Analysis of the Expression of Fas, FasL and Bcl-2 in the Pathogenesis of Autoimmune Thyroid Disorders

Shenren Chen[1], S. M. Fazle Akbar[2], Zhichao Zhen[1], Yiping Luo[1], Lijuan Deng[1], Haihua Huang[1], Linxin Chen[1] and Wei Li[1]

To investigate the expression of apoptosis-related protein (Fas, FasL, and Bcl-2) in the pathogenesis of autoimmune thyroid disorders (ATDs), immunohistochemical staining was performed on 20 Hashimoto's thyroiditis (HT), 20 Graves' disease (GD), and 20 thyroid follicular adenoma (TFA, as control). All the cases expressed Fas, mainly on the cell surface and cytoplasm. FasL was found in 17 cases of the TFA. Bcl-2 was detected in 15 cases of HT, 19 of GD and 17 of TFA. In TFA, a moderate Fas expression and a minimal or no FasL expression was detected on follicular cells. In HT, the follicles adjacent to infiltrating lymphocytes showed increased levels of Fas and FasL expression. A weaker staining of Fas and FasL was exhibited on infiltrating lymphocytes than on thyrocytes. In a comparison of GD with HT, thyrocytes and lymphocytes showed similar Fas staining, but for FasL the staining was rather weaker in HT. The expression of Bcl-2 was nearly identical in GD and TFA, but much weaker on the follicular cells in vicinity of lymphocytes and on the lymphocytes located in germinal centers of HT tissues. The expression of Fas, FasL, Bcl-2 in Hashimoto's thyroiditis and Graves' disease were almost same. FasL strong expression and Bcl-2 weak expression on the follicles in HT may induce apoptosis. These results provided evidence for expression of Fas, FasL and Bcl-2 in the pathogenesis of autoimmune thyroid disease. The lymphocytes seem not to be directly engaged in the process *via* their own FasL, but they may provide some cytokines that, in turn, upregulate Fas and/or FasL expression to induce apoptosis. *Cellular & Molecular Immunology*. 2004;1(3):224-228.

Key Words: ATD, HT, GD, Fas, FasL, Bcl-2

Introduction

Hashimoto's thyroiditis (HT) and Graves' disease (GD) are considered to be autoimmune thyroid disorders (ATDs). In HT, infiltration of lymphocytes and destruction of thyrocytes are prominent histological features, often resulting in hypothyroidism. In contrast, GD is characterized by the hyperplasia of thyrocytes that results from stimulation with autologous anti-TSH receptor antibodies. There is little evidence of thyrocyte injury in GD, and lymphocytic infiltrates are less frequent than HT (1).

The Fas receptor (Fas, CD95, and APO-1) and its ligand (FasL, CD95L) are transmembrane proteins that belong to the TNF family of receptors and ligands. Fas and FasL interaction triggers apoptosis in various systems (2, 3), whereas the Bcl-2 protooncogene is the prototype of a family that inhibit apoptotic cell death induced by various stimuli, such as growth factor deprivation (4).

There is considerable evidence that Fas and FasL interaction play a role in the pathogenesis of HT (5, 6), as well as GD (1). And, the expression of Bcl-2 which could render thyrocytes resistant to Fas/FasL-mediated apoptosis, may thus be involved in the pathogenesis of HT and GD (1, 5). However, in the previous studies, the results are controversial. As to the expression of Fas on thyrocytes, Tanimoto et al (7), Kawakami et al (8) and Hiromatsu et al. (1) reported the constitutive expression of Fas on thyrocytes of GD and normal controls. On the other hand, Giordano et al (5) reported increased expression of Fas on the surface of thyrocytes in HT, but not on normal controls and non-autoimmune thyroid disease. In thyroid diseases, Giordano et al. (5) and Mitsiades et al. (6) reported the increased expression in HT and they postulated that the Fas/FasL system might cause thyrocyte damage in HT. However, Stokes et al. (9) and Fiedler et al. (10) failed to show the expression of FasL mRNA on normal and

[1]The Second Hospital of Shantou University Medical College, Guangdong, Shantou 515041, China.

