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Enhanced cellular proliferative activity and cell death in chronic thyroiditis and thyroid papillary carcinoma

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Abstract For the analysis of cellular proliferative activity and cell death in thyroid diseases, the Ki-67 labeling index, bcl-2 protein expression and cell death of follicular epithelia by immunohistochemistry and in situ DNA nick-end labeling methods were evaluated in normal thyroid tissues as well as in surgical specimens from cases of Hashimoto's disease (16 cases), focal lymphocytic thyroiditis (13 cases), Graves' disease (15 cases), follicular adenoma (20 cases) and papillary carcinoma (43 cases). Cellular proliferative activity and cell death were both enhanced in cases of thyroiditis, including Hashimoto's disease and focal lymphocytic thyroiditis. Thyroids from patients with follicular adenoma and papillary carcinoma also showed increased cellular proliferative activity and cell death. In addition, predominant high cellularity and partial loss of bcl-2 protein expression in papillary carcinoma suggested that the overgrowth and dedifferentiation were associated with malignancy.

Key words Thyroiditis · Papillary carcinoma · Cell proliferation · Ki-67 · Cell death · Apoptosis · bcl-2 · Cellularity

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Introduction

In addition to cellular proliferative activity (Shimizu et al. 1993), cell death is an important consideration in tumor development (Bowen and Sarraf 1986; Arends et al. 1994) and the pathogenesis of autoimmune inflammation. Furthermore, the balance between the two competing phenomena, cellular proliferative activity and death, provides important information about epithelial cell growth (Gerdes et al. 1984; Shaw et al. 1992). The recent development of a Ki-67 immunostaining method made it possible to identify cellular proliferative activity in histological sections (Brown and Gatter 1990; Barbareschi et al. 1994). On the other hand, apoptotic cell death is demonstrated by using an in situ DNA nick-end labeling method (Gavrieli et al. 1992). Moreover, it has been reported that bcl-2 protein down-regulates apoptotic cell death resulting in long-lived post-mitotic cells (Hockenbery et al. 1991; LeBrun et al. 1993). It is, therefore, worthwhile to follow bcl-2 protein expression of follicular epithelia in relationship to cell proliferation and death in thyroid tumors and chronic lymphocytic thyroiditis.

Recently we have determined a definite association between chronic lymphocytic thyroiditis and papillary carcinoma in surgically resected thyroids of Japanese and white and black Americans. The possibility was then raised that autoimmune thyroiditis may predispose to development of papillary thyroid carcinoma (Okayasu et al. 1995).

In this paper, we examine the possibility that chronic thyroiditis may induce malignant transformation by enhancement of cell mitosis and proliferation of follicular epithelia via cell death due to chronic lymphocyte infiltration. For this purpose, the correlation between cellular proliferative activity and cell death was compared in various thyroid lesions, including Hashimoto's disease, focal lymphocytic thyroiditis, Graves' disease, follicular adenoma, and papillary carcinoma, by measuring Ki-67 by immunohistochemistry and apoptosis by in

situ DNA nick-end labeling. In addition, bcl-2 protein expression was analyzed immunohistochemically for its possible role in regulating cellular proliferative activity and cell death in these diseases of the thyroid.

Materials and methods

Cases

A total of 108 cases of surgically resected thyroid glands were selected and reviewed from Departments of Pathology located at Kitasato University Hospital and Tokyo Medical and Dental University Hospital. Histological observations were performed on 4-μm-thick sections of 10% formalin-fixed and paraffin-embedded tissues. The specimens collected were classified into six lesions, according to the WHO criteria (Hedinger and Sobin 1974). Normal thyroid tissues without remarkable changes were selected from 14 thyroids resected for possible follicular adenoma. Of the remaining specimens, 16 represented Hashimoto's disease (struma lymphomatosa), 13 focal lymphocytic thyroiditis, 15 Graves' disease, 20 follicular adenoma, and 43 papillary carcinoma. Waterhouse and Doniach's histological criteria for focal lymphocytic thyroiditis were used; i.e., more than 40 foci per standard representative section (2 cm^2). A 'focus' was defined as an aggregate of 50 or more lymphocytes. Adenomatous goiter, follicular carcinoma, and medullary carcinoma were excluded from this study.

