CLINICAL STUDY

Fas and Bcl-2 protein expression in thyrocytes of patients with nodular goiter

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Abstract

Objective: The relative expression of the apoptotic protein Fas and the anti-apoptotic protein Bcl-2 were investigated in thyrocytes from patients with non-toxic nodular goiter (NTG, $n = 20$) and Hashimoto's thyroiditis (HT, $n = 5$), who underwent fine-needle aspiration biopsy for diagnostic reasons. On the basis of the clinical and cytological findings, the patients with NTG were sub-classified into the group of those with colloid nodules ($n = 9$), degenerative nodules ($n = 6$) and adenomatous nodules ($n = 5$).

Methods: Fine-needle biopsy aspirates were examined by immunocytochemistry for Fas and Bcl-2 expression, using specific monoclonal antibodies. For the evaluation of Fas and Bcl-2 immunoreactivity, an expression index, based on the number of cells with positive staining, was used: grade 1 included samples with positive staining in $<20\%$ of cells; grade 2 included samples with 20–50% positive cells; and grade 3 included samples with $>50\%$ positive cells.

Results: Fas protein expression was generally low (grade 1) in patients with nodular goiter, in contrast to patients with HT, in whom high expression was detected (grade 3). Only in aspirates from degenerative nodules (four out of six), and in which lymphocytes were also present, was Fas expressed at an intermediate level (grade 2). On the other hand, Bcl-2 protein was differentially expressed among the nodule subtypes. It was low in colloid and degenerative nodules (grade 1) but high in adenomatous ones (grades 2 and 3). Bcl-2 expression was also low in patients with HT (grade 1).

Conclusion: It is concluded that in comparison to HT, where there is up-regulation of Fas and down-regulation of Bcl-2 protein, Fas expression is low in human goiter, indicating low apoptotic activity. The regulation of Bcl-2 protein differs between adenomatous and colloid nodules, suggesting that this protein may play a role in the differentiation of thyroid nodules.

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Introduction

Human thyroid cells proliferate during development and in adult life in response to physiological and pathological stimuli. It is estimated that under normal conditions during adulthood, thyroid cells divide about 5–10 times (1). The size of the thyroid gland remains relatively constant, however, because cell proliferation is balanced by cell death. This balance may be disturbed in human nodular goiter, which is the result of focal hyperplasia of thyroid follicular cells at one or more (often multiple) sites within the thyroid gland (2).

Previous studies have focused mainly on the role of thyroid-cell proliferation in goitrogenesis. Few studies have examined the other side of the coin, namely the role of apoptosis in human goiter. Recent evidence has emphasized that apoptosis plays a complementary but

opposite role, with respect to mitosis, in the regulation of tissue homeostasis (3, 4). When this balance is disturbed, abnormal cellular accumulation in the form of hyperplasia and neoplasia may result.

Apoptosis (programmed cell death) is highly orchestrated, and is usually induced through activation of special genes. One of these genes, known as Fas, has been well described (5, 6). Fas antigen, a trans-membrane glycoprotein, can mediate apoptosis upon forming complexes with Fas ligand (FasL) in a variety of tissues, including the thyroid gland (7, 8). On the other hand, the apoptotic process is regulated by members of the Bcl-2 family of proteins. The Bcl-2 gene is an anti-apoptotic gene, preventing cell death and promoting cell longevity (9–11).

Several recent studies have demonstrated the contribution of apoptosis, particularly that via the Fas

pathway and the regulatory protein Bcl-2, to autoimmune thyroid disease and neoplasia (12–17). Only a few studies have investigated the role of Fas and Bcl-2 proteins in rat models of goiter (18–20); none has investigated this aspect of human goiter.

In the present study, we have investigated the presence of Fas and Bcl-2 proteins in thyrocytes from patients, with goiter, who underwent fine-needle aspiration (FNA) biopsies for diagnostic reasons.

