Circulating Soluble Fas Ligand Correlates With Disease Activity in Graves' Hyperthyroidism

Chih-Yuan Wang, Wen-Bin Zhong, Tien-Chun Chang, and Yuan-Feen Tsai

Apoptosis of thyrocytes may play an important role in the pathogenesis of autoimmune thyroiditis. Meanwhile, the Fas/Fas ligand (FasL) apoptosis pathway has received much attention in physiological homeostasis and immune regulation in various thyroid diseases, including Graves' hyperthyroidism (GD). FasL is a type II membrane protein of the tumor necrosis factor family, and induces apoptosis when it binds to Fas. Soluble FasL (sFasL) may also exert cytotoxic activity against Fas-expressing cells by producing trimerization of Fas molecule, but soluble Fas (sFas) is an apoptotic inhibitor. To determine the role of circulating sFas/sFasL in modulating disease activity of GD, we examined the circulating levels of sFas/sFasL in GD patients with various levels of anti–thyrotropin-stimulating hormone (TSH) receptor antibodies (TRAb). Serum samples were obtained from 22 consecutive untreated hyperthyroid GD patients with higher TRAb level (63.8% ± 12.5%, group I) and 22 treated euthyroid GD patients, who were in a state of disease remission, with lower TRAb level (7.9% ±5.9%, group II). The levels of sFas were significantly higher in group I (1.56 ± 0.26 ng/mL) than in group II (0.76 ± 0.26 ng/mL, $P < .01$). The levels of sFasL were also significantly higher in group I patients (0.153 ± 0.018 ng/mL) than in group II patients (0.126 ± 0.012 ng/mL, $P < .01$). A significant correlation was found between sFasL levels and TRAb activity in all GD patients ($r = 0.69$, $P < .01$). Because changes in sFasL levels and TRAb levels occur in parallel, increased serum sFasL in patients with GD may contribute to homeostasis in the thyroid. Serum sFasL may be considered to be a candidate marker for evaluating disease aggression or regression in GD.

THE APOPTOSIS of thyroid follicular cells may play a pivotal role in the pathogenesis of autoimmune thyroiditis.[1] The Fas/Fas ligand (FasL) apoptosis pathway has received much attention in terms of a potential role in physiologic homeostasis and immune regulation in various thyroid diseases, including Hashimoto's thyroiditis, Graves' disease (GD), and thyroid cancer.[2-4] Giordano et al[2] even suggested that the constitutive expression of FasL on thyrocytes leads to apoptosis in normal and Hashimoto's thyrocytes, thereby resulting in clinical hypothyroidism. However, the result was questioned because they used nodular goiter thyrocytes as controls, and the specificity of the polyclonal FasL-specific antibodies was also criticized.[5] Recently, Hiromatsu et al[3] and Mitsiades et al[4] found that Fas is present in normal thyroid, whereas FasL is only expressed in diseased thyroid, including GD and thyroid carcinomas. It is very important that the expression of FasL on thyrocytes seemed to be associated with the immune-privileged effect in GD. Hiromatsu's study also showed that FasL on Graves' thyrocytes had functional activity in that it induced apoptosis in target cells transfected with human Fas antigen, whereas FasL on normal thyrocytes did not.[3] The increased expression of FasL in Graves' thyrocytes may try to improve homeostasis in the thyroid by eliminating infiltrating lymphocytes and hyperplastic thyrocytes via Fas-mediated apoptosis.[3] This result raises the important question whether Graves' thyrocytes use FasL expression to escape immune attack and whether FasL expression regulates disease activity of hyperplastic thyrocytes via apoptosis.[6]

FasL is a type II membrane protein of the tumor necrosis factor family, and induces apoptosis when binds to Fas antigen.[7] Membrane-bound FasL is converted to a soluble form (sFasL) by a metalloproteinase, and sFasL also exerts cytotoxic activity against Fas-expressing cells by causing trimerization of Fas molecules.[8-10] Thus, it is likely that sFasL acts as a cytotoxic agent in peripheral autoimmune response and induces apoptosis in thyrocytes and lymphocytes.