[2]Third Department of Internal Medicine, Ehime University, School of Medicine, Ehime, Japan.

[3]Corresponding to: Dr. Shenren Chen, The Second Hospital of Shantou University Medical College, Guangdong, Shantou 515041, China. Tel: +86-754-8355431, Fax: +86-754-8346543, E-mail: Chen-Shenren@163.com.

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Abbreviations: HT, Hashimoto's thyroiditis; GD, Graves' disease; ATD, autoimmune thyroid disorder; EAT, experimental autoimmune thyroiditis; TFA, thyroid follicular adenoma.

thyroiditis tissue samples. The discrepancies in Fas and FasL and their mRNA expression in thyroid tissue may be due to the differences in methods. Batteux et al. (11) provided evidence that direct injection of DNA expression vectors encoding FasL into inflamed thyroid of experimental autoimmune thyroiditis (EAT) inhibited development of lymphocytic infiltration of the thyroid and induced death of infiltrating T cells. Their results are in agreement with the hypothesis of Dayan et al. (12) that FasL expression on thyrocytes avoided autoimmune thyroiditis, and they considered that FasL expression on thyrocytes might have a curative effect on ongoing EAT by inducing death of pathogenic autoreactive infiltrating T lymphocytes. FasL expressed on normal human thyrocytes is still a matter of debate. Bcl-2 protooncogene has been known to inhibit apoptotic cell death. That has been reported over-expression of Bcl-2 in thyrocytes from patients with GD (1) and down-regulation of Bcl-2 in thyroid follicles of HT (6).

Whether Fas/FasL interaction plays a damage pathogenic role and results in hypothyroidism or has a curative effect to avoid autoimmune thyroiditis, what role Bcl-2 plays in the pathogenesis of ATDs, is the objective of the present study.

Materials and Methods

Thyroid tissue collection

Thyroid tissues specimens were obtained during operation. All patients attended the Second Affiliated Hospital of Shantou University Medical College, Shantou, China between 1993 and 2000. All samples were fixed in 10% buffered formalin, embedded in paraffin, and cut into 4 μm sections. 20 patients with HT (female, aged 18-46 years) and 20 patients with GD (female, aged 21-45 years) were diagnosed according to Fisher criteria (1975) by clinical criteria and confirmed by appropriate laboratory tests (TSH, T3, T4, TPO, TGA, TMA) and histological findings (hematoxylin and eosin staining). At the time of surgery all patients were euthyroid. Normal thyroid tissue adjacent to thyroid follicular adenoma (TFA) was obtained from 20 subjects with follicular adenoma (female, aged 28-64 years, as control).

Immunohistochemical stain

Rabbit anti-human polyclonal antibody to Fas (Sc-714-G, Santa Cruz) and to FasL (Sc-534-G, Santa Cruz), and mouse anti-human monoclonal antibody to Bcl-2 (SC-509, Santa Cruz) were used as primary antibodies. Biotin-labled antibody and HRP-avidin were used. Staining procedure performed the introduction of the manufacturer. A known sample from a patient with breast cancer was used as a positive control, and negative control slides were processed with PBS liquid instead of the primary antibody, but included all other steps of the procedure. Diaminobenzidine (DAB)-hydrogen peroxide was employed as chromogen.

Analysis of the expression of Fas, FasL and Bcl-2

Expression of Fas, FasL and Bcl-2 on membrane and/or in cytoplasm of thyroid follicular cells and/or infiltrating lymphocytes, containing brown granules was analyzed. The number of cells in the whole specimen was enumerated. Fas-, FasL-, and Bcl-2-positive thyrocytes and infiltrating lymphocytes were counted over the whole specimens, respectively.

