

Expression of CD40 and Apoptosis Related Molecules in Autoimmune Thyroid Diseases

Jeong-Hae Kie¹, Min-Sun Cho², and Woo-Ick Yang¹

¹Department of Pathology, Yonsei University College of Medicine, Seoul, Korea;

²Department of Pathology, Ehwa Woman's University College of Medicine, Seoul, Korea.

Apoptosis is responsible for the loss of thyrocytes in autoimmune thyroiditis. Recent investigations into the pathogenesis of apoptosis have revealed that the important roles of suicide molecules expression on both thyrocytes and cytotoxic T-lymphocytes. To study the mechanism of thyrocyte loss in various forms of thyroiditis, we evaluated in situ expression patterns of CD40, Fas, and Fas-L on thyrocytes and infiltrating inflammatory cells by immunohistochemical staining of thyroid samples obtained from 49 patients (Graves' disease, n=10; Hashimoto's thyroiditis, n=14; nonspecific lymphocytic thyroiditis, n=11; subacute granulomatous thyroiditis, n=11; normal, n=3). The role of cytotoxic T-lymphocytes was also evaluated by analyzing the expression of granzyme B along with their phenotypic characteristics. CD40 was not expressed on thyrocytes of normal controls while they showed a diffuse expression of Fas and a scattered focal expression of Fas-L. The plump thyrocytes proximal to the inflammatory infiltrates showed more intense expressions of these three molecules in various forms of thyroiditis and a close correlation was found between CD40 and Fas-L expression on thyrocytes. Unlike Fas, which was expressed on infiltrating lymphocytes in all groups, Fas-L was not expressed on infiltrating lymphocytes, except those in subacute granulomatous thyroiditis. Granzyme B expressing activated cytotoxic T-lymphocytes occupied a negligible proportion of CD8+ T-lymphocytes in various forms of thyroiditis, and no difference was found in terms of their proportions according to the type of thyroiditis. These results show the acquisition of CD40, Fas and Fas-L molecules on thyrocytes proximal to inflammatory cell aggregates

and the negligible expression of granzyme B and Fas-L on the infiltrating lymphocytes, and suggest that Fas and Fas-L mediated apoptosis of thyrocytes (fratricide) may be more important than T cell-mediated cytotoxicity in various forms of thyroiditis.

Key Words: CD40, Fas, Fas-L, granzyme-B, apoptosis, thyroiditis

INTRODUCTION

Apoptosis and necrosis are two different pathways of cell death. Necrosis is a passive phenomenon caused by cell damage, and is characterized by the loss of membrane integrity, cell swelling and eventual cell lysis. In contrast, apoptosis is an organized active phenomenon. Morphologically, apoptosis shows membrane blebbing, cell shrinkage by condensation of cytoplasm and chromatin fragmentation with the ultimate formation of apoptotic bodies. Although apoptosis plays an important role in a multitude of normal physiologic functions, such as embryogenesis, morphogenesis, and immune regulation, it is also involved many pathologic conditions. There is growing evidence to suggest that apoptosis plays an important role in autoimmune diseases including SLE, scleroderma, and diabetes mellitus.¹⁻³ As is the case in the aforementioned autoimmune diseases, apoptosis is now well established as the main mechanism of cell death in various forms of thyroiditis. Several studies show that apoptotic thyrocytes are increased in Hashimoto's thyroiditis while they are decreased in Graves' disease.⁴⁻⁶ There are two major underlying mechanisms of parenchymal

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Reprint address: requests to Dr. Woo-Ick Yang, Department of Pathology, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea. Tel: 82-2-361-5262, Fax: 82-2-362-0860, E-mail: wiyang9660@yumc.yonsei.ac.kr

cells apoptosis in autoimmune diseases; one Fas-based and the other perforin-granzyme-based. Fas and Fas-L expression on thyrocytes has been reported using various detection methods, but there are many discrepancies about their degrees of expression as well as their roles in the autoimmune process.⁶⁻¹⁰ Therefore, in this study we evaluated the *in situ* expression pattern of CD40, Fas, and Fas-L on thyrocytes and infiltrating inflammatory cells along with their phenotypic characteristics to study the underlying mechanisms of apoptosis in various forms of thyroiditis.

MATERIALS AND METHODS

Thyroid tissue

This study was performed upon 49 thyroid samples from patients with Graves' disease ($n = 10$), Hashimoto's thyroiditis ($n = 14$), nonspecific lymphocytic thyroiditis ($n = 11$ cases), and subacute granulomatous thyroiditis ($n = 11$). Three of normal controls were included. The clinical findings and laboratory data were reviewed and clinicopathologic correlation performed. Graves' disease (GD) was defined as: an elevated T4 and T3, a depressed TSH level, the presence of ophthalmopathy and dermopathy, and positivity for autoantibody. Hashimoto's thyroiditis (HT) was defined as: a depressed T4 and T3, an elevated TSH level, high titers of auto-antibodies (especially to thyroglobulin or thyroid peroxidase), and the presence of dense parenchymal lymphocytic infiltrates. Nonspecific lymphocytic thyroiditis (NLT) was defined as: histologic findings of scattered lymphocytic aggregates without clinical or laboratory findings supporting the diagnosis of GD or HT. Subacute granulomatous thyroiditis (SGT) was histologically defined by granulomatous thyroiditis without evidence of infectious microorganisms on special stains (Ziehl-Neelsen and Periodic Acid Schiff stains). Normal thyroid tissue without inflammatory cell infiltrates was obtained as a control from 3 patients who received lobectomy due to follicular adenoma.