Cellularity

As a measure of cellularity, epithelial cells in 4-μm-thick sections stained by hematoxylin/eosin were counted under the light microscope. The number of epithelial cells per 0.25 mm^2 was described in each case.

Immunohistochemistry

To detect the presence of Ki-67-positive cells and bcl-2 protein in the specimens examined, microwave-processing pretreatment in citrate buffer (pH 6.0) was carried out three times for 5 min each. The conventional streptavidin/biotin method was used for detection of Ki-67-positive cells in 4-μm-thick-sections of formalin-fixed and paraffin-embedded tissues. Briefly, after routine deparaffinization and blocking of endogenous peroxidase with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min at room temperature, sections were incubated overnight at 4° C with rabbit anti-(human Ki-67) (× 150 dilution, Dako, Copenhagen, Denmark). After washing with phosphate-buffered saline (PBS), pH 7.4, streptavidin/biotin/peroxidase (Histofine SAB kit, Nichirei, Tokyo, Japan) was used. Counterstaining for nuclei was achieved with 0.3% methyl green solution. Ki-67 labeling indices were determined by random counting of 1000 epithelial nuclei. For the detection of bcl-2 protein, the sandwich detection method was used (Toth et al. 1994). Briefly, 4-μm-thick deparaffinized sections were incubated overnight at 4° C with mouse monoclonal anti-(bcl-2 protein) (× 100 dilution, Dako) after pretreatment to block endogenous peroxidase. After washing with PBS, the sections were each incubated, at room temperature for 30 min, with the peroxidase-conjugated $F(ab')_2$ fragment of rabbit anti-(mouse IgG) (× 25 dilution, Dako) peroxidase/antiperoxidase complex (mouse monoclonal) (× 100 dilution, Dako), and peroxidase-conjugated $F(ab)_2$ fragments of rabbit anti-(mouse IgG) (× 25 dilution) respectively. After washing with PBS, the diaminobenzidine reaction was performed for color development. Counterstaining of nuclei was achieved with 0.3% methyl green solution. Positivity of bcl-2 protein in follicular epithelia was defined as diffusely positive, less diffusely positive, scattered positive and negative.

Cell death

Death of epithelial cells was determined indirectly by detection of apoptotic cells in 4-μm-thick deparaffinized sections (Barbareschi et al. 1994). For apoptosis, the in situ DNA nick-end labeling method (Apop Tag in situ apoptosis detection kit peroxidase; Oncor, Gaithersburg, USA) was used. Briefly, after deparaffinization and blocking of endogenous peroxidase with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min at room temperature, incubation with 100 μg/ml proteinase K (Sigma, USA) was performed for 15 min at room temperature. After prehybridization, terminal deoxynucleotidyltransferase with digoxigenin-11-dUTP and dATP was applied to the sections, with incubation in a moist chamber for 60 min at 37° C. Anti-digoxigenin-antibody/peroxidase was employed for 30 min at room temperature to detect digoxigenin-11-dUTP labeling, followed by color development with 3,3'-diaminobenzidine containing H_2O_2 solution. Counterstaining was achieved with 0.3% methylgreen solution. The cell death rate was calculated after counting, in each case, 100000 epithelial nuclei in randomly selected fields under the light microscope.

Statistical analysis

The statistical significance of the difference between two groups was determined by Student's *t*-test.

Results

Cellular proliferative activity

The Ki-67 labeling index (the number of Ki-67-positive cells per 1000 epithelial nuclei) was compared for the six groups (Figs. 1, 2). A significant increase of labeling index was observed in the specimens of all of the thyroid diseases, including Hashimoto's disease, focal lymphocytic thyroiditis, Graves' disease, follicular adenoma, and papillary carcinoma, when compared to normal thyroid tissues. In particular, thyroiditis, including Hashimoto's disease or focal lymphocytic thyroiditis, showed a significant increase of Ki-67 labeling. Furthermore, an increase of labeling index was seen in both neoplastic lesions of follicular adenoma and papillary carcinoma, being significantly higher ($P < 0.05$) in papillary carcinoma than in follicular adenoma.

