Immunohistochemical Analysis of Bcl-2, Bax and Bak Expression in Thyroid Glands from Patients with Graves' Disease

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Abstract. In order to clarify the role of apoptosis and the expression of Bcl-2 family proteins in the pathology of Graves' disease (GD), we evaluated the apoptosis by *in situ* end-labeling of fragmented DNA and the expression of Bcl-2, Bax and Bak by immunohistochemistry in thyroid tissues from 20 patients with GD and in normal thyroid tissues from 6 patients with follicular adenoma (N). Apoptotic nuclei were found in thyrocytes and in germinal center of lymphoid follicles. Bcl-2 was strongly expressed in both GD and N thyrocytes. Bax was not expressed in either GD or N thyrocytes. Bak was expressed in thyrocytes from 5 of 20 patients with GD, while it was detected in all N thyrocytes. In lymphoid follicles Bcl-2 was expressed in the mantle zone, while Bax and Bak were both expressed in the germinal center. The percentage of apoptotic nuclei in GD thyrocytes was low (0–3.6%), and negatively correlated with the weight of the thyroid glands resected ($r_s = -0.43$, $P < 0.05$). It was greater in Bak-positive GD thyrocytes than in Bak-negative ones (mean $\pm$ SD; $1.7 \pm 0.7\%$ vs. $0.7 \pm 0.9\%$, $P < 0.05$). These findings suggest that the differential expression of Bcl-2 family proteins in both thyrocytes and lymphoid follicles may be involved in the pathology of GD.

Key words: Apoptosis, Bcl-2, Bax, Bak, Graves' disease

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APOPTOSIS is a type of cell death that has been shown to contribute to cell destruction and proliferation [1]. Fas/Fas ligand and Bcl-2 family interactions are important mechanisms to control apoptosis [1, 2]. The ratio of the expression of death antagonists (Bcl-2, Bcl-XL, Mcl-1) to death agonists (Bax, Bak, Bcl-Xs, Bad) determines the survival or death of cells in physiological and pathological conditions such as cancer growth [2–4], neuronal apoptosis following cerebral

artery occlusion [5] and regeneration of various cells [6, 7]. Apoptosis have been recently demonstrated to be involved in the pathogenesis of Hashimoto's thyroiditis [8–15], although the Fas ligand expression in thyrocytes is controversial [12–20]. Graves' disease (GD) is an autoimmune thyroid disorder and hyperplasia of thyroid glands by stimulation with anti-thyrotropin receptor antibody (TRAb) is a histological hallmark [21, 22]. We have previously reported the increased level of serum soluble Fas in patients with GD [23] and functional expression of Fas ligand in thyrocytes of GD patients [19], and may render thyrocytes resistant to Fas-mediated apoptosis. On the other hand, there are a few studies on the expression of Bcl-2 in GD [9–11, 14, 15, 19], and only one report on Bax expression [24], but no report regarding the expression

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of the other Bcl-2 family proteins.

The aim of the present study was to determine the presence of apoptosis and the expression of Bcl-2, Bax and Bak in thyroid tissues from patients with GD.

Materials and Methods

Thyroid tissue

We obtained thyroid specimens from 20 patients with GD (all women, aged 18 to 45 yr, mean age: 26 ± 8 yr). The diagnosis of GD was made by the presence of hyperthyroidism, detection of TRAb in serum and/or increased ^{123}I uptake ratio. Free T_3 , free T_4 , TSH, and anti-thyrotropin antibody (TRAb) were also determined, as previously reported [23, 25]. Anti-thyroglobulin antibody and anti-microsomal antibody were determined using commercial kits (HTG antibody kit, CIS Bio International, France; Serodia AMC, Fujirebio Inc., Tokyo, respectively). Clinical characteristics of

the patients were shown in Table 1. Normal thyroid tissue was also obtained from 6 patients with thyroid adenoma, all women aged 23 to 42 yr (34 ± 8 yr), and used as control. The study protocol was approved by the institutional review board of the Koike Hospital and informed consent was obtained from all subjects after the explanation of the study.