GD, an autoimmune thyroid disorder, is characterized by hyperplasia of thyrocytes that results from binding of anti–thyroid-stimulating hormone (TSH) receptor antibodies (TRAb) to TSH receptors, and disease activity is clearly associated with the level of TRAb.[11] However, Kawakami et al[12] reported that TRAb in Graves' patients may act in the same way as TSH to inhibit Fas/FasL-mediated apoptosis, and Hiromatsu et al[3] reported increased expression of FasL in Graves' thyrocytes and the downregulation of Fas expression by TSH or TRAb. Since TRAb levels are highly correlated with disease activity in GD, it would be valuable to investigate the role of circulating sFas/sFasL in disease regulation in GD patients with various levels of TRAb.

In the present study, we examined the levels of circulating sFas/sFasL in GD patients with various levels of TRAb to investigate the possible role of circulating sFasL in modulating disease activity.

MATERIALS AND METHODS

Patients

Between January 2000 and December 2000, serum samples were obtained from 2 groups of GD patients and 1 group of controls. Group I consisted of 22 consecutive untreated GD patients (6 men and 16 women; age range, 20 to 45 years; mean ± SD, 37.2 ± 10.9 years) with

From the Graduate Institute of Physiology, and the Division of Endocrinology, Department of Internal Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University and Far-Eastern Memorial Hospital, Taipei, Taiwan.

Submitted August 31, 2001; accepted November 14, 2001.

Supported by National Science Council of the Republic of China Grant No. NSC-89-2314-B002-517.

Address reprint requests to Tien-Chun Chang, MD, Division of Endocrinology, Department of Internal Medicine, College of Medicine, National Taiwan University Hospital, 7, Chung-Shan South Rd, Taipei, Taiwan.

0026-0495/02/5106-0028$35.00/0

doi:10.1053/meta.2002.32034

Table 1. Demographic Characteristics of Patients With Graves' Disease

Characteristics	Untreated GD High TRAb (n = 22) (≥ 50%)	Treated GD Low TRAb (n = 22) (≤ 20%)
Age (yr)	37.2 ± 10.9	42.9 ± 14.3
Men/women	6/16	1/21
sFas (ng/mL)*	1.56 ± 0.26	0.76 ± 0.26
sFasL (ng/mL)*	0.152 ± 0.018	0.126 ± 0.012
TRAb(%)*	63.8 ± 12.5	7.9 ± 5.8

*Statistically significant ($P < .01$).

higher TRAb level (≥50% inhibition of TSH binding; range, 50.0% to 87.4%; mean ± SD, 63.8% ± 12.5%). Group II consisted of 22 consecutive GD patients (1 man and 21 women; age range, 26 to 65 years; mean ± SD, 42.9 ± 14.3 years), who were euthyroid after antithyroid drug (methimazole or propylthiouracil) treatment for 1 year, and had low TRAb levels (<25% inhibition of TSH binding; range, 0.7% to 21.7%; mean ± SD, 7.9% ± 5.9%; all patients in group II had TRAb levels > 15% before treatment). The control group (group III) consisted of 22 normal subjects (8 men and 14 women; age range, 22 to 45 years; mean ± SD, 33.8 ± 8.9 years). All patients gave their informed consent before participation in this study.

Patients who had undergone radioiodine therapy or surgery were excluded from the study. The diagnosis of GD was made on the basis of clinical and laboratory criteria. GD patients had elevated concentrations of free thyroid hormones and undetectable or clearly reduced TSH levels in the serum, had TRAb (thyrotropin-binding inhibitory immunoglobulin [TBII]; normal range, <15% inhibition of TSH binding) in the serum, and showed diffuse increased uptake of radionuclide on the scintiscan.

Antibodies to the TSH Receptor

Anti-TSH receptor antibodies in patients' sera were assayed using a thyrotropin receptor autoantibody kit (RSR Ltd, Cardiff, UK) according to the manufacturer's instructions.

sFas/sFasL Enzyme-Linked Immunosorbent Assay

Detection of sFas/sFasL was performed using sandwich enzyme-linked immunosorbent assay (ELISA) (Medical & Biological Laboratories, Nagoya, Japan) on serum samples kept frozen at -20°C.[13,14] The sFas assay uses Fas antibodies against 2 different epitopes. One of the antibodies was a polyclonal antibody and recognized the intracellular domain (No. 305-319 amino acid), while the other was a monoclonal antibody and recognized the extracellular domain (No. 110-120 amino acid). All reactions were at room temperature. In wells coated with anti-Fas polyclonal antibody, 1:4 diluted serum samples or standards were incubated for 1 hour. After washing, a peroxidase-conjugated anti-Fas monoclonal antibody was added to the microwell and incubated for 1 hour. After another washing, a chromogenic substrate was added and allowed to incubate for 30 minutes. The reaction was stopped and absorbance at 450 nm was measured. A standard curve was prepared from sFas calibrators, and the concentration of sFas in serum samples was determined by interpolation. All samples were assayed in duplicate. The intra-assay coefficient of variation was less than 8% and the interassay coefficient of variation was less than 9%.