Cell death

The cell death rate in various lesions was determined by calculating the number of apoptotic cells per 100000 epithelial nuclei (Figs. 3, 4). The cell death was significantly enhanced ($P < 0.001$) in Hashimoto's disease, focal lymphocytic thyroiditis, follicular adenoma, and papillary carcinoma, when compared to normal

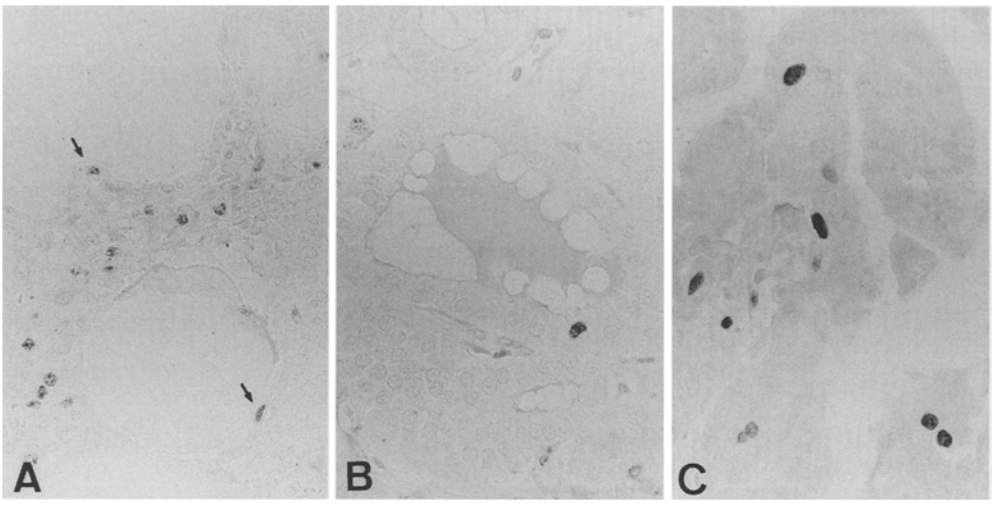

Fig. 1A–C Ki-67-positive cells in Hashimoto's disease (**A**), follicular adenoma (**B**), and papillary carcinoma (**C**). Note the positive nuclei of follicular epithelia (*arrows*) and lymphoid cells (**A**), and scattered positive nuclei of tumor cell (**B**, **C**) (streptavidin/biotin immunostaining with anti-Ki-67 antibody, × 540)

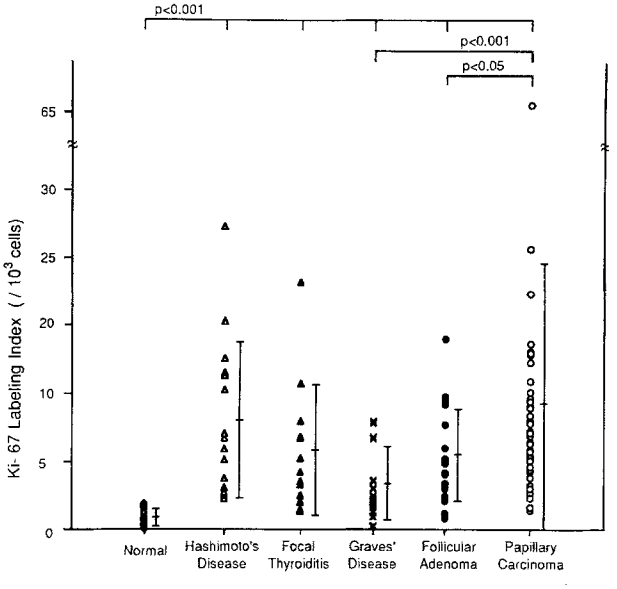

Fig. 2 Ki-67 labeling index of epithelia in thyroid diseases. The labeling index is significantly higher ($P < 0.001$) in all of the thyroid diseases than in normal thyroid tissue. Papillary carcinoma shows a significantly higher Ki-67 labeling index than Graves' disease ($P < 0.001$) and follicular adenoma ($P < 0.05$)

thyroids. Cell death was particularly noticeable in thyroiditis, including Hashimoto's disease and focal lymphocytic thyroiditis. Papillary carcinomas showed significantly higher cell death rates than follicular adenomas ($P < 0.05$). No significant increase of cell death was observed in Graves' disease when compared to normal thyroids.