Detection of apoptosis

Apoptotic cells were detected by *in situ* end-labeling of fragmented DNA using assay kits (Apop TagTM, *in situ* apoptosis detection kit, peroxidase, Oncor Inc., Gaithersburg, MD) as previously described [26]. The paraffin-embedded tissue sections were deparaffinized by xylene and ethanol. Sections were then treated with proteinase K (20 $\mu\text{g}/\text{ml}$) for digesting protein in tissue. Digoxigenin-dUTP were added to the 3'-OH ends of DNA by terminated deoxynucleotidyl transferase (TdT). After incubating with anti-digoxigenin antibody conjugated with peroxidase, the sections were stained with

Table 1. Clinical characteristics of patients with Graves' disease and expression of Bax, Bak and Bcl-2 and apoptosis in thyrocytes

Case	Age (yr)	Sex	FT ₃ (pg/mL)	FT ₄ (ng/dL)	TRAb (%)	TgAb (U/ml)	MicAb	Duration of medication (months)	Current treatment	Weight of the resected thyroid (g)	Thyrocytes			
											Expression ^a of			Apoptosis ^b (%)
											Bax	Bak	Bcl-2	
1	20	F	2.4	0.3	44.9	1800	102400	7	MMI 30 mg	30.9	0	1	2	2.45
2	29	F	3.5	0.5	22.8	5.7	ND	108 ^c	MMI 20 mg	38.3	0	0	2	0.57
3	19	F	3.7	0.8	3.5	242	102400	4	β -blocker	33.6	0	0	2	0.63
4	36	F	7.4	1.7	38.7	1800	<100	7	PTU 450 mg	29.3	0	1	2	1.99
5	23	F	3.9	0.8	23.2	0.9	<100	8	PTU 200 mg	28.3	0	0	2	0.51
6	29	F	5.5	1.5	0.5	120.9	102400	1	PTU 300 mg	23.1	0	0	2	0.51
7	45	F	4.2	0.3	77.3	232	102400	8 ^c	MMI 15 mg	21.0	0	1	2	1.32
8	16	F	3.3	0.9	9.2	1.6	102400	5	PTU 150 mg	29.0	0	1	2	0.73
9	25	F	4.6	0.9	42.9	154.9	102400	2	PTU 300 mg	10.5	0	0	2	3.60
10	34	F	5.7	1.6	45.6	25.6	102400	4	PTU 450 mg	10.2	0	0	2	1.40
11	26	M	6.8	2.1	25.3	1.6	6400	3	MMI 20 mg	34.2	0	0	2	0.00
12	37	M	4.9	1.9	68.5	0.3	6400	2	PTU 600 mg	58.2	0	0	1	0.20
13	26	F	4.4	1.1	65.8	1.3	ND	1	PTU 150 mg	11.5	0	0	2	1.33
14	22	F	7.3	1.7	31.2	5.9	51200	4	MMI 30 mg	32.1	0	0	2	0.33
15	18	F	5.1	1.5	36.5	1800	102400	4	Lithium 1200 mg	45.2	0	0	2	0.40
16	23	M	3.5	0.7	40	301	6400	1	PTU 450 mg	23.7	0	0	2	0.66
17	22	F	3.5	1.0	17.0	750.4	102400	1	MMI 30 mg	18.2	0	0	2	0.33
18	19	M	2.1	0.7	70.2	11.0	102400	1	MMI 30 mg	52.0	0	2	2	1.80
19	29	F	4.8	1.6	NT	8.7	ND	12 ^c	MMI 30 mg	38.9	0	0	2	0.33
20	18	F	4.8	1.5	39.4	9.1	12800	3	MMI 30 mg	56.9	0	0	2	0.10

FT₃: free T₃, FT₄: free T₄, MMI: Methimazole, PTU: Propylthiouracil, TRAb: Anti-thyrotropin receptor antibody

TgAb: Anti-thyroglobulin antibody, MicAb: Anti-microsomal antibody, ND: not determined

^aExpression of Bax, Bak and Bcl-2 was assessed as strong positive (2), positive (1) or negative (0) staining by immunohistochemistry.

^bApoptosis was assessed as percentages of apoptotic nuclei of thyrocytes. ^cindicate the periods after the recurrence of GD.

diaminobenzidine (DAB) and were counterstained with methyl green. The mean percentage of positive staining of nuclei was calculated after counting positively or negatively stained nuclei of 500 thyrocytes in several fields under light microscope by 2 independent observers.

Immunohistochemistry

Immunohistochemical staining was performed using mouse monoclonal antibodies against human Bcl-2 (IgG1 κ ; Dako A/S, Glostrup, Denmark), Bak (IgG2a κ ; Oncogene Research Products, Cambridge, MA) and CD23 (MHM6, IgG1 κ , Dako), and rabbit polyclonal antibody against Bax (IgG; Oncogene), and mouse IgG1 (Dako), mouse IgG2a (Dako) and normal rabbit immunoglobulin (Dako) as negative controls. Positive reactivity was identified using a streptavidin-biotin-peroxidase detection system (Dako catalyzed signal amplification system, Dako) as previously reported [26]. Briefly, the paraffin-embedded tissue sections were deparaffinized, and then put in DAKO (R) Target Retrieval Solution (Dako) in order to amplify the signal, and placed in a hot water bath (95–97°C) for 20–40 min and then cooled for 20 min at room temperature. After blocking endogenous peroxidase activity with a 3% hydrogen peroxidase solution for 10 min, intrinsic biotin with an Endogenous Avidin/Biotin Blocking kit (Nichirei Corporation, Tokyo Japan), and nonspecific binding by serum-free protein, the tissue was incubated with prediluted mouse monoclonal antibodies (10 μ g/ml) or rabbit polyclonal antibodies (5 μ g/ml) for 15 min, followed by 15-min incubation with a biotinylated rabbit anti-mouse IgG + M + A antibody or goat anti-rabbit IgG antibody and for 15-min incubation with streptavidin-biotin-peroxidase complex, biotinyl tyramide and hydrogen peroxide, and streptavidin conjugated with peroxidase. Staining was completed with DAB for 5 min, and the specimens were counterstained with Mayer's hematoxylin for 2 to 5 min.

