotin-labeled anti-Fas APO-1 antibody (Kamiya Biomedical Company, Seattle, WA, 2 μg/ml) according to the instructions of the manufacturer. Twenty-four hours later, CH11 (50 ng/mL) was added to 24-well plates and transfected with the help of Oligofectamine (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA with oligo(dT) primer using Superscript II reverse transcriptase (Invitrogen). The cDNA products were amplified for 35 cycles using Taq DNA polymerase (Invitrogen) using the exon 3 primer FasF3' (5'-GGCTGAACTGAC-CTGCCGT-3') and the exon 7 primer FasR7' (5'-CTGCAATGTTCTCTACTCC-3'), both of which are common to transmembrane Fas and the alternatively spliced, shorter, soluble Fas transcript. The primer sequences for caspase-8 were CaspF8', 5'-GCATTAGGGACAGGAAT- GGA-3', and CaspR8', 5'-TTATTCACAGTGGCCATCCC-3', and CaspF8R', 5'-GACTTCAGCAGACATCCTAC-3' as described above, and visualized by enhanced chemiluminescence.

**Transfection of antisense FLIP and control oligonucleotides.** To delineate the role of FLIP as a negative regulator of Fas-mediated apoptosis in thyroid carcinoma cells, we transfected FRO cells with fully phosphorothioated single-stranded antisense oligonucleotide directed against the human FLIP translation initiation codon (sequence, 5'-GACTTCAGGACAGCATCCT-3') or control phosphorothioate oligodeoxynucleotide (sequence, 5'-TGGATCGAGCATGTCAGA-3') as previously described by Perlman et al. (23). FRO cells were plated in 24-well plates and transfected with the help of Oligofectamine (Invitrogen). Twenty-four hours later, CH11 (50 ng/mL) was added to appropriate wells and the cells were incubated for an additional 18 hours. Cell death was quantified by MIT as above.

**BrdUrd incorporation assay.** Cell proliferation in cells treated, in the presence of BrdUrd, with the Fas cross-linking antibody CH11 (100 or 1,000 ng/mL for 48 hours) was quantified by measuring the amount of BrdUrd incorporated into nuclear DNA using the BrdUrd incorporation assay (Oncogene Research, Cambridge, MA) according to the instructions of the manufacturer.

**Quantification of NF-kB and AP-1 activity in vitro.** The DNA binding activity of NF-kB and AP-1 was quantified by ELISA using the Trans-AM NF-kB (p65) and Trans-AM AP-1 (c-Jun) Transcription Factor Assay Kits (Active Motif North America, Carlsbad, CA) according to the instructions of the manufacturer as previously described (24, 25). Briefly, nuclear extracts were prepared as previously described (26) from control and CH11-treated cells SW579 cells and incubated in 96-well plates coated with immobilized double-stranded oligonucleotides (5'-AGTTGAGGACCTTTCCAGGC-3' and 5'-CGCT-TGATGACTGGCGGAA-3', respectively) containing a consensus binding site (underlined sequences) for NF-kB or AP-1, respectively. Transcription factor binding to the respective target oligonucleotide was detected by incubation with primary antibody specific for the activated form of p65 or c-Jun, respectively (Active Motif North America). Followed by anti-immunoglobulin G horseradish peroxidase conjugate and developing solution, and quantified at 450 nm with a reference wavelength of 655 nm. To monitor the specificity of the assay, background binding was calculated by adding in selected wells the respective consensus oligonucleotides in excess (20 pmol/well) as soluble competitors that prevented transcription factor binding to the probe immobilized on the plate. The resulting values were subtracted from the values obtained in wells with immobilized oligonucleotides alone. Results were normalized for nuclear extract protein content. This method has significantly higher sensitivity than electrophoretic mobility shift assays and allows high-throughput automated quantification of transcription factor activity in our model in a format amenable to repeated measurements and statistical analysis (25–28).

**Statistical analysis.** Quantitative comparisons of immunohistochemical variables between different patient groups were examined with the Mann-Whitney U test. Potential correlations between individual immunohistochemical variables were evaluated with the calculation of the Spearman’s correlation coefficient. Statistical significance was set at 0.05.

**Results.**

**Lack of inactivating mutations in the Fas DD of thyroid carcinoma cell lines.** Mutations in the Fas DD have been described in various other models (29–31) and have been blamed for tumor cell resistance to Fas-mediated apoptosis. Sequencing of the death domains of Fas in our thyroid carcinoma cell line panel did not reveal any mutations.