Cellularity

Cellularity was determined by counting the epithelial nuclei per 0.25 mm^2 under the light microscope (Fig. 5). Cellularity was significantly higher ($P < 0.001$–0.05) in all cases of thyroid diseases, including Hashimoto's disease, focal lymphocytic thyroiditis, Graves' disease, follicular adenoma, and papillary carcinoma than in nomal thyroids. Papillary carcinoma showed a particularly high cellularity compared with the five other lesions described.

bcl-2 protein expression

Baseline levels of bcl-2 protein were expressed in the thyroid follicular epithelia (Fig. 6; Table 1). However, follicular epithelia negative for bcl-2 protein expression were found in a focal distribution in Hashimoto's disease, focal thyroiditis, and Graves' disease. Cells degenerating through lymphocyte infiltration were negative for bcl-2 protein in these thyroid diseases. The appearance rate of epithelia positive for bcl-2 protein decreased in follicular adenoma and papillary carcinoma. It is of special note that, of 43 cases of papillary carcinoma, 8 were negative, 8 were scattered positive, and 27 were less diffusely positive.

Discussion

Ki-67-positive cells in thyroid tissues were found in very low numbers, particularly in normal thyroid tissues. This observation is common in the endocrine organs, in which the basal cellular proliferative activity is very low (Shimizu et al. 1993). In comparison with normal thyroid epithelia, the significant increase of Ki-67 labeling index of follicular epithelia and neoplastic cells in chronic thyroiditis (Hashimoto's disease and focal lymphocytic thyroiditis), follicular adenoma, and papillary carcinoma reflects enhanced cellular

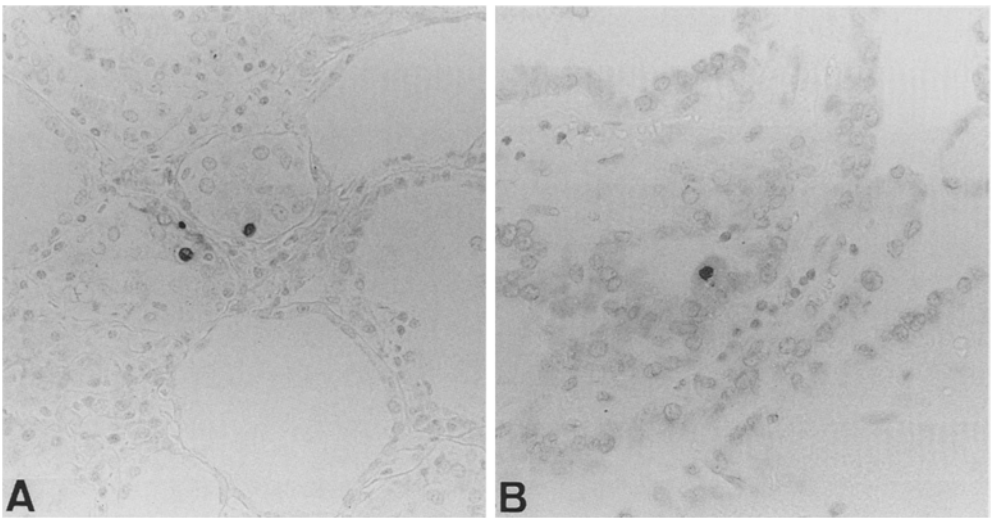

Fig. 3A,B Cell death in Hashimoto's disease (**A**) and papillary carcinoma (**B**). Single, epithelial cell death is scattered in Hashimoto's disease and papillary carcinoma (in situ nick-end labeling method, × 550)