Immunohistochemical stain

Immunohistochemical staining was performed using rabbit polyclonal antibodies against human Fas (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), Fas-L (Santa Cruz Biotechnology Inc.), CD40 (Santa Cruz Biotechnology Inc.), and mouse monoclonal antibodies against CD4 (Novocastra Laboratories Ltd. Newcastle upon Tyne, UK), CD8 (Novocastra Laboratories Ltd.), T-cell restricted intracellular antigen (TIA, Coulter Immunology, Hialeah, FL, USA), and granzyme B (Monosan, Am Uden, Netherlands). Immunoreactivity was identified using an EnVision™ kit (Dako Corp. A/S, Glostrup, Denmark) for CD40, Fas and Fas-L and a Universal LSAB2 kit (Dako Corp.) for monoclonal antibodies. Microwave-based epitope retrieval (320mg sodium EDTA/L, pH 8.0) was performed before immunostaining for CD4 and CD8, and citric acid buffer (0.01M, pH 6.0) was used for microwave-based epitope retrieval for Fas, Fas-L, CD40, TIA, and granzyme B.

CD40, Fas, and Fas-L expressions were semi-quantitatively assessed for the number of positively stained cells and the intensity of the positive reaction. The number of positively stained thyrocytes was classified into five categories: 0, no positive thyrocytes; 1, < 25% of thyrocytes positive; 2, between 25% and 50% of thyrocytes positive; 3, between 50% and 75% of thyrocytes positive; 4, > 75% of thyrocytes positive. The intensity of CD40 and Fas expression was graded by comparing their immunostaining intensities with that of germinal center staining as a control: 1, fainter staining intensity; 2, same staining intensity; 3, stronger staining intensity.¹¹ The intensity of Fas-L expression was graded in an identical manner but it was compared with that of the plasma cell.¹² CD4, CD8, TIA, and granzyme B reactive T-lymphocytes were counted on 5 high power fields in areas of dense lymphocytic infiltration sparing the lymphoid follicles.

Statistical analysis

The Kruskal-Wallis test was used to compare the data and correlation was assessed using the Spearman' signed ranks test. *p* value of less than 0.05 was considered significant.

RESULTS

CD40 expression

CD40 was not expressed on thyrocytes from the normal controls (Fig. 1A) and in 5 cases of GD showing sparse lymphocytic infiltration. Its expression pattern on thyrocytes was membranous. HT showed the most prominent immunoreactivity followed by SGT, NLT and GD (Table 1). The number of thyrocytes expressing CD40 correlated well with the numbers of CD4+ and CD8+ lymphocytes in all groups (correlation coefficient: 0.767 with the number of CD4+ lymphocytes and 0.760 with the number of CD8+ lymphocytes) and there were no differences among groups (Tables 2 and 3). CD40 was more strongly expressed on plump thyrocytes proximal to dense lymphocytic infiltration (Fig. 1B). Germinal centers of the lymphoid follicles, some lymphocytes of the mantle layers and some endothelial cells of the vessels revealed immunoreactivity for CD40. In SGT, macrophages and multinucleated giant cells also showed immunoreactivity for CD40 and plump thyrocytes near granulomatous inflammation also expressed

stronger CD40 (Fig. 1C).

Fas expression

Fas was constitutively expressed on nearly all thyrocytes even in the normal controls (Fig. 2A) and there were no statistical differences in positivities and intensities among all groups (p value > 0.05) (Table 1). Moreover, there was no correlation between Fas expression and the number of either CD4+ or CD8+ lymphocytes. However, plump thyrocytes in areas of dense lymphocytic infiltration in various forms of autoimmune thyroiditis (Fig. 2B) and in areas near the granulomas in SGT showed convincingly stronger membranous expression (Fig. 2C). Infiltrating lymphocytes showed diffuse cell surface immunoreactivity for Fas in various types of autoimmune thyroiditis. In SGT, macrophages and multinucleated giant cells as well as infiltrating lymphocytes revealed strong immunoreactivity (Fig. 2C).

Fas-L expression

As in Fas, Fas-L was also constitutively expressed in normal controls but only a few scat-

Table 1. The Expression of CD40, Fas, and Fas-L on Thyrocytes in Various Types of Thyroiditis

Type of thyroiditis	CD40		Fas		Fas-L	
	Positivity by number (Mean \pm SD)	Positivity by intensity (Mean \pm SD)	Positivity by number (Mean \pm SD)	Positivity by intensity (Mean \pm SD)	Positivity by number (Mean \pm SD)	Positivity by intensity (Mean \pm SD)
Graves' disease	0.9 \pm 1.04	1.3 \pm 1.34	3.8 \pm 0.40	2.9 \pm 0.30	1.8 \pm 0.87	1.4 \pm 0.66
Hashimoto's thyroiditis	2.5 \pm 0.63	2.7 \pm 0.79	3.9 \pm 0.26	2.9 \pm 0.26	3.0 \pm 0.65	2.4 \pm 0.62
Nonspecific thyroiditis	2.2 \pm 0.93	2.4 \pm 0.48	4.0 \pm 0.00	2.9 \pm 0.29	2.3 \pm 0.75	2.1 \pm 0.67
Subacute granulomatous thyroiditis	2.3 \pm 0.62	2.5 \pm 0.49	4.0 \pm 0.00	3.0 \pm 0.00	1.9 \pm 0.51	2.2 \pm 0.45

SD, standard deviation.