Patients and methods

Patients

In total, 25 patients (aged 19–74 years) participated in the study. These included 20 patients with non-toxic uninodular or multinodular goiter (NTG) and five patients with goitrous Hashimoto's thyroiditis (HT). The clinical diagnosis was based on physical examination, ultrasound and isotope scanning of the thyroid, and thyroid-function tests (for free tetra-iodothyronine, tri-iodothyronine, thyrotropin, and for antithyroid antibodies), which were performed routinely in our institution. From each patient, the dominant or single palpable nodule (usually >2 cm in diameter) was aspirated using a 22G needle with a biopsy pistol (Cameco Ltd, London, UK), and 5–6 aspirates were taken. All aspirated nodules were 'cold' in thyroid isotope scanning. In the case of nodules with cystic degeneration, the fluid was first aspirated and then cellular samples were taken from the solid part of the nodule. All patients gave informed consent for the FNA procedure.

Cytology

Additional information on the nodule was obtained from cytopathology reports. Smears from FNA aspirates were either air-dried and stained with May–Grünwald–Giemsa or were fixed immediately in 95% ethanol and then stained using the Papanicolaou method. The FNA aspirates were sub-classified according to the relative amounts of colloid and cellularity (as well as the pattern of cellularity). The presence of macrophages and lymphocytes was recorded. Thus, benign aspirates were classified as colloid, adenomatous, degenerative (cystic degeneration of nodules) and characteristic of HT.

Immunocytochemistry

FNA samples were collected on glass slides that were air-dried and kept frozen at temperatures below –80 °C. Prior to investigation, slides were air-dried overnight, fixed for 10 min in Zamboni's fixative (Ylem, Rome, Italy) and washed twice with Tris-buffered saline (pH 7.6) prior to labeling. Immunocytochemical investigations were performed by using the biotin–

streptavidin–alkaline phosphatase technique, using the Sandon Kwik Kit (as recommended by the manufacturer) in the Sequenza coverplate immunostaining system (Sandon Inc., Pittsburgh, PA, USA). The primary antibodies used for Fas detection were the clone C-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); for Bcl-2 detection, the clone 100/D5 (Ylem). The antibodies were diluted 1:50 in Tris-buffered saline (pH 7.6) and applied for 1 h. The reaction was revealed by fast red chromogen, and slides were counterstained with hematoxylin and mounted with glycerol gelatin. Positive cells appeared red in color. Negative controls were made by omitting the primary antibody.

Evaluation of immunoreactivity

For evaluation of Fas and Bcl-2 immunoreactivity, at least five fields ($\times 200$) from each slide were examined by two independent observers. Thus, an expression index was created by classifying the samples into three categories based on the percentage of positive cells in the total number of cells counted per field. Grade 1 included the samples with less than 20% positive cells, grade 2 samples had 20–50% positive cells, and grade 3 samples showed more than 50% positive cells.

Before the experiments were conducted, the antibodies were tested against different positive controls, including paraffin tissue sections, smears, and FNA samples from patients positive for each gene.

Results

Cytological findings

The nodules biopsied from the patients with NTG were reported as benign, and included lesions with cytological features associated with colloid nodules ($n = 9$), nodules with cystic degeneration ($n = 6$), and adenomatous nodules ($n = 5$). The cytology also confirmed the clinical diagnosis in the five patients with HT.

Fas and Bcl-2 expression in NTG

The results of Fas and Bcl-2 protein expression, indexed according to cytological sub-classification, for the group of patients with nodular goiter are shown in Table 1 and Fig. 1. Fas protein was present in thyrocytes from all patients with NTG. However, the Fas protein expression index was generally low (grade 1) in all subtypes of nodule, with the exception of nodules with cystic degeneration, in which four out of six samples showed intermediate levels of Fas protein expression (grade 2). Cytological examination of aspirates from these nodules also showed the presence of lymphocytes.