Statistical analysis

The data of frequencies of different positive cells were expressed as mean ± SD. The statistical analysis was done by unpaired or paired *t*-test, when indicated. Mann-Whitney's U-test and the Wilcoxon rank-sum test were also used when unpaired and paired t-tests were not indicated respectively. The prevalences were shown as the number of cases positive for Fas, FasL and Bcl-2 in HT, GD and TFA, and the data were analyzed by χ^2 test. *p*-value less than 0.05 were considered to indicate statistical significance. Statistical calculations were performed using the SPSS statistical program.

Results

Fas was presented in TFA, HT and GD

In HT sections, follicles located in the vicinity of lymphocytes exhibited Fas (prevalence: 20/20, 100%, frequency: 76.31 ± 15.79%, Figure 1A) as well as the control follicles (TFA). The pattern of staining for Fas was mainly cytoplasmic but membranous as well. The lymphocytes exhibited weaker positive staining (prevalence: 20/20, 100%, frequency: 63.42 ± 22.85%). In GD sections, thyrocytes and infiltrating lymphocytes were stained positively for Fas as nearly well as in HT (Figure 1B). Thyrocytes from normal thyroid tissues adjacent to TFA (control) were stained positive for Fas (prevalence: 20/20, 100%, frequency: 75.75 ± 12.89%, exhibited moderate, Figure 1C).

FasL was upregulated in follicles with HT

TFA were stained weakly or negative for FasL (prevalence: 17/20, 85%, frequency: 26.08 ± 20.73%, Figure 2D). In

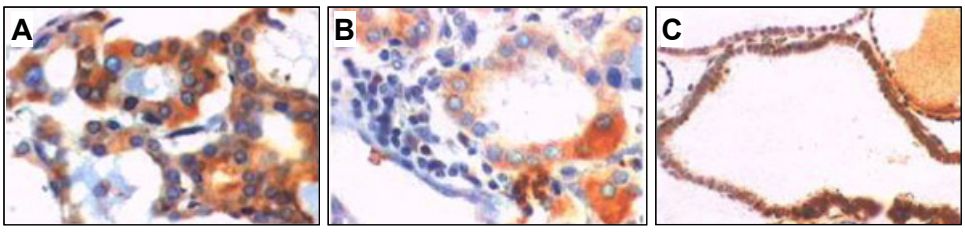

Figure 1. Representative staining pattern of Fas in Hashimoto's thyroiditis (A, ×400), Graves' disease (B, ×400) and Thyroid follicular adenoma (C, ×100).

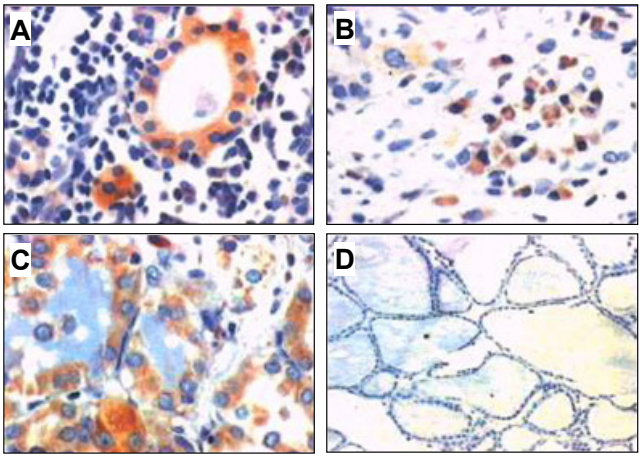

Figure 2. Representative staining pattern of FasL in thyroid follicles of Hashimoto's thyroiditis (A, ×400), in lymphocytes of Hashimoto's thyroiditis (B, ×400), in Graves' disease (C, ×400) and negative in thyroid follicular adenoma (D, ×100).

contrast, thyrocytes from HT sections stained strongly positive for FasL, exhibited mainly a membranous, but also a cytoplasmic, pattern of staining. The positive prevalence and frequency were even higher in follicles from areas adjacent to lymphocytes infiltrates (prevalence: 20/20, 100%, frequency: 46.10 ± 25.80%, Figure 2A) than that away from lymphocytes infiltrates. Staining of infiltrating lymphocytes for FasL was weaker than staining of follicles (Figure 2B). In GD sections, thyrocytes and infiltrating lymphocytes were stained for FasL rather weaker than that in HT (Figure 2C).