Table 2. Correlation between CD40 Expression on Thyrocytes and Number of CD4+ Lymphocytes in Various Types of Thyroiditis

Type of thyroiditis	Correlation coefficient between CD40 by number and CD4+ lymphocytes (p -value)	Correlation coefficient between CD40 by intensity and CD4+ lymphocytes (p -value)
Graves' disease	0.732 (0.016)	0.763 (0.010)
Hashimoto's thyroiditis	0.714 (0.003)	0.912 (0.000)
Nonspecific thyroiditis	0.659 (0.027)	0.774 (0.005)
Subacute granulomatous thyroiditis	0.816 (0.002)	0.866 (0.006)

Table 3. Correlation between CD40 Expression on Thyrocytes and Number of CD8+ Lymphocytes in Various Types of Thyroiditis

Type of thyroiditis	Correlation coefficient between CD40 by number and CD8+ lymphocytes (<i>p</i> -value)	Correlation coefficient between CD40 by intensity and CD8+ lymphocytes (<i>p</i> -value)
Graves' Disease	0.686 (0.028)	0.794 (0.016)
Hashimoto's thyroiditis	0.683 (0.007)	0.911 (0.000)
Nonspecific thyroiditis	0.773 (0.005)	0.646 (0.032)
Subacute granulomatous thyroiditis	0.826 (0.001)	0.751 (0.008)

Table 4. Correlation between CD40 and Fas-L Expression on Thyrocytes in Various Types of Thyroiditis

Type of thyroiditis	Correlation coefficient between CD40 and Fas-L by number (<i>p</i> -value)	Correlation coefficient between CD40 and Fas-L by intensity (<i>p</i> -value)
Graves' Disease	0.727 (0.017)	0.471 (0.169)
Hashimoto's thyroiditis	0.693 (0.019)	0.551 (0.041)
Nonspecific thyroiditis	0.783 (0.004)	0.856 (0.000)
Subacute granulomatous thyroiditis	0.799 (0.002)	0.149 (0.661)

Table 5. Correlation between Fas-L Expression on Thyrocytes and Number of CD4+ Lymphocytes in Various Types of Thyroiditis

Type of thyroiditis	Correlation coefficient between Fas-L by number and CD4+ lymphocytes (<i>p</i> -value)	Correlation coefficient between Fas-L by intensity and CD4+ lymphocytes (<i>p</i> -value)
Graves' Disease	0.582 (0.077)	0.494 (0.146)
Hashimoto's thyroiditis	0.406 (0.149)	0.479 (0.083)
Nonspecific thyroiditis	0.735 (0.009)	0.773 (0.005)
Subacute granulomatous thyroiditis	0.673 (0.023)	0.387 (0.239)

tered thyrocytes expressed Fas-L (Fig. 3A). As in CD40, HT showed the most intense and extensive immunoreactivity (Table 1). Its topographic expression correlated well with CD40 in all groups (Table 4). A tendency towards stronger membranous expression on thyrocytes proximal to inflammatory aggregates was also noted, as in CD40 and Fas. Infiltrating lymphocytes in various forms of autoimmune thyroiditis showed negligible expression of Fas-L, while plasma cells revealed strong cytoplasmic expression. Some plump endothelial cells strongly expressed Fas-L. In SGT, infiltrating lymphocytes as well as macrophages and multinucleated giant cells revealed strong immunoreactivity for Fas-L (Fig. 3C). The correlation coefficients between the number of thyrocytes expressing Fas-L and the number of either CD4+ or CD8+ lymphocytes are 0.497 and 0.522, respectively in all cases irrespective of the group

and there were no statistical differences among the groups (Tables 5 and 6). Compared with CD40, Fas-L expression on thyrocytes displayed a low correlation with the number of either CD4+ or CD8+ lymphocytes (Tables 2, 3, 5, and 6).

CD4, CD8, TIA, and granzyme B expression in infiltrating lymphocytes

The mean ratios of CD4+ (Fig. 4A) and CD8+ lymphocytes (Fig. 4B) in various forms of thyroiditis were as follows: 0.97 (GD), 0.67 (HT), 0.74 (NLT), and 0.66 (SGT). Therefore, the relative number of CD4+ lymphocytes was higher in GD than in the others. The mean percentages of infiltrating lymphocytes positive for TIA (Fig. 4C) and granzyme B (Fig. 4D) per CD8+ lymphocytes were 70.94% and 2.28% respectively and no statistically significant difference was found in