**Expression of Fas signaling pathway components in thyroid carcinoma cell lines.** We have previously reported widespread expression of Fas among thyroid carcinoma specimens and cell lines (10). In the present study, we investigated the expression of several apoptosis mediators involved in the Fas pathway (Fas, procaspase-8, FLIP, FADD, procaspase-3, and procaspase-9) at the mRNA and protein levels (Fig. 1A and B). Fas and procaspase-8 mRNA were present in normal thyroid tissue and throughout our cell line panel; in fact, several cell lines expressed significantly higher Fas and/or procaspase-8 mRNA than normal thyroid tissue. Spliced mRNA (encoding for soluble Fas) was detected as a very faint band only in cell lines with very high levels of full-length Fas mRNA, constituted only a very small fraction of the total Fas mRNA, and, overall, its expression in thyroid cell lines was not higher than that in other Fas-sensitive cell lines (e.g., SK-N-MC cells; ref. 32). Thus, overall, the presence of spliced Fas mRNA encoding for soluble Fas does not seem to be able to explain the resistance of thyroid carcinoma cell lines to Fas-mediated apoptosis. A prior study has suggested that transgenic mice that carry the mutant form of Gs α-subunit gene (gsp) downstream of the bovine thyroglobulin promoter develop thyroid neoplasms that express low levels of FADD, leading to suppression of Fas-mediated apoptosis (33). In our study, FADD was found in all thyroid carcinoma cell lines (Fig. 1B) and in...
FLIP expression was also widely distributed among the thyroid carcinoma cell lines of our panel (Fig. 1A and B) and tumor specimens (Fig. 2A and D).

Immunohistochemical detection of FLIP and procaspase-8 expression and caspase-8 cleavage in thyroid carcinoma tumor specimens. Group A (good prognosis) papillary thyroid carcinoma specimens exhibited higher immunoreactivity for cleaved (activated) caspase-8 than group B (poor prognosis) tumors (1 ± 0.8 versus 0.2 ± 0.4, respectively; P = 0.018, Mann-Whitney U test). Group A tumors tended to have more procaspase-8 (inactive proenzyme) and less FLIP immunoreactivity than group B (1.3 ± 0.6 versus 0.8 ± 0.8 and 0.8 ± 0.6 versus 1.1 ± 0.8, respectively) but the difference did not reach statistical significance (P > 0.05, Mann-Whitney U test), suggesting that procaspase-8 and FLIP expression cannot fully explain independently the patient’s prognosis. However, composite index (procaspase-8 immunoreactivity – FLIP immunoreactivity) was higher in group A than in group B tumors (0.5 ± 0.8 versus –0.3 ± 0.9, respectively; P = 0.021, Mann-Whitney U test) and correlated with cleaved (active) caspase-8 immunostaining (P = 0.001, Spearman’s correlation coefficient), suggesting that it is the balance between procaspase-8 and FLIP levels that controls the activation of caspase-8 and the apoptotic fate of the cell (Fig. 2).

Proteasome inhibition does not overcome the resistance of thyroid carcinoma cells to Fas-mediated apoptosis. In another prior study, it was shown that thyrocytes from goiters have increased proteasome activity and that proteasome inhibitors sensitize them to TRAIL-induced apoptosis (34). In our study, we investigated whether a similar mechanism protects thyroid carcinoma cells from Fas-mediated apoptosis. Proteasome inhibitors suppress the degradation of the NF-κB inhibitor IκB, thus resulting in IκB accumulation and NF-κB inhibition. As the transcription factor NF-κB promotes survival in numerous models by stimulating the transcription of several antiapoptotic genes, such as cIAP-2 and A1 (25, 35, 36), proteasome inhibitors induce apoptosis in several types of cancer cells (37) and may promote sensitivity to Fas-mediated apoptosis (37). We therefore investigated the effect of the proteasome inhibitors MG132 and PS-341 (bortezomib) on Fas-mediated apoptosis in thyroid carcinoma cells. However, we did not find any sensitizing effect on Fas-mediated apoptosis in primary thyroid carcinoma specimens (Fig. 1C and D). Immunostaining for FADD was strong in thyroid carcinoma cells and with intensity comparable to that of normal thyroid tissue and without any difference between prognosis groups.