In contrast, Bcl-2 protein expression was different in the different subtypes of NTG (Table 1 and Fig. 1). It

Table 1 Fas and Bcl-2 expression index (grades 1–3) in thyrocytes from patients with goiter.

No.	Gender (M/F)	Age (years)	Fas protein (grade)	Bcl-2 protein (grade)	Type of nodule (cytology)
1	F	60	–	1	Colloid
2	F	45	1	1	Colloid
3	F	45	1	1	Colloid
4	M	53	1	1	Colloid
5	F	25	1	1	Colloid
6	F	25	1	1	Colloid
7	F	74	1	–	Colloid
8	F	32	1	1	Colloid
9	F	49	1	1	Colloid
1	F	50	2	1	Degenerative*
2	F	20	2	1	Degenerative
3	F	70	1	1	Degenerative
4	F	72	2	1	Degenerative
5	F	65	1	1	Degenerative
6	F	45	2	1	Degenerative
1	F	45	1	3	Adenomatous
2	F	20	1	2	Adenomatous
3	F	40	1	3	Adenomatous
4	F	25	1	3	Adenomatous
5	F	39	1	2	Adenomatous

*Grade 2 Fas protein expression was seen in FNA samples in which lymphocytes were also present.

Figure 1 Fas and Bcl-2 immunostaining observed in FNA samples from patients with nodular goiter. (A) Grade 3 Fas expression on thyrocytes from HT. (B) Grade 1 Fas expression on thyrocytes from a colloid nodule. (C) Grade 3 Bcl-2 expression on thyrocytes from an adenomatous nodule. (D) Grade 1 Bcl-2 expression on thyrocytes from a colloid nodule.

Table 2 Fas and Bcl-2 protein expression in thyrocytes from patients with HT.

No.	Gender (M/F)	Age (years)	Fas protein (grade)	Bcl-2 protein (grade)
1	F	49	3	1
2	F	60	3	1
3	F	43	3	1
4	F	72	2	1
5	M	48	3	1

was low (grade 1) in thyroid cells from nodules of the colloid type and in degenerative nodules, but was high (grades 2 and 3) in cells from nodules of the adenomatous type.

Fas and Bcl-2 expression in HT

Thyroid cells from all five patients with HT showed high levels of Fas protein expression (grades 2 and 3) and low levels of Bcl-2 protein expression (grade 1), as shown in Table 2 and Fig. 1.

Discussion

In the present study, the expression of the apoptotic Fas protein and the anti-apoptotic Bcl-2 protein was investigated in FNA samples from patients with NTG. The findings were compared with those obtained from a group of patients with HT.

The involvement of apoptosis through activation of the Fas–FasL pathway in HT has been well documented in previous studies (14, 15, 21). These studies suggest that, in HT, thyroid follicular cells undergo apoptosis by up-regulation of the Fas protein and down-regulation of the Bcl-2 protein.

The findings, from the present study, for patients with HT are in agreement with previous reports. Fas protein was found to be highly expressed on thyrocytes from untreated patients with HT, whereas Bcl-2 expression was low. This is the first study (to our knowledge) to demonstrate these changes in FNA samples.

In contrast to the findings for HT, a different picture was seen in patients with NTG with regard to Fas and Bcl-2 expression. Fas protein was expressed in thyrocytes from NTG, but at a low level (grade 1). Only in some patients with degenerative nodules was Fas expressed at an intermediate level (grade 2). On the other hand, Bcl-2 expression was found to differ between the different subtypes of nodules. It was low in thyrocytes from nodules of the colloid type and in thyrocytes from degenerative nodules, but a higher level of expression was evident in thyrocytes from adenomatous nodules (grades 2 and 3).