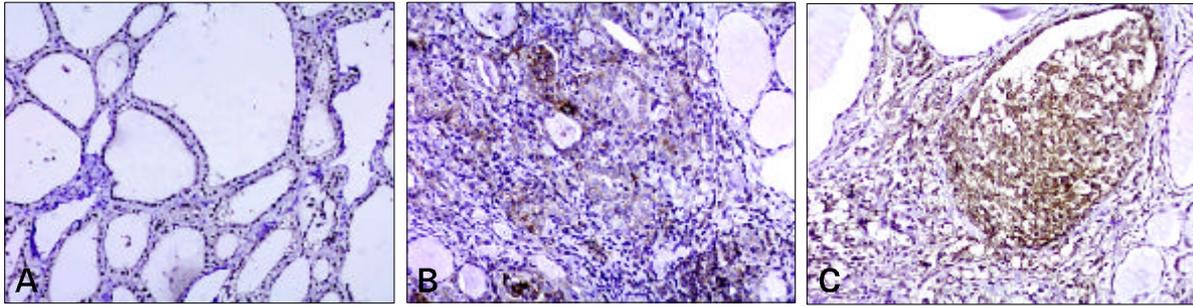


Fig. 1. CD40 expression in controls (A), Hashimoto's thyroiditis (B), and subacute granulomatous thyroiditis (C). Immunoreactivity was not detected on thyrocytes of the normal controls while thyrocytes and lymphocytes of Hashimoto's thyroiditis and macrophages of subacute granulomatous thyroiditis showed immunoreactivity.

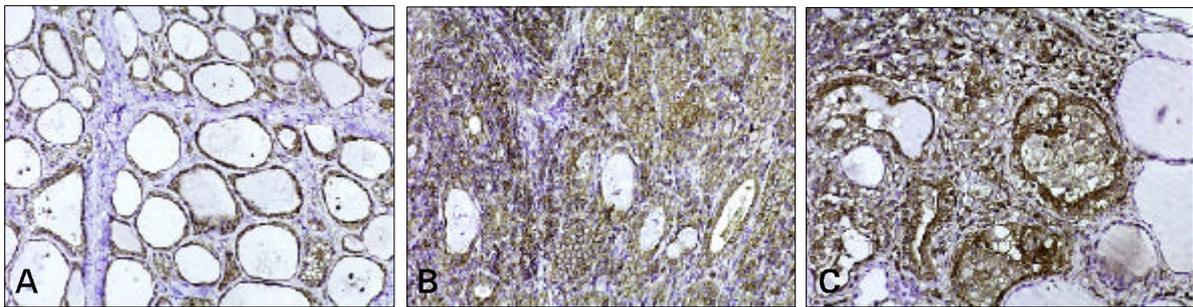


Fig. 2. FAS expression in a control (A), Hashimoto's thyroiditis (B), and subacute granulomatous thyroiditis (C). Diffuse immunoreactivity was detected on thyrocytes of the normal controls. Plump thyrocytes proximal to inflammatory cell aggregates in Hashimoto's thyroiditis and subacute granulomatous thyroiditis membranous displayed positivity. Lymphocytes of Hashimoto's thyroiditis and macrophages of subacute granulomatous thyroiditis also showed immunoreactivity.

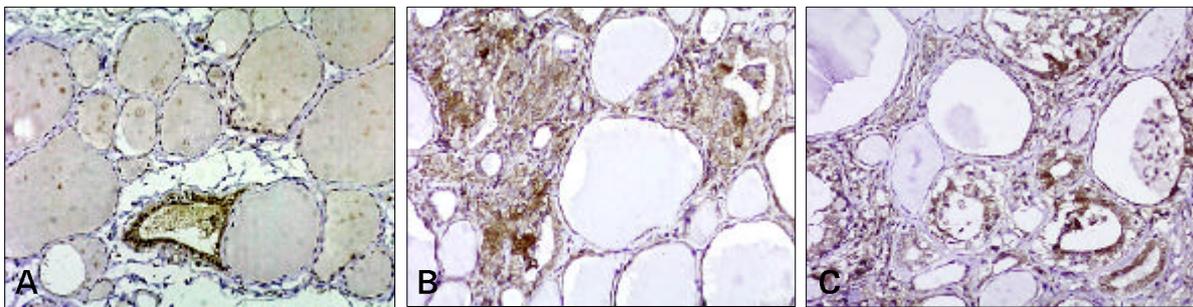


Fig. 3. FAS-L expression in a control (A), Hashimoto's thyroiditis (B), and subacute granulomatous thyroiditis (C). Immunoreactivity was detected on a few scattered thyrocytes of normal controls and on plump thyrocytes proximal to lymphoid aggregates in Hashimoto's thyroiditis and granulomas in subacute granulomatous thyroiditis showed strong immunoreactivity. Infiltrating lymphocytes and macrophages of subacute granulomatous thyroiditis also showed positive reaction.

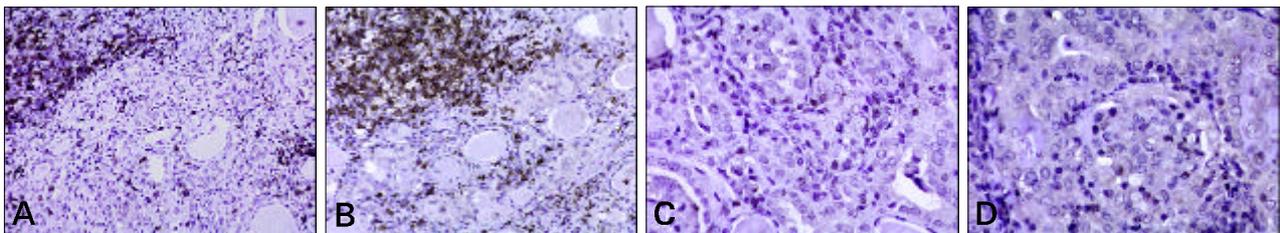


Fig. 4. CD4 (A), CD8 (B), TIA (C), and granzyme B (D) expressing lymphocytes in a representative case of Hashimoto's thyroiditis.