Fig. 1. Expression of Fas pathway mediators in thyroid carcinoma cells. A, reverse transcription-PCR for Fas (and soluble Fas), (pro)caspase-8, and FLIP in normal thyroid tissue (from two different patients) and our panel of thyroid carcinoma cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is also shown for comparison. B, immunoblotting for (pro)caspase-8, FLIP, FADD, (pro)caspase-3, and (pro)caspase-9 expression in our panel of thyroid carcinoma cell lines. Tubulin is shown as a control. C and D, FADD is strongly expressed in thyroid carcinoma specimens. Immunostaining of a papillary (C) and a medullary (D) thyroid carcinoma for FADD. Magnification, ×125.

Fig. 2. Immunohistochemical detection of DISC components in thyroid carcinoma specimens. Two papillary thyroid carcinomas, one group A (good prognosis; A–C) and one group B (poor prognosis; D–F), immunostained for FLIP (A and D), procaspase-8 (B and E), and cleaved (active) caspase-8 (C and F). Magnification, ×125.
thyroid carcinoma cells (Fig. 3A and B). The proteasome inhibitor MG132 had a sensitizing effect on TRAIL-induced apoptosis in this model, serving as a positive control (Fig. 3A).

**FLIP is recruited to the DISC complex on Fas cross-linking in thyroid carcinoma cell lines.** We have previously reported that caspase-8 is not recruited to the DISC complex in thyroid carcinoma cells on Fas cross-linking (10). In this study, we investigated the recruitment of FLIP in the DISC. Thyroid carcinoma cells were stimulated with or without anti-Fas before cell lysis. Precipitation of Fas resulted in isolation of the DISC complex, which was then studied by immunoblotting. We found that cross-linking of Fas in thyroid carcinoma cells promotes FLIP recruitment to the DISC (Fig. 4A). In some thyroid carcinoma cell lines, FLIP was found to interact with Fas even in the absence of exogenous Fas triggering (Fig. 4A). On the contrary, the SK-N-MC cells, which are very sensitive to Fas-mediated apoptosis (19, 20) and served as controls, exhibit strong recruitment of pro-caspase-8 (32), but not of FLIP (Fig. 4A), to the DISC. These findings suggest that the resistance of thyroid carcinoma cells to Fas-mediated apoptosis is determined at the level of the DISC by FLIP. They also confirm the structural integrity and surface localization of Fas (because Fas cross-linking resulted in FLIP recruitment), the presence of adequate amounts of FADD (because FLIP recruitment requires FADD), as well as the lack of significant soluble Fas presence (because soluble Fas would inhibit all intracellular signaling).

**Treatment with FLIP antisense oligonucleotide results in sensitization to Fas-mediated apoptosis.** The strong recruitment of FLIP to the DISC complex of thyroid carcinoma cells implicates it as an inhibitor of Fas-mediated apoptosis in this model. To confirm this role, we transfected FRO cells with a FLIP antisense oligonucleotide or control oligonucleotide for 24 hours, followed by treatment with the Fas-cross-linking antibody CH11. FLIP antisense oligonucleotide–treated FRO cells were sensitized to apoptosis induced by CH11 (P < 0.01). Control oligonucleotide–treated FRO cells remained resistant to CH11-induced apoptosis (Fig. 4B). It should be noted that whereas short incubation of FRO cells with FLIP antisense oligonucleotide had no toxic effect on its own, prolonged exposure (>48 hours) resulted in FLIP-specific cell death (not shown). This indicates that inhibition of FLIP expression specifically leads to thyroid carcinoma cell death. This in turn could indicate that baseline FLIP expression is necessary for thyroid carcinoma cells to suppress constitutive apoptotic signaling (e.g., from the interaction of endogenous Fasl with Fas).

**Fas cross-linking stimulates proliferation in thyroid carcinoma cells.** SW579 cells were treated, in the absence of serum, with the Fas-cross-linking antibody CH11 for 48 hours in the presence of BrdUrd, and their proliferation rate was estimated using a BrdUrd incorporation assay. The rate of BrdUrd incorporation in cells treated with 100 or 1,000 ng/mL CH11 was 203 ± 6% and 304 ± 81%, respectively, that of control cells (P < 0.05 for both cases; Fig. 5A).