The sFasL assay was identical to the above assay. In wells coated with anti-FasL monoclonal antibody, 4H9, 1:2 diluted serum samples or standards were incubated for 1 hour. After washing, a peroxidase-conjugated anti-FasL monoclonal antibody, 4A5, was added to the microwell and incubated for 1 hour. After another washing, a chromogenic substrate was added and allowed to incubate for 30 minutes. The reaction was stopped and absorbance at 450 nm was measured. A standard curve was prepared from sFasL calibrators, and the concentration of sFas in serum samples was determined by interpolation. All samples were assayed in duplicate. The intra-assay coefficient of variation was less than 4.2% and the interassay coefficient of variation was less than 7.3%.

Statistics

Results were analyzed by paired Student's *t* test. *P* values less than .05 were considered statistically significant. Linear regression test was used to examine the correlation between sFasL levels and other parameters.

RESULTS

sFas and sFasL Serum Level

The demographic characteristics of patients with GD are shown in Table 1.

As shown in Fig 1, serum levels of sFas were significantly higher in the untreated GD patients with higher TRAb level (group I; mean ± SD, 1.56 ± 0.26 ng/mL) than in the GD patients with low levels of TRAb (group II; 0.76 ± 0.26 ng/mL, $P < .01$) or in control subjects (group III; 0.79 ± 0.24 ng/mL, $P < .01$).

As shown in Fig 2, serum levels of sFasL were also significantly higher in group I patients (mean ± SD, 0.153 ± 0.018 pg/mL) than in group II patients (0.126 ± 0.112 pg/mL, $P <$ .01). Serum concentrations of sFasL in control subjects (group III) were all below the level of 0.1 ng/mL (mean ± SD, 0.076 ± 0.010 pg/mL).

Correlation Between sFas, sFasL, and TRAb

A significant correlation was seen between serum levels of sFas, sFasL, and TRAb. There was also a significant correlation between sFasL levels and TRAb activity in group I patients (n = 22, $r = 0.43$, $P < .05$). When group II patients were included in this analysis, a prominent correlation was found (n = 44, $r = 0.69$, $P < .01$; Fig 3).

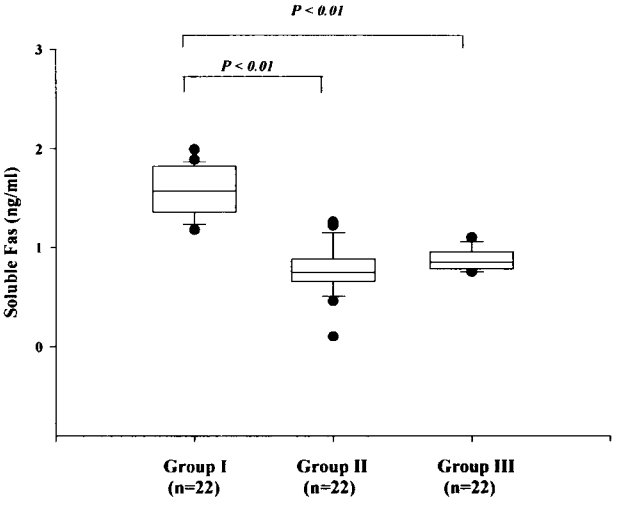

Fig 1. Serum levels of sFas in untreated GD patients (group I) with higher TRAB level, treated GD patients with low levels of TRAb (group II), and control subjects (group III).