Statistical analysis

Differences of the expression of Bcl-2 family proteins and percentages of apoptotic cells between groups were evaluated using Mann-Whitney U test, Student's *t* test or Fisher's exact probability test. Correlations were assessed by Spearman's signed ranks test. A

probability level of less than 0.05 was considered significant.

Results

Apoptotic cells in thyroid tissues

Apoptotic nuclei were observed in thyrocytes (Fig. 1A) and in the germinal center of lymphoid follicles (Fig. 1B). In normal thyroid glands, apoptotic nuclei were rarely found in follicular cells (Fig. 1C). The mean percentage of apoptotic thyrocytes tended to be greater in GD than in N, but the difference was not statistically significant ($1.0 \pm 0.9\%$ in GD vs. $0.4 \pm 0.3\%$ in N, Mann-Whitney U test, $P = 0.14$).

Expression of Bcl-2, Bax and Bak in thyroid tissues

Bcl-2 was expressed in the cytoplasm of thyrocytes from both GD (Fig. 2A) and N (Fig. 2E). Bak was expressed in all N thyrocytes (Fig. 2F), while it was not detected in thyrocytes from 15 out of 20 GD patients (Fig. 2B). Bax was not expressed in thyrocytes from either GD (Fig. 2C) or N (Fig. 2G).

In lymphoid follicles, Bcl-2 was expressed in the mantle zone (Fig. 2I), while both Bax and Bak were expressed in the germinal center (Fig. 2J, 2K). CD23-positive cells were detected in the germinal center (Fig. 2L).

Relation of apoptosis and Bcl-2 family protein expression in thyroid to the clinical parameters of GD

The percentage of apoptotic cells was negatively correlated with the weight of resected thyroid glands, with free T_4 level (Fig. 3). The percentage of apoptotic cells was significantly greater in Bak-positive specimens than Bak-negative specimens ($1.7 \pm 0.7\%$ vs. $0.7 \pm 0.9\%$, respectively, Table 2). Other clinical parameters including TRAb did not relate with apoptosis or expression of Bcl-2, Bak or Bax of thyrocytes.

Discussion

We have addressed the involvement of apoptosis and the expression of Bcl-2 family proteins, namely, Bcl-2, Bax and Bak, in thyroid tissue from patients

Fig. 1. *In situ* end-labeling of fragmented DNA in thyroid tissues from patients with GD (A, B) and a control subject (C). Arrows indicate apoptotic nuclei.

Fig. 2. Immunohistochemical staining with Bcl-2 (A, E, I), Bak (B, F, J), Bax (C, G, K), CD23 (L) and control immunoglobulins (D, mouse IgG2; H, normal rabbit immunoglobulin) in thyroid tissues from patients with GD (A~D, I~L) and a control subject (E~H). In lymphoid follicles, Bcl-2 protein was detected in the mantle zone, while CD23-positive cells were detected in the germinal center.

Fig. 3. Relationships between apoptosis of thyroid follicular cells and clinical parameters in Graves' disease (A, weight of the resected thyroid glands; B, serum free T_4 level).

Table 2. Relationships between Bak expression in thyrocytes and clinical parameters of Graves' disease.

	Bak expression in thyrocytes		P value
	Positive	Negative	
Apoptosis (%)	1.66 ± 0.66	0.73 ± 0.89	$P < 0.05$
Weight of the resected thyroid glands (g)	32.4 ± 11.6	30.9 ± 15.3	NS
Free T_3 (pg/mL)	3.9 ± 2.1	4.8 ± 1.2	NS
Free T_4 (ng/dL)	0.78 ± 0.58	1.28 ± 0.48	$P = 0.07$
TRAb (%)	48.1 ± 27.2	33.0 ± 19.9	NS

Values are expressed as mean ± SD.