This study is the first to examine *in vivo* the expression of Fas and Bcl-2 proteins, the two key

proteins involved in the induction and regulation of apoptosis in human goiter. We neither directly assessed the level of apoptosis, as such, in our patients, nor were we able to obtain data on Fas and Bcl-2 expression from normal thyroids (for ethical reasons). Nevertheless, by comparing the degree of Fas expression in thyrocytes in NTG with that in HT, which is characterized by high apoptotic activity, we can infer that apoptosis, via the Fas death pathway, is generally low in human goiter. Only in nodules with cystic degeneration and the presence of lymphocytes is there moderate Fas expression and (presumably) apoptotic activity. Local release of inflammatory cytokines by the immune cells may induce Fas expression on the thyrocytes in the degenerative nodules. Indeed, the expression of Fas has been shown to increase in thyrocytes and other cell types after treatment with inflammatory cytokines (22, 23).

On the other hand, the anti-apoptotic protein Bcl-2 is differentially expressed in the different subtypes of nodular goiter. It is up-regulated in adenomatous nodules and down-regulated in colloid and degenerative nodules. The pathobiological significance of this differential regulation of Bcl-2 protein is not clear, but it may be associated with the differentiation of thyroid nodules into colloid and adenomatous ones. Interestingly, in thyroid tumors, expression of Bcl-2 appears to be related to the degree of differentiation of the cells and is reported to be high in thyroid follicular adenomas and oxyphilic tumors. It is believed that adenomatous nodules are more likely to develop into neoplastic tumors than are colloid nodules. Thus, by preventing apoptosis and promoting cell longevity, Bcl-2 may cause a shift in the tissue kinetics towards preservation of genetically aberrant cells, thereby facilitating tumor progression (16).

Previous immunohistochemical studies of thyroid tissue sections found Fas protein present on thyroid follicular cells from healthy individuals and from patients with thyroid carcinomas, Graves' disease and HT, although the extent and the specific disorders associated with this expression differed between the studies (15, 21, 22, 24). In thyrocyte cultures, Fas protein was detected either by antibodies against Fas antigen or by Western blotting of cell lysates (25, 26). However, it was recently reported that FasL, but not Fas, is constitutively expressed in normal thyroids and that Fas expression is induced on thyrocytes only under the influence of inflammatory cytokines (22). The discrepancies between the latter study and previous studies may have resulted from differences in the reagents used (13).

Finally, the role of apoptosis and the involvement of the Fas pathway were previously investigated in rat models of goiter during the stages of goiter formation and involution (18, 19). The major findings of these studies were an increased number of apoptotic cells paralleled by increased number of Fas-positive cells

during the development of goiter and the early stage of involution and constitutive expression of FasL throughout the experiment. These findings suggest that Fas expression may serve as a limiting factor for the induction of Fas-mediated apoptosis. On the other hand, the study of Patel *et al.* (20) revealed high levels of Bcl-2 immunoreactivity (in normal and goitrous rat thyroids) that were significantly reduced during goiter involution. They also showed an absence of apoptosis within control thyroids but an increased number of apoptotic cells within goitrous glands.

It is difficult, however, to make direct comparisons between our human data and data from animal models of goiter. The process of goitrogenesis was faster in experimental goiter under the influence of trophic factors (i.e. iodine and thyrotropin) and it was studied in the stages of goiter formation and involution (with the presence and withdrawal of trophic factors), whereas our patients had long-standing, slowly growing nodular goiter with normal thyrotropin levels.

In conclusion, the *in vivo* expression of Fas protein is low in human nodular goiter, indicating reduced thyroid cell apoptosis via the Fas death pathway. Therefore, the regulation of Fas protein expression may play a role in the pathogenesis of goiter by upsetting the normal equilibrium between thyroid-cell proliferation and apoptosis in favor of the former process. On the other hand, regulation of expression of the anti-apoptotic Bcl-2 protein in adenomatous nodular goiter is different from that in colloid-type goiter. The latter protein may have a particular role in the development of adenomatous nodules and/or their differentiation from colloid nodules in human nodular goiter.

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