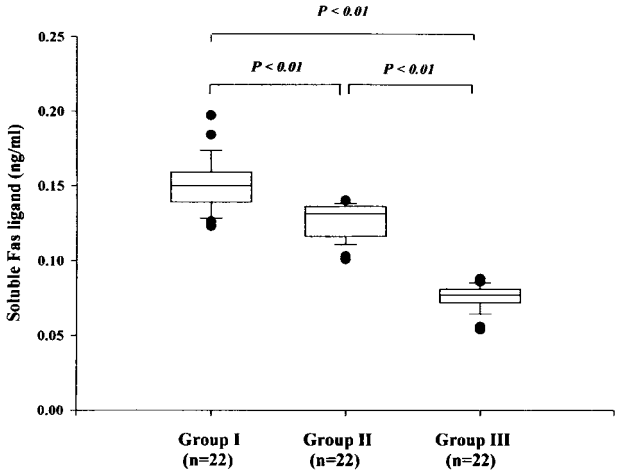

Fig 2. Serum levels of sFasL in untreated GD patients (group I) with higher TRAb level, treated GD patients with low levels of TRAb (group II), and control subjects (group III).

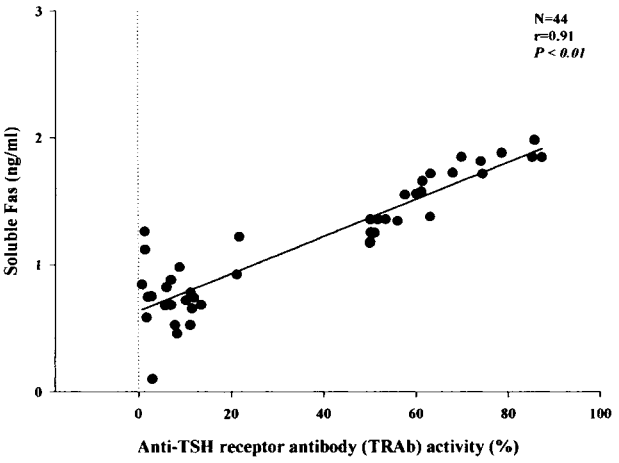

Fig 4. Correlation between serum sFas and serum TRAb activity in all patients with GD ($r = 0.91$, $P < .01$).

Figure 4 shows that there was also a significant correlation between sFas levels and TRAb activity in GD patients (n = 44, $r = 0.91$, $P < .01$).

Since sFas is an apoptosis inhibitor while sFasL induces apoptosis, it is interesting that a significant correlation was observed between serum levels of sFas and sFasL in GD patients (n = 44, $r = 0.71$, $P < .01$; Fig 5).

DISCUSSION

FasL, a member of the tumor necrosis factor family that can induce apoptosis of Fas-bearing cells, was first reported to be expressed in activated T cells and natural killer cells.[15] Although membrane-associated FasL is more efficient than sFasL in aggregating Fas, sFasL also has cytotoxic activity against Fas-expressing cells, as it causes trimerization of Fas molecules, thus inducing apoptosis.[9,16]

Recently, sFasL was reported to induce epithelial cell apoptosis in acute lung injury,[17] and other reports indicated that serum sFasL levels increase with the severity of heart failure in patients with myocarditis[18] and in patients with advanced congestive heart failure.[19] However, although FasL expression in GD thyrocytes has been shown to have functional activity in inducing apoptosis of Fas-bearing target cells,[3] the cytotoxic effect of circulating sFasL requires further investigation. On the other hand, sFas had been reported to be a pathologic factor in GD.[14] In the present study, we analyzed circulating sFasL levels in GD patients with different disease activity and found that serum sFasL may be a marker for evaluating disease activity in GD.

The pathogenesis of GD is associated with serum TRAb, and the disease activity is closely correlated with the level of TRAb.[11] We found that the levels of circulating sFas and sFasL correlated closely with to TRAb levels, especially in GD patients with higher TRAb levels (>50%). Although Hiromatsu

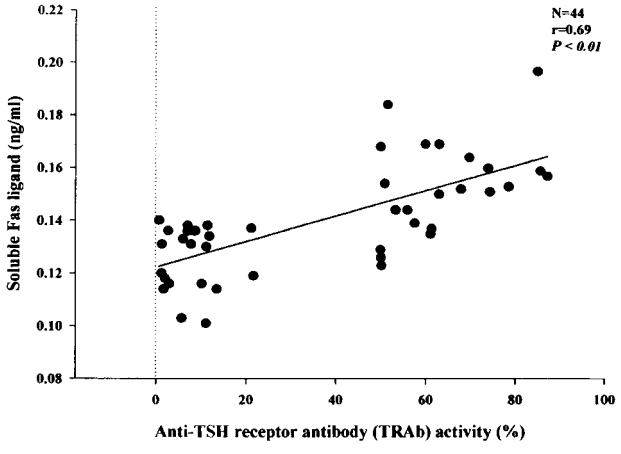

Fig 3. Correlation between serum sFasL levels and serum TRAb activity in all patients with GD ($r = 0.69$, $P < .01$).