Table 6. Correlation between Fas-L Expression on Thyrocytes and Number of CD8+ Lymphocytes in Various Types of Thyroiditis

Type of thyroiditis	Correlation coefficient between Fas-L by number and CD8+ lymphocytes (p-value)	Correlation coefficient between Fas-L by intensity and CD8+ lymphocytes (p-value)
Graves' disease	0.421 (0.224)	0.629 (0.051)
Hashimoto's thyroiditis	0.405 (0.149)	0.606 (0.021)
Nonspecific thyroiditis	0.588 (0.056)	0.864 (0.001)
Subacute granulomatous thyroiditis	0.614 (0.046)	0.193 (0.568)

their ratios for the various forms of thyroiditis.

DISCUSSION

In this study, we evaluated the in situ expression pattern of CD40, Fas, and Fas-L on thyrocytes and infiltrating lymphocytes by immunohistochemical staining in various types of autoimmune thyroiditis, to investigate their roles in the apoptotic processes of thyrocytes. CD40, a member of TNF receptor family, is found on the surface of B-lymphocytes and plays an important role in their interaction with T helper lymphocytes. By binding to CD40 on antigen specific B-lymphocytes, activated T-lymphocytes transiently expressing CD40-L transduce signals essential for B-lymphocytes growth and differentiation by secreting variable cytokines.¹³ In addition to B-lymphocytes, CD40 expression was also demonstrated on many non-lymphoid cells including thymic epithelial cells, endothelial cells, keratinocytes, fibroblasts and thyrocytes.¹⁴⁻²⁰ Fas is a 45 kDa membrane type I protein belonging to the tumor necrosis factor receptor (TNFR) superfamily, which also includes CD40.²¹⁻²³ Fas has a 70 amino acid intracellular "death domain" that transduces signals for apoptotic cell death and Fas mediated apoptosis by cross-linking Fas with anti-Fas antibodies.²⁴ Fas-L is a 40kDa type II membrane protein expressed as a membrane bound form that is processed into a soluble form that retains biologic activity.^{25,26} As in the case of CD40, Fas is also expressed in various non-lymphoid tissues and it may be directly involved in the apoptosis of some non-lymphoid cells.^{6-10,27,28} In contrast to Fas, Fas-L expression is known to be tightly regulated, though low levels of Fas-L expression have been reported in some non-lymphoid organs.^{7,9,25}

Our results show that the degree of CD40 expression on thyrocytes is closely correlated with the intensity of lymphocyte infiltration. In contrast to Fas and Fas-L, CD40 was not expressed on the thyrocytes of normal controls or in GD that show no lymphocyte infiltrates. There have been several reports on the expression of CD40 in thyroid tissue. Faure et al.¹⁸ and Metcalfe et al.¹⁹ reported CD40 expression in GD and multinodular goiter. However, these two reports did not evaluate CD40 expression in normal thyroid tissue, nor did they assess the relationship between its expression and lymphocytic infiltration. Smith et al.²⁰ reported that CD40 was expressed on various cells of the thyroid gland including thyrocytes, lymphocytes, macrophages, endothelial cells, and fibroblast-like cells. They stated that, though CD40 mRNA was invariably detected in the normal and the pathologic thyroid, CD40 protein expression on thyrocytes was detected only in inflammatory areas by immunohistochemistry, which is in line with our results.

In autoimmune diseases, the natural unresponsiveness to self antigen is abrogated which causes inflammatory cell infiltration.² Cytokines, such as INF γ , TNF α , and IL-1 α , released by infiltrating inflammatory cells then induce CD40 expression on thyrocytes. The CD40 expressing thyrocytes can reciprocally react with CD40-L on T-lymphocytes, resulting in the activation of B-lymphocytes as well as cytotoxic T-lymphocytes (CTLs). Furthermore, the induction of cell surface Fas-L expression by CD40 activation has been demonstrated by a recent study on the apoptosis of hepatocytes during allograft rejection.²⁹ Moreover, cytokines, such as IL-6, was shown to be transmitters of this reaction.³⁰⁻³² Therefore, CD40 expressed on thyrocytes may play dual roles in thyrocyte apoptosis by inducing Fas-L expression

on themselves as well as by recruiting CTLs.