SW579 cells were treated with CH11 (1,000 ng/mL) for 0, 5, 15, 30, 60, and 120 minutes. Protein lysates were assayed by immunoblotting for phospho-MEK, phospho-ERK,
and glyceraldehyde-3-phosphate dehydrogenase. The levels of phosphorylated MEK were increased on CH11 treatment, peaking at 60 minutes, and were followed by increased levels of phosphorylated ERK (Fig. 5B).

Fas cross-linking activates NF-κB and AP-1 and exerts an antiapoptotic effect in thyroid carcinoma cells. In SW579 cells treated for 1 to 4 hours with CH11 (500 ng/mL) in the absence of serum, the DNA binding activity of the transcription factors NF-κB and AP-1 was stimulated for up to 11-fold and 6.6-fold, respectively (Fig. 6A). This suggests that Fas cross-linking activates NF-κB and AP-1.

As NF-κB activity plays a protective role against several apoptotic stimuli, such as TRAIL (21), we next investigated the effect of CH11 (500 ng/mL) on TRAIL-induced apoptosis (100 ng/mL TRAIL-LZ for 24 hours). We found that Fas-cross-linking had a modest, yet statistically significant (*), protective effect against TRAIL-induced apoptosis in SW579 cells (Fig. 6B).

Discussion

Several populations of activated immune cells express the apoptosis-inducing ligand FasL. Fas-mediated cell death is an effector pathway for cell-mediated cytotoxicity and participates in the immune response against neoplastic cells (38). Therefore, the emergence of resistance to this pathway contributes to neoplastic cell escape from immune surveillance and is an important step during the carcinogenesis process (39). We have previously reported that thyroid carcinoma cells are resistant to Fas-mediated apoptosis (10). In the present study, we investigated the mechanism of this resistance and dissected the Fas signaling pathway in thyroid cancer in vitro and in vivo. We found that thyroid carcinoma cells not only avoid Fas-mediated apoptosis but also divert the Fas signaling pathway from cell death to proliferation. The balance between procaspase-8 and FLIP levels emerged as a key regulator of thyroid carcinoma cell fate.

Several potential mechanisms for resistance to Fas-mediated apoptosis have been proposed in cancer cells, including down-regulation of Fas protein expression, intracytoplasmic sequestration and failure of the receptor to translocate to the cell surface, production and secretion of a soluble form of “decoy” receptor [either an alternatively spliced form of Fas or another soluble inhibitor of Fas activation (decoy receptor 3)], or mutations of Fas, especially in the death domain (for a detailed review, see ref. 40). However, none of these mechanisms is applicable to thyroid cancer. Thyroid carcinoma cells express full-length Fas on their surface. As shown in this study, the presence of spliced mRNA encoding for soluble Fas cannot explain resistance to apoptosis in thyroid carcinoma cells. Moreover, no death domain–inactivating mutations were found in thyroid carcinoma cell lines.

A prior study has suggested that transgenic mice that carry the mutant form of Gsα-α-subunit gene (gsp) downstream of the bovine thyroglobulin promoter develop thyroid neoplasms that express low levels of FADD, leading to suppression of Fas-mediated apoptosis (33). It should be pointed out, however, that in humans, gsp mutations are associated with well-differentiated hyperfunctioning thyroid nodules and not with
clinically invasive thyroid cancer. Our study showed abundant FADD expression throughout thyroid carcinoma cell lines and specimens without any difference between prognosis groups. In another prior study, it was shown that thyrocytes from goiters have increased proteasome activity and that proteasome inhibitors sensitize them to TRAIL-induced apoptosis (34). In our present study, we investigated whether a similar mechanism protects thyroid carcinoma cells from Fas-mediated apoptosis. However, we did not find any sensitizing effect of the proteasome inhibitors MG132 and bortezomib (PS-341, Velcade) on Fas-mediated apoptosis in thyroid carcinoma cells.