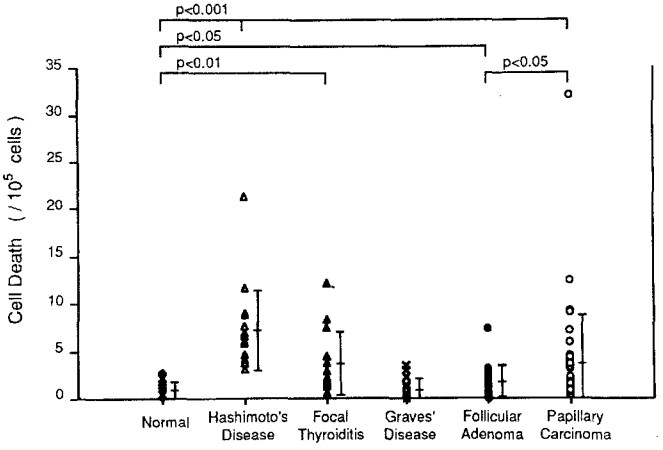

Fig. 4 Cell death rate in thyroid diseases. Epithelial cell death rate is significantly higher ($P < 0.05$–0.001) in Hashimoto's disease, focal lymphocytic thyroiditis, follicular adenoma, and papillary carcinoma than in normal epithelia. Papillary carcinoma shows a significantly higher cell death rate ($P < 0.05$) than follicular adenoma

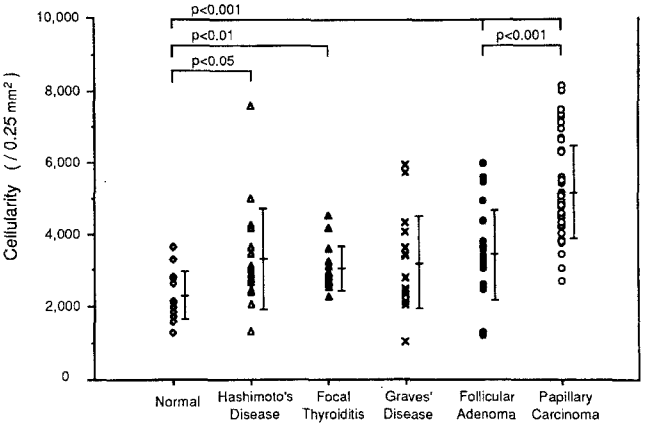

Fig. 5 Cellularity of epithelia in thyroid diseases. The cellularity of epithelia is significantly higher in all of the thyroid diseases, including Hashimoto's disease ($P < 0.05$), focal lymphocytic thyroiditis ($P < 0.01$), Graves' disease ($P < 0.05$), follicular adenoma ($P < 0.001$), and papillary carcinoma ($P < 0.001$) than in normal epithelia. Papillary carcinoma shows a significantly higher cellularity ($P < 0.001$) than follicular adenoma

proliferative activity. With respect to chronic thyroiditis, the enhanced cellular proliferative activity suggests attempts at regeneration of follicular epithelia following cell death or damage due to lymphocytic infiltration and autoimmune damage. On the other hand, both follicular adenoma and papillary carcinoma show the enhanced cellular proliferative activity characteristic of neoplasia. Furthermore, it is plausible that papillary carcinoma was significantly higher in its Ki-67 labeling index than was follicular adenoma, because of its record of positive correlation with activity or malignancy of cancer in other organs (Mcgurrin et al. 1987; Weidner et al. 1994). The relatively low value of the Ki-67 labeling index in thyroid papillary carcinoma compared to the indices of carcinomas of other organs, particularly of the gastrointestinal tract (Shepherd et al. 1988), suggests that well-differentiated papillary carcinomas of the thyroid are slow-growing tumors. Thus, these results confirm clinical observations on the growth of thyroid papillary carcinomas.