There appears to be a basal level of apoptosis even in the normal thyroid gland and this may be related to the ongoing turnover of cells and the maintenance of the cellular population.^{4,6,33,34} In the present study, Fas was constitutively expressed diffusely on thyrocytes including normal controls, and its expression was stronger on plump thyrocytes around the lymphocytic infiltrates. Whereas only a few scattered thyrocytes expressed Fas-L in the normal controls, its expression was increased in accord with the amount of lymphocytic infiltration in various types of thyroiditis. Several reports have shown the expressions of Fas and Fas-L on thyrocytes, but their results have not been identical, and this has caused considerable debate, especially concerning Fas-L expression.^{6-10,33} Giordano et al.⁷ reported the presence of Fas-L on thyrocytes at the gene and protein levels, while Arscott and Baker³³ could not detect the mRNA of Fas-L by a ribonuclease protection assay or by RT-PCR; this group also questioned the specificity of the anti-Fas antibodies used by Giordano et al.⁷ based on their immunoblot results. However, Arscott and Baker³³ concurred with Giordano et al.⁷ on the expression of Fas-L in cases of GD and HT by immunohistochemical staining. From the technical standpoint, the microwave pretreatment used for enhancing antigenicity sometimes induces non-specific cytoplasmic staining in tissues with abundant endogenous avidin. Thyroid is known to have abundant endogenous avidin activity, for this reason we chose to use the EnVision™ kit from Dako for CD40, FAS, and Fas-L. Pseudo-positive immunostaining from various sources is known to be mainly cytoplasmic, therefore we consider that stronger membranous expressions of Fas and Fas-L proximal to inflammatory cell aggregates strongly favors the true expression of these molecules on thyrocytes. Until recently, it had been understood that parenchymal cell loss in autoimmune diseases is mainly caused by cytotoxic reactions of infiltrating lymphocytes.^{35,36} However, several recent reports have indicated that apoptosis of thyrocytes by surface expressed Fas and Fas-L (fratricide) plays a more important role than CTL mediated cytotoxicity in the destruction of thyrocytes.^{7,33,37} Therefore, our results

demonstrating the presence of Fas and Fas-L on thyrocytes in all forms of thyroiditis support this Fas and Fas-L mediated mechanism of thyrocytes apoptosis (fratricide).

The other important mechanism of thyrocyte apoptosis, CTL mediated cytotoxicity of thyrocytes, was also evaluated by in situ immunophenotypic analyses of the infiltrating lymphocytes. CTLs mediate cytolysis by two different pathways. One is Fas-L mediated cytolysis of Fas expressing target cells and the other is cytotoxic granule mediated cytolysis through perforin-created "holes".³⁸ CD4+ T-lymphocytes do not usually demonstrate the perforin-granzyme-based mechanism of lysis and seem to lyse mainly via the Fas-based pathway, while CD8+ T-lymphocytes usually adopt both mechanisms. Although there were indications that perforin is a main molecule required for CTL mediated cytotoxicity in the mid-1980s, granzymes now appear to be the main molecules. Granzyme B is a type of serine esterase, and is found in granules of activated CTLs while TIA is expressed on all CTLs irrespective of their activation status.^{38,39} Our results show a good correlation between the number of TIA+ lymphocytes and CD8+ lymphocytes in all forms of thyroiditis. However, the number of granzyme B+ activated CTLs occupied a negligible proportion of CD8+ lymphocytes in all groups and no differences in their proportions were found in the various types of thyroiditis. Furthermore, Fas-L was not distinctively expressed in infiltrating lymphocytes of various forms of thyroiditis except some of those in SGT. Therefore, CD8+ T-lymphocytes seem not to act as killers of thyrocytes but rather as regulators of the inflammatory process in autoimmune thyroid diseases, as Ludgate et al. suggested.⁴⁰ However, we cannot completely exclude the possibility that a small number of Fas-L or granzyme B expressing lymphocytes might be functionally involved in the apoptotic process of thyrocytes, because GD and NLT usually show small numbers of apoptotic thyrocytes.⁴

The present study includes 11 cases of SGT, on the assumption that SGT may have a somewhat different pathogenesis and the data of SGT were analyzed in comparison with those of autoimmune thyroiditis. SGT is understood to be a virus induced disease.^{41,42} Most virus specific CTLs are

CD8+ T-lymphocytes that recognize cytosolic antigens in association with class I MHC molecules on any nucleated cells. Moreover, full activation of CD8+ CTLs requires cytokines produced by CD4+ helper lymphocytes.⁴³ Because thyrocytes do not express B7 or B7-2, costimulators of T-lymphocytes,⁴⁴ CD40 expressed on thyrocytes seems to be a pivot in this sequence. In contrast to the other types of thyroiditis studied, Fas-L was expressed on infiltrating T-lymphocytes in SGT. However, granzyme B+ CTLs occupied a negligible proportion of the CD8+ T-lymphocytes, as found in other types of thyroiditis. These results suggest that virus infected Fas expressing thyrocytes may be efficiently eliminated by Fas-L expressed on the thyrocytes and CTLs. Macrophages and multinucleated giant cells have been reported to express CD40, Fas, and Fas-L, as was found in the present study.^{13,45,46} These cells are involved in the apoptosis observed in granulomas⁴⁷ and may also induce the Fas-L mediated apoptosis of bystander lymphocytes and thyrocytes.⁴⁶

In conclusion, our results display the acquisition of CD40, Fas, and Fas-L on thyrocytes proximal to inflammatory cell aggregates and the negligible expression of granzyme B and Fas-L on the infiltrating lymphocytes, and suggest that the Fas and Fas-L mediated apoptosis of thyrocytes (fratricide) may be more important than T cell-mediated cytotoxicity in various forms of autoimmune thyroiditis.