Bcl-2 was down regulated in HT than in GD

Thyrocytes from TFA were stained for Bcl-2 in a cytoplasmic pattern (Figure 3C). In HT sections, follicular cells in vicinity of lymphocytes exhibited significantly weaker staining for Bcl-2 (Figure 3A). But, follicular cells away from lymphocytes were stained with intensity equal to that of TFA. In addition, lymphocytes located in germinal centers of HT tissues were weak for Bcl-2, whereas those located in the interfollicular areas were moderate positive (Figure 3A). Thyrocytes and infiltrating lymphocytes in GD were stained for Bcl-2 and exhibited equal intensity to that of normal tissues (Figure 3B).

The prevalence and frequency of positive staining in TFA and in tissues from HT and GD for all the proteins mentioned above are summarized in Table 1 and Table 2.

Discussion

Over the past some years, numberous reports had showed that most of the cells, especially some epithelial cells expressing Fas rapidly underwent apoptopsis upon engagement with FasL. Thus, the immune privilege of the anterior chamber of the eye (13), the testis (14), the brain (15) and the placenta (16) has been linked to the expression of FasL on certain cells of those tissues. The interaction of Fas and FasL may constitute a common pathogenic mechanism mediating target destruction in organ-specific autoimmunity and some autoimmune diseases, such as acinar epithelial cells in salivary gland from patients with Sjogren' syndrome (17), autoimmune type 1 diabetes (18, 19), intestinal epithelial cells in uncreative colitis (20) and multiple sclerosis (21). Bcl-2 is another apoptosis-related molecule. Recently, Bcl-2 was shown to be down-regulated during the early events leading to programmed cell death (22). Bcl-2 protein might be as an inhibiting molecule and play an important role in the balance between apoptosis promotion and inhibition.

We studied the role of the apoptosis-related molecules Fas, FasL, and Bcl-2 in ATDs such as HT and GD. We found that follicular cells from normal thyroid tissues adjacent to TFA expressed moderate Fas and minimal FasL, and that had been corroborated by four other studies (6-8, 23). Although another study (5) showed absence of Fas and presence of FasL in non-autoimmune thyroids, this discrepancy might be explained by the use of different non-toxic goiters as normal thyroid tissues. Follicles adjacent to lymphocytes in thyroid glands with HT showed an increased level of FasL (prevalence and frequency). Infiltrating lymphocytes in HT exhibited weaker positive staining for Fas and FasL than thyrocytes. In GD, thyrocytes and lymphocytes showed nearly similar positive staining for Fas and FasL with HT, but rather weaker for FasL than HT. Immunostaining for Bcl-2 (prevalence and frequency) was similar in GD and TFA, but follicular cells in vicinity of lymphocytes and lymphocytes located in germinal centers of HT tissues exhibited weaker staining.

The concomitant upregulation of FasL in affected areas of HT tissues suggested that the Fas/FasL system was important to the apoptotic process in this setting and in the pathogenesis of HT. The co-expression of Fas and FasL by throcytes could lead them to a suicidal and/or fratricidal death, as shown for activated T lymphocytes (3). In contrast, the lymphocytes infiltrating the glands exhibited weaker staining for FasL than thyrocytes, which implied that infiltrating lymphocytes were not directly engaed in the killing of thyrocytes with their own FasL (not directly

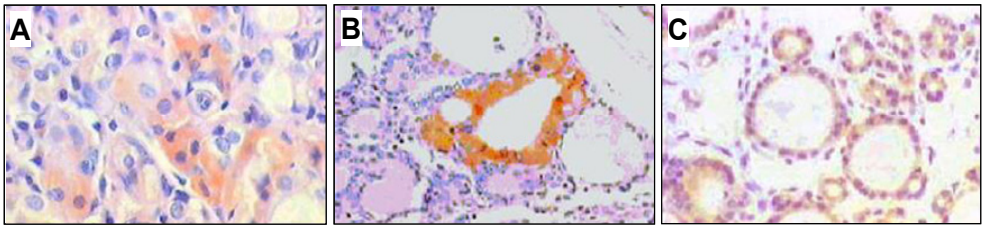

Figure 3. Representative staining pattern of Bcl-2 in Hashimoto's thyroiditis (A, ×400), Graves' disease (B, ×200) and thyroid follicular adenoma (C, ×200).