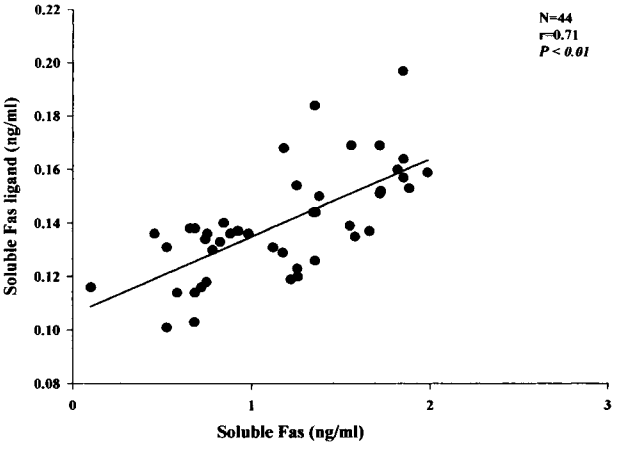

Fig 5. Correlation between serum levels of sFas and sFasL in all patients with GD ($r = 0.71$, $P < .01$).

et al[14] have reported a significant correlation between sFas and TRAb levels, our study is the first report of a correlation between sFasL and TRAb levels in GD. In addition, a significant correlation was seen between serum levels of sFas and sFasL, suggesting that sFas/sFasL is related to disease activity of GD patients.

Conventionally, Fas, FasL, and sFasL are thought to induce apoptosis, while sFas is an apoptotic inhibitor. In Hiromatsu's study,[14] and our own, circulating sFas levels and TRAb were simultaneously increased. Thus, sFas may play a role, together with TRAb in preventing the Fas/FasL-mediated apoptosis of thyrocytes, thus leading to thyroid hyperplasia and the increased disease activity. However, although Fas is expressed in both normal and diseased thyroid, FasL is only expressed in diseased thyroid. Generally, FasL is expressed in immune-privileged sites, such as retina and testis, where it affords protection against specifically activated lymphocytes.[20,21] Recent reports even indicated the greater the expression of FasL, the more downregulation of disease activity was seen in autoimmune thyroiditis[22]; in addition, FasL expression on thyrocytes may have curative effect on ongoing experimental autoimmune thyroiditis by inducing apoptosis of autoreactive infiltrating T lymphocytes in an animal model.[23]

Production of FasL can be used to induce specific tolerance by apoptosis and clonal deletion of antigen-reactive T cells,[24-26] but FasL can also cause local damage. In our study, sFasL was only detected in GD patients; in addition, sFasL levels were significantly correlated with TRAb and sFas levels. This suggests that sFasL may have a dual effect in modulating disease activity in GD, acting at immune-privileged sites to destroy infiltrating activated lymphocytes and also inducing apoptosis of hyperplastic thyrocytes. The explanation provided by Hiromatsu et al,[3] that increased expression of FasL in GD thyrocytes may help in maintaining thyroid homeostasis, appears to be more important. Generally, higher levels of TRAb are indicative of more aggressive disease activity in GD. Since we noted higher sFasL levels in those GD patients with higher TRAb (>50%), it was expected that thyrocytes of these patients would show higher expression of apoptosis. However, the immune-privileged effect on activated infiltrating lymphocytes may be more important, as the conventional Fas/FasL-mediated apoptosis of thyrocytes is usually prevented by TRAb,[12] or blocked by a protein inhibitor in thyrocytes.[27]

On the other hand, in our study and in other reports,[13,14,28] although circulating sFas can be readily detected in both GD patients and normal subjects, serum levels of sFasL in normal subjects are lower than 0.1 ng/mL. These results are compatible with the finding that FasL is only expressed in diseased thyroid, as in GD and thyroid carcinoma. Therefore, circulating level of sFasL may be a potential marker for evaluating disease activity in GD, since changes in sFasL levels and TRAb levels occur in parallel. In conclusion, increased serum sFasL in patients with GD may contribute to homeostasis in the thyroid. Serum sFasL may be useful as a marker for evaluating disease aggression or regression in GD.