REFERENCES

- McNally J, Yoo DH, Drappa J, Chu JL, Yagita H, Friedman SM, et al. Fas ligand expression and function in systemic lupus erythematosus. *J Immunol* 1997;159:4628-36.
- Miller JF, Flavell RA. T cell tolerance and autoimmunity in transgenic models of central and peripheral tolerance. *Curr Opin Immunol* 1994;6:892-9.
- Sgonc R, Gruschwitz MS, Dietrich H, Recheis H, Gershwin ME, Wick G. Endothelial cell apoptosis in a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *J Clin Invest* 1996;98:785-92.
- Tanimoto C, Hirakawa S, Kawasaki H, Hayakawa N, Ota Z. Apoptosis in thyroid diseases: a histochemical study. *Endocr J* 1995;42:193-201.
- Kotani T, Aratake Y, Hirai K, Fukazawa Y, Sato H, Ohtaki S. Apoptosis in thyroid tissue from patients with Hashimoto's thyroiditis. *Autoimmunity* 1995;20:231-6.
- Hammond LJ, Lowdell MW, Cerrano PG, Goode AW, Bottazzo GF, Mirakian R. Analysis of apoptosis in relation to tissue destruction associated with Hashimoto's autoimmune thyroiditis. *J Pathol* 1997;182:138-44.
- Giordano C, Stassi G, De Maria R, Todaro M, Richiusa P, Papoff G, et al. Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. *Science* 1997;275:960-3.
- Xerri L, Devilard E, Hassoun J, Mawas C, Brig F. Fas ligand is not only expressed in immune privileged human organs but is also coexpressed with Fas in various epithelial tissues. *Mol Pathol* 1997;50:87-91.
- Mitsiades M, Poulaki V, Kotoula G, Mastorakos G, Tseleni-Balafouta S, Koutras DA, et al. Fas/Fas ligand up-regulation and Bcl-2 down-regulation may be significant in the pathogenesis in Hashimoto's thyroiditis. *J Clin Endocrinol Metab* 1998;83:2199-203.
- Hiromatsu Y, Bednarczuk T, Soyejima E, Miyake I, Yang D, Fukazawa H, et al. Increased serum soluble Fas in patients with Graves' disease. *Thyroid* 1999;9:341-5.
- Yoshino T, Kondo E, Cao L, Takahashi K, Hayashi K, Nomura S, et al. Inverse expression of bcl-2 protein and Fas antigen in lymphoblasts in peripheral lymph nodes and activated peripheral blood T and B lymphocytes. *Blood* 1994;83:1856-61.
- Straer J, Mariani SM, Walczak H, Ruker FG, Leithauser F, Krammer PH, et al. CD95 ligand (CD95L) in normal human lymphoid tissues: a subset of plasma cells are prominent producers of CD95L. *Am J Pathol* 1999;154:193-201.
- Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, et al. The CD40 antigen and its ligand. *Annu Rev Immunol* 1994;12:881-922.
- Galy AH, Spits H. CD40 is functionally expressed on human thymic epithelial cells. *J Immunol* 1992;149:775-82.
- Hollenbaugh D, Mischel-Petty N, Edwards CP, Simon JC, Denfeld RW, Kiener PA, et al. Expression of functional CD40 by vascular endothelial cells. *J Exp Med* 1995;182:33-40.
- Gaspari AA, Sempowski GD, Chess P, Gish J, Phipps RP. Human epidermal keratinocytes are induced to secrete interleukin-6 and co-stimulate T lymphocyte proliferation by a CD40-dependent mechanism. *Eur J Immunol* 1996;26:1371-7.
- Fries KM, Sempowski GD, Gaspari AA, Blieden T, Looney RJ, Phipps RP. CD40 expression by human fibroblasts. *Clin Immunol Immunopathol* 1995;77:42-51.
- Faure GC, Bensoussan-Lejzerowicz D, Bene MC, Aubert V, Leclere J. Coexpression of CD40 and class II antigen HLA-DR in Graves' disease thyroid epithelial cells. *Clin Immunol Immunopathol* 1997;84:212-5.
- Metcalf RA, McIntosh RS, Marelli-Berg F, Lombardi G, Lechler R, Weetman AP. Detection of CD40 on human thyroid follicular cells: analysis of expression and