Table 1. *The prevalence of positive Fas, FasL and Bcl-2 in HT, GD and TFA.*

	Hashimoto's thyroiditis		Graves' disease		Normal thyrocytes
	Thyroid follicles	Lymphocytes	Thyroid follicles	Lymphocytes	TFA
Fas	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
FasL	100% (20/20)*	100% (20/20)	100% (20/20)*	100% (20/20)	85% (17/20)
Bcl-2	75% (15/20)	65% (13/20)	95% (19/20)**	95% (19/20)**	85% (17/20)

* Compared with TFA respectively, $p<0.05$;
** Compared with HT, $p<0.01$.

Table 2. *The frequency of positive Fas, FasL and Bcl-2 in HT, GD and TFA.*

	Hashimoto's thyroiditis		Graves' disease		Normal thyrocytes
	Thyroid follicles (%)	Lymphocytes (%)	Thyroid follicles (%)	Lymphocytes (%)	TFA (%)
Fas	76.31 ± 15.79	63.42 ± 22.85	74.26 ± 22.53	68.42 ± 22.85	75.75 ± 12.89
FasL	60.25 ± 26.51**	46.10 ± 25.80*	50.10 ± 28.36**	40.10 ± 26.10	26.08 ± 20.73
Bcl-2	9.75 ± 10.40$^{\#}$	6.78 ± 11.39*	14.30 ± 17.20	7.17 ± 16.73	14.80 ± 21.26

* Compared with thyroid follicles of HT, $p<0.05$;
** Compared with TFA, $p<0.01$;
$^{\#}$ Compared with GD and TFA, $p<0.05$.

involved in thyrocytes death during HT), but rather induced thyrocytes apoptosis *via* production of cytokines. The latter might stimulate the follicular cells to express high levels of Fas and/or FasL, autocrine/paracrine Fas-FasL interation of the thyroid follicular cells of HT was a major mechanism in HT autoimmune thyrocytes destruction. It was in agreement with the report of Batteux et al. (11).

In our study, it is very interesting that Fas and FasL were expressed on various follicular cells and lymphocytes. On the other hand, Bcl-2 expressed only on the normal or hyperplasic cells but not those destructed cells or hypoplasic cells.

These data suggested that in ATDs such as HT, elevated Fas/FasL-mediated apoptosis and down-regulated Bcl-2 might contribute to the pathophysiology, as a participant in the destruction of thyroid follicular cells in the disease. But, in GD, another ATD, the expression of Fas and FasL on thyrocytes was nearly as strong as that in HT, but the thyrocytes apoptosis was far less than that in HT and GD which was characterized by the hyperplasia of thyrocytes, showed mainly hyperthyroidism in fact. We considered it might be resulted from down-regulation of Fas/FasL interation by TSH or TSH receptor auto-antibody, and the higher expression of Bcl-2, which could render thyrocytes resistant to Fas/FasL-mediated apoptosis, might thus be involved in the pathogenesis of GD (1).

According to the results and conclusion of our study, if gene therapy of EAT by *in vivo* administration of plasmid DNA coding for FasL has a curative effect to avoid autoimmune thyroiditis by inducting death of infiltrating lymphocytes (11), it may be more beneficial that direct injection of DNA expression vectors encoding FasL into infiltrating T lymphocytes of HT to induce their death, and at the same time, injecting DNA encoding Bcl-2 into follicular cells of HT to avoid FasL-mediated apoptosis of thyrocytes.

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