A more widespread mechanism of resistance to Fas-mediated apoptosis described in several models is the overexpression of antiapoptotic proteins. FLIP is overexpressed in several types of cancers and confers resistance to Fasl- and TRAIL-induced apoptosis (14). FLIP is a short-lived protein, which correlates with the ability of the protein synthesis inhibitor cycloheximide to restore Fas sensitivity in thyroid carcinoma cells and other models. In our study, FLIP was recruited to the DISC in thyroid carcinoma cells, instead of caspase-8. Down-regulation of FLIP with a specific antisense oligonucleotide sensitized thyroid carcinoma cells to Fas-mediated apoptosis. Taken together, these data define the DISC as the key regulator of caspase activation and consequent apoptosis in thyroid cancer cells. In thyroid carcinoma specimens, group A (good prognosis) papillary thyroid carcinoma specimens exhibited higher immunoreactivity for cleaved (activated) caspase-8 than group B (poor prognosis) tumors. Group A tumors tended to have more procaspase-8 (inactive proenzyme) and less FLIP immunoreactivity than group B, but the difference for each protein alone did not reach statistical significance, suggesting that procaspase-8 and FLIP expression cannot fully explain independently the patient’s prognosis (and probably the apoptotic fate of the cell). However, a composite index (procaspase-8 immunoreactivity – FLIP immunoreactivity) was higher in group A than in group B tumors and correlated with cleaved caspase-8 immunostaining intensity, suggesting that it is the balance between procaspase-8 and FLIP levels that controls caspase-8 activation and regulates the apoptotic fate of the cell.

The finding that Fas itself is always present and structurally intact in thyroid carcinoma cells, although it does not lead to apoptosis, becomes even more intriguing given the prior observation that papillary cancer cells actually overexpress Fas compared with adjacent normal follicles (15). This observation was also confirmed in our thyroid tumor specimens. One potential explanation for these findings is that Fas expression may confer a survival advantage to the neoplastic cell. Indeed, we found that Fas cross-linking stimulated BrdUrd incorporation and activated the MEK/ERK, AP-1, and NF-κB pathways in SW579 cells.

Other members of the tumor necrosis factor receptor family, such as tumor necrosis factor receptor 1, TRAIL-R1, and TRAIL-R2, have long been known to activate NF-κB (41), yet little is known about similar actions of Fas, especially in cancer cells. Fas has been reported to transduce an activation/proliferation signal in normal T lymphocytes (42) and human fibroblasts (43, 44). Similarly to our current findings in thyroid carcinoma cells, Kataoka et al. have shown that, in human T lymphocytes, FasL activates NF-κB, AP-1, and the ERK signaling pathways in a FLIP-dependent manner (45–47). Moreover, Fas cross-linking has been reported to accelerate liver regeneration after partial hepatectomy in mice (48). In a study of a large panel of solid tumor cell lines, a growth-stimulating effect of Fas cross-linking was reported in a melanoma, a pancreatic carcinoma, and an epidermoid carcinoma cell line (49). In a study of malignant B cells isolated from patients with chronic B lymphocytic leukemia, most patient cells underwent apoptosis after Fas cross-linking but cells from one of eight patients responded with proliferation (50). We have previously reported that Fas cross-linking partially protects retinoblastoma cells from apoptosis induced by the NF-κB inhibitor SN50, suggesting that it may activate an antiapoptotic pathway (32). In our present study, Fas-cross-linking had a modest, yet statistically significant, protective effect against TRAIL-induced apoptosis in SW579 cells.

As tumor-infiltrating lymphocytes express FasL, on their cell surface in an effort to induce tumor cell apoptosis, it is conceivable that the thyroid carcinoma cell distorts this signal into a growth stimulus and even uses it as a protective mechanism against other tumor-infiltrating lymphocyte cyto-toxic ligands such as TRAIL. This survival benefit conferred by Fas explains why thyroid carcinoma cells overexpress Fas and do not harbor death domain mutations or high levels of soluble (decoy) Fas. As thyroid carcinoma cells express their own FasL (11), this pathway may even function as an autocrine growth/survival loop. This hypothesis is supported, in our study, by the detection of constitutive FLIP binding to the DISC of some unstimulated thyroid carcinoma cell lines and by the apoptosis-inducing effect of prolonged exposure to a FLIP antisense oligonucleotide.

In conclusion, our study shows that, in thyroid carcinomas, the proteolytic cleavage and activation of caspase-8 depends on the balance between expression levels for procaspase-8 and FLIP, and correlates with favorable clinical prognosis. FLIP is recruited to the DISC complex instead of caspase-8, thus inhibiting caspase-8 activation, allowing thyroid carcinoma cells to avoid Fas-mediated apoptosis and diverting the Fas pathway signaling from apoptosis to proliferation and survival. Inhibition of FLIP expression resensitizes thyroid carcinoma cells to Fas-mediated apoptosis. In thyroid carcinomas, Fas is structurally intact and may actually confer a survival advantage to the neoplastic cell.

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