For an indirect estimate of cell death, apoptotic cell death was determined by the in situ DNA nick-end labeling method, which has been described by Gavrieli et al. (1992). This method utilizes terminal transferase to incorporate biotinylated nucleotides, thereby permitting the in situ visualization of apoptotic cell nuclei containing fragmented DNA. According to this method, values of apoptotic cell death rate show indirect cell death in histological sections (Bursch et al. 1990). Usually, apoptotic cell nuclei in histological

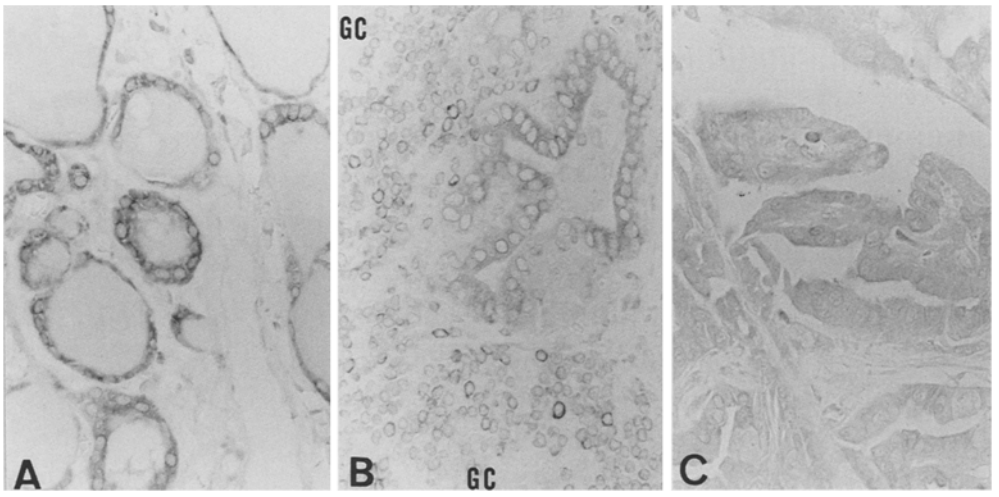

Fig. 6A–C bcl-2 protein expression of the normal thyroid epithelia (**A**), in Hashimoto's disease (**B**), and in papillary carcinoma (**C**). bcl-2 expression is constantly seen in the normal follicular epithelia (**A**), possibly suggesting regeneration (**B**), *GC* germinal center. (**C**) Partial loss or reduction of bcl-2 expression is identified in papillary carcinoma (sandwich immunostaining with anti-bcl-2 antibody, × 530)

Table 1 bcl-2 Protein expression in the follicular epithelium of the thyroid gland. *Diffusely positive* diffusely positive cells, *Less diffusely positive* rather diffusely positive cells but a few negative cells, *Scattered positive* few positive cells. The number indicates the number of cases

Normal	Hashimoto's disease	Focal thyroiditis	Graves' disease	Follicular adenoma	Papillary carcinoma
Diffusely positive: 14	Less diffusely positive: 16	Less diffusely positive: 13	Less diffusely positive: 15	Diffusely positive: 12	Less diffusely positive: 27
				Less diffusely positive: 8	Scattered positive: 8
					negative: 8

sections were detected in a dispersed pattern and cell death was not frequently found in normal thyroid tissues with this method. Cell death was enhanced in Hashimoto's disease, focal lymphocytic thyroiditis, follicular adenoma, and papillary carcinoma. In both Hashimoto's disease and focal thyroiditis, the enhancement of cell death is considered to be caused by lymphocytic infiltration targeting follicular epithelia due to autoimmune phenomena. Accordingly, enhanced cell death in chronic thyroiditis might include cell necrosis as well as apoptotic cell death. On the other hand, in neoplastic lesions, including follicular adenoma and papillary carcinoma, the enhanced apoptosis contributes to the high rates of cell death. This proclivity for enhanced apoptotic cell death in tumors is similar to results reported by others concerning neoplasms in the stomach (Kasagi et al. 1994; Saegusa et al. 1995) and breast (Allan et al. 1992).

It is likely that the cell cycle is quite slow and that cell death, occurring at low frequency in thyroid tissue, is kept at a constant state; accordingly, both the Ki-67 labeling index and the cell death rate were very low in the present study. From the viewpoint of a correlation between cellular proliferative activity and cell death, it is conceivable that the enhanced proliferative activity in chronic thyroiditis occurs as a reaction to the high cell death rate by epithelial destruction due to autoimmune mechanisms. Thyrotropin stimulation, possibly due to hypothyroidism in lymphocytic thyroiditis, might have a role in the enhancement of proliferative activity, although no information concerning thyroid function was available in the present study (Tunbridge et al. 1981; Lazarus et al. 1984; Rosenthal et al. 1987). Furthermore, both enhanced cellular proliferative activity and apoptotic cell death in neoplasia are generally accepted phenomena, as documented in carcinoma of the breast and the gastrointestinal tract. This positive correlation of malignant neoplasms and apoptosis was a consistent observation.