Apoptosis are assessed as percentages of apoptotic nuclei of thyrocytes. TRAb: anti-thyrotropin receptor antibody

NS: not significant

with GD. In autoimmune thyroid diseases, the increased apoptosis has been reported in Hashimoto's thyroiditis [8–14], while it has been controversial in GD [8–11, 18, 19, 22]. In the present study we demonstrated apoptotic nuclei in thyroid follicular cells. The percentage of apoptotic follicular cells ranged from 0% to 3.6%. It was smaller compared to Hashimoto's thyroiditis [8–13], and statistically not different from normal. Similar values were reported by Hammond *et al.* (<1.0–3.5%) [11], whereas Kotani *et al.* [8] reported much higher values (7–14%), and Okayasu *et al.* (0–5/10⁵) [10] and Tanimoto *et al.* (0%) [9] reported smaller values. Recently Labat-Moleur *et al.* [24] reported that apoptosis was more often found in thyroid parenchyma showing epithelial hy-

perplasia, cellular hypertrophy and little colloid, than that returning to regular shape lined by cuboidal or even flat cells and densely filled with colloid. Therefore, the discrepancies among the reports might be attributed to the differences in treatment for GD (anti-thyroid drugs or ¹³¹I therapy) or disease activity of GD. Furthermore, we demonstrated that the mean percentage of apoptotic follicular cells was negatively correlated with weight of the resected thyroid glands and with free T_4 level. These results suggest that hyperplasia may be inversely related with apoptosis of thyrocytes, although the preoperative administration of iodine and anti-thyroid drugs might also affect the results. Vitale *et al.* [27] reported that iodide excess induces apoptosis in human thyroid cells through a p53-independent mechanism. The close relationship between apoptosis and goiter formation in rat model supports our observation in human [28]. Kawakami *et al.* [29] reported that GD immunoglobulins inhibited the Fas-mediated apoptosis *in vitro*. However, we could not show a correlation between apoptosis of thyrocytes and serum TRAb levels.

It is known that Bcl-2 family proteins regulate apoptosis [2]. The ratio of the expression of death antagonists (Bcl-2, Bcl-XL) to death agonists (Bax, Bak) determines the survival or death of cells in physiological and pathological conditions [2]. The present study confirmed that normal thyroid follicular cells expressed Bcl-2 [9–11, 13] and Bak [30], suggesting that Bcl-2 and Bak expression in normal thyroid follicular cells

may regulate apoptosis in the physiological state. There is no report on the expression of Bak in GD thyrocytes. The present study showed that Bak was expressed in 5 of 20 GD thyroid glands. Although there were no correlations between Bak expression in thyrocytes and free T₃, free T₄ or TRAb levels, the mean percentage of apoptotic thyrocytes was significantly greater in Bak-positive thyroid glands than in Bak-negative thyroid glands. However, there was no difference in the resected weight of thyroid glands between Bak-positive and Bak-negative groups. The failure of the relation of Bak expression with weight of the thyroid or free T₄ levels may be due to the small number of patients or small percentage of apoptotic nuclei. Therefore, it remains unclear whether the down-regulation of Bak expression in GD contributes to the hyperplasia which is characteristic of GD. Future analysis on the regulation of the expression of Bcl-2 family proteins in thyrocytes may provide a new insight into the pathophysiology of GD.

With respect to the Bax expression in thyroid follicles, Labat-Moleur *et al.* [24] reported that Bax was localized as a paranuclear dot in both GD and normal thyrocytes and also expressed finely and diffusely during early apoptosis, while neither the previous reports [3, 4] nor the present study found Bax expression in normal thyrocytes. The discrepancies among the reports might be due to the differences in treatment for GD or disease activity and to the technical differences of tissue fixation and/or staining procedures including antibodies against Bax.

This is the first report on the expression of Bcl-2

family proteins in lymphoid follicles in GD. Bax and Bak, apoptotic agonists, were expressed in the germinal center of lymphoid follicles in all patients with GD, while Bcl-2, an apoptotic antagonist, was expressed in the mantle zone of lymphoid follicles. By contrast, apoptotic nuclei were more often found in the germinal center. These findings were similar to findings on tonsils reported by Martinez-Valdez *et al.* [31]. They reported the expression of Bax, but not Bcl-2, in the germinal center of tonsils. Since germinal center formation is a dynamic process during the humoral immune response [32], the differential expression of Bcl-2 family proteins in lymphoid follicles may play a role in the humoral immune process in GD.

In conclusion, the expression of Bak in thyrocytes may be related to the sensitivity to apoptosis of GD thyrocytes. However, it remains unclear whether the down-regulation of Bak expression in GD reduces the apoptosis of thyrocytes and thus allows the hyperplasia of thyroid glands stimulated with TRAb associated with GD. It is also uncertain whether the differential expression of Bcl-2 family proteins in lymphoid follicles plays a role in the formation of the germinal center in GD. Further studies are indicated to clarify these issues.

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