- function. *J Clin Endocrinol Metab* 1998;83:1268-74.
20. Smith TJ, Sciakly D, Phipps RP, Jennings TA. CD40 expression in human thyroid tissue: evidence for involvement of multiple cell types in autoimmune and neoplastic disease. *Thyroid* 1999;9:749-55.
 21. Armitage RJ. Tumor necrosis factor receptor superfamily members and their ligands. *Curr Biol* 1994;4:407-13.
 22. Schulze-Osthoff K. The Fas/APO-1 receptor and its deadly ligand. *Trends Cell Biol* 1994;4:421-5.
 23. Nagata S, Golstein P. The Fas death factor. *Science* 1995;267:1449-56.
 24. Debatin KM, Goldman CK, Bamford R, Waldmann TA, Krammer PH. Monoclonal antibody-mediated apoptosis in adult T cell leukaemia. *Lancet* 1990;335:497-500.
 25. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 1993;75:1169-78.
 26. Tanaka M, Suda T, Takahashi T, Nagata S. Expression of the functional soluble form of human Fas ligand in activated lymphocytes. *EMBO J* 1995;14:1129-35.
 27. Leithauser F, Dhein J, Mechttersheimer G, Koretz K, Bruderlein S, Henne C, et al. Constitutive and induced expression of APO-1, a new member of nerve growth factor superfamily in normal and neoplastic cells. *Lab Invest* 1993;69:415-29.
 28. Suzuki A, Enari M, Eguchi Y, Matsuzawa A, Nagata S, Tsujimoto Y, et al. Involvement of Fas in regression of vaginal epithelia after ovariectomy and during an estrous cycle. *EMBO J* 1996;15:211-5.
 29. Afford SC, Randhawa S, Eliopoulos AG, Hubscher SG, Young LS, Adams DH. CD40 activation induces apoptosis in cultured human hepatocytes via induction of cell surface fas ligand expression and amplifies fas-mediated hepatocyte death during allograft rejection. *J Exp Med* 1999;189:441-6.
 30. Choi C, Park JY, Lee J, Lim JH, Shin EC, Ahn YS, et al. Fas ligand and Fas are expressed constitutively in human astrocytes and the expression increases with IL-1, IL-6, TNF-alpha, or IFN-gamma. *J Immunol* 1999;162:1889-95.
 31. Arnold R, Seifert M, Asadullah K, Volk HD. Crosstalk between keratinocytes and T lymphocytes via Fas/Fas ligand interaction; modulation by cytokines. *J Immunol* 1999;162:7140-7.
 32. Eliopoulos AG, Davies C, Knox PG, Gallagher NJ, Afford SC, Adams DH, et al. CD40 induces apoptosis in carcinoma cells through activation of cytotoxic ligands of the tumor necrosis factor superfamily. *Mol Cell Biol* 2000;20:5503-15.
 33. Arcsott PL, Baker JR Jr. Apoptosis and thyroiditis. *Clin Immunol Immunopathol* 1998;87:207-17.
 34. Sreelekha TT, Pradeep VM, Vijayalakshmi K, Belthazar A, Chellam VG, Nair MB, et al. In situ apoptosis in the thyroid. *Thyroid* 2000;10:117-22.
 35. Del Prete GF, Maggi E, Mariotti S, Tiri A, Vercelli D, Parronchi P, et al. In vivo activated T cells in the thyroid infiltrate of patients with Hashimoto's thyroiditis. *Clin Exp Metab* 1987;64:818-24.
 36. Kawakami A, Matsuoka N, Tsuboi M, Koji T, Urayama S, Sera N, et al. CD4+ T cell-mediated cytotoxicity toward thyrocytes; the importance of Fas/Fas ligand interaction inducing apoptosis of thyrocytes and the inhibitory effect of thyroid stimulating hormone. *Lab Invest* 2000;80:471-84.
 37. Stassi G, Todaro M, Bucchieri F, Stoppacciaro A, Farina F, Zunmo G, et al. Fas/Fas ligand-driven T cell apoptosis as a consequence of ineffective thyroid immunoprivilege in Hashimoto's thyroiditis. *J Immunol* 1999;162:263-7.
 38. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Ann Rev Immunol* 1996;14:207-32.
 39. Bontkes HJ, de Gruijl TD, Walboomers JM, van den Muysenberg AJ, Gunther AW, Scheper RJ, et al. Assessment of cytotoxic T-lymphocyte phenotype using the specific markers granzyme B and TIA-1 in cervical neoplastic lesions. *Br J Cancer* 1997;76:1353-60.
 40. Ludgate M, Jasani B. Apoptosis in autoimmune and non-autoimmune thyroid disease [editorial]. *J Pathol* 1997;182:123-4.
 41. Sato M. Virus-like particles in the follicular epithelium of the thyroid from a patient with subacute thyroiditis (De Quervain). *Acta Pathol Jpn* 1975;25:499-501.
 42. Werner J, Gelderblom H. Isolation of foamy virus from patients with de Quervain thyroiditis [letter]. *Lancet* 1979;2:258-9.
 43. Ploegh HL. Viral strategies of immune evasion. *Science* 1998;280:248-53.
 44. Matsuoka N, Eguchi K, Kawakami A, Tsuboi M, Nakamura H, Kimura H, et al. Lack of B7-1/BB1 and B7-2/B70 expression on thyrocytes of patients with Graves' disease. Delivery of costimulatory signals from bystander professional antigen-presenting cells. *J Clin Endocrinol Metab* 1996;81:4137-43.
 45. Kiener PA, Davis PM, Starling GC, Mehlin C, Klebanoff SJ, Ledbetter JA, et al. Differential induction of apoptosis by Fas-Fas ligand interactions in human monocytes and macrophages. *J Exp Med* 1997;185:1511-6.
 46. Brown SB, Savill J. Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes. *J Immunol* 1999;162:480-5.
 47. Koga M, Hiromatsu Y, Jimi A, Toda S, Koike N, Nonaka K. Immunohistochemical analysis of Bcl-2, Bax, and Bak expression in thyroid glands from patients with subacute thyroiditis. *J Clin Endocrinol Metab* 1999;84:2221-5.