Although follicular epithelia showed a significant increase of Ki-67 labeling index in Graves' disease over normal thyroid tissues, the apoptotic cell death rate was almost the same as that in normal thyroid tissue. Apoptosis might depend on whether chemotherapy was applied before surgical removal. In the present study, cases of Graves' disease were not reviewed with respect to chemotherapy.

The cellularity of follicular epithelia in histological sections revealed a significant increase in all varieties of thyroid diseases, including Hashimoto's disease, focal

lymphocytic thyroiditis, Graves' disease, follicular adenoma, and papillary carcinoma in comparison with normal thyroid tissues. This might explain the greater or lesser enlargement of the thyroid usually seen in these diseases of the thyroid. Papillary carcinoma, in particular, shows the overgrowth as a malignant neoplasm with a predominantly high cellularity.

Baseline levels of bcl-2 protein are expressed in the thyroid follicular epithelia. This suggests that thyroid epithelia require strict protection from cell death, including apoptosis, as reported in the literature (Korsmeyer 1992; Gompel et al. 1994; Haldar et al. 1994; Pietenpol et al. 1994; Pilotti et al. 1994). Accordingly, apoptotic cell death was rarely found in normal thyroid tissues in the present study. Its expression, however, was lost in degenerated follicular epithelia associated with lymphocytic infiltration in chronic thyroiditis including, Hashimoto's disease and focal lymphocytic thyroiditis. Inversely, regenerating follicular epithelia expressed bcl-2 protein in their cytoplasms as clearly as did the differentiating epithelia of skin or intestines and estrogen-receptor-positive breast tissue (Bhargava et al. 1994). In particular, papillary carcinoma showed a focal or partial loss of its expression. This result suggests that some of the thyroid cancer cells escaped from the cell control by bcl-2 regulation, resulting in the overgrowth of epithelial cells.

In cases of papillary carcinoma, bcl-2 expression appeared to be down-regulated. The apoptotic cell death rate was significantly enhanced. This result corresponds with the fact that the *bcl-2* gene inhibits apoptosis. The abnormal activation of *bcl-2* has been described to be an early event that can inhibit apoptosis in vivo and accelerate tumor progression in colorectal tumorigenesis (Sinicrope et al. 1994; Bronner et al. 1995). Further, an inverse correlation was described between bcl-2 and p53 protein expression in colorectal adenomas and breast cancer cells (Sinicrope et al. 1994; Halder et al. 1994). In breast cancer, bcl-2 protein is frequently expressed, and its expression is associated with favorable clinicopathological features (Joensuu et al. 1994). An association of bcl-2 down-regulation with progression of thyroid cancer should be discussed with further analysis in several different categories such as tumor size, invasive growth, and lymph node metastasis. On the other hand, bcl-2 expression correlates well with cell differentiation (LeBrun et al. 1993; Novack and Korsmeyer 1994). Accordingly, the down-regulation of bcl-2 expression in thyroid papillary carcinoma might correlate with dedifferentiation of thyroid follicular epithelial cells (Pilotti et al. 1994). Further analysis to detect changes of other regulatory factors, including Bax (bcl-2-associated X protein) expression, p53 mutation, and thyrotropin receptor expression for thyroid cancer cells is necessary for this consideration.

In conclusion, our results revealed enhanced cellular proliferative activity and cell death in chronic thyroiditis, including Hashimoto's disease and focal lymphocytic thyroiditis and thyroid neoplasms, particularly papillary carcinoma. The results also indicated that the cell cycle of follicular epithelia in chronic thyroiditis is accelerated by the enhanced cell death due to lymphocytic infiltration. Further studies are needed to determine possible genetic changes, including oncogene expression and genetic instability, in thyroiditis and thyroid carcinoma for the evaluation of causal relationships in association with both lesions.

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