REFERENCES

1. Arscott PL, Baker JR Jr: Apoptosis and thyroiditis. Clin Immunol Immunopathol 87:207-217, 1998
2. Giordano C, Stassi G, De Maria R, et al: Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. Science 275:960-963, 1997
3. Hiromatsu Y, Tomoaki H, Yagita H, et al: Functional fas ligand expression in thyrocytes from patients with Graves' disease. J Clin Endocrinol Metab 84:2896-2902, 1999
4. Mitsiades N, Poulaki V, Mastorakkpsi G, et al: Fas ligand expression in the thyroid carcinoma: A potential mechanism of immune evasion. J Clin Endocrinol Metab 84:2924-2932, 1999
5. Stokes TA, Rymaszewski M, Arscott PL, el al: Constitutive expression of FasL in thyrocytes. Science 279:2015, 1998
6. Baker Jr JR: Editorial: Dying (apoptosing?) for a consensus on the Fas death pathway in the thyroid. J Clin Endocrinol Metab 84: 2593-2595, 1999
7. Suda T, Takahashi, Golstein P, et al: Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 75:1169-1178, 1993
8. Kayagaki N, Kawasaki A, Ebata T, et al: Metalloproteinase-mediated release of human Fas ligand. J Exp Med 182:1777-1783, 1995
9. Tanaka M, Suda T, Takahashi T, et al: Expression of the functional soluble form of human Fas ligand in activated lymphocytes. EMBO J 14:1129-1135, 1995
10. Tanaka M, Suda T, Haze K, et al: Fas ligand in human serum. Nature Med 2:317-322, 1996
11. Oertel JE, LiVolsi VA: Pathology of thyroid disease, in Braverman LE, Utiger RD (eds): Werner and Ingber's, The Thyroid (ed 6). Philadelphia, PA, Lippincott, 1989, pp 603-642
12. Kawakami A, Eguchi K, Matsuoka N, et al: Modulation of Fas-mediated apoptosis of human thyroid epithelial cells by IgG from patients with Graves' disease (GD) and idiopathic myxoedema. Clin Exp Immunol 110:434-439, 1997
13. Shimaoka Y, Hidaka Y, Okumura M, et al: Serum concentration of soluble Fas in patients with autoimmune thyroid diseases. Thyroid 8:43-47, 1998
14. Hiromatsu Y, Bennarczuk T, Soyejima E, et al: Increased serum soluble Fas in patients with Graves' disease. Thyroid 9:341-345, 1999
15. Nagata S, Golstein P: The Fas death factor. Science 267:1449-1456, 1995
16. Seig S, Smith D, Kaplan D: Differential activity of soluble versus cellular Fas ligand: Regulation by an accessory molecule. Cell Immunol 195:89-95, 1999
17. Matute BG, Liles WC, Steinberg KP, et al: Soluble Fas ligand induces epithelial cell apoptosis in human with acute lung injury (ARDS). J Immunol 163:2217-2225, 1999
18. Toyozaki T, Hiroe M, Tanaka M, et al: Levels of soluble Fas ligand in myocarditis. Am J Cardiol 82:246-248, 1998
19. Yamaguchi S, Yamaoka M, Okuyama M, et al: Elevated circulating and cardiac secretion of soluble Fas ligand in patients with congestive heart failure. Am J Cardiol 83:1500-1503, 1999
20. Griffith TS, Brunner T, Fletcher SM, et al: Fas ligand-induced apoptosis as a mechanism of immune privilege. Science 270:1189-1119, 1995
21. Bellgrau D, Gold D, Selawry H, et al: A role for CD95 ligand in preventing graft rejection. Nature 377:630-632, 1995
22. Batteux F, Lores P, Bucchini D, et al: Transgenic expression of Fas ligand on thyroid follicular cells prevents autoimmune thyroiditis. J Immunol 164:1681-1686, 2000

23. Batteux F, Tourneur L, Charreire J, et al: Gene therapy of experimental autoimmune thyroiditis by in vivo administration of plasmid DNA coding for Fas ligand. J Immunol 162:603-608, 1999

24. Watanabe-Fukunaga R, Brannan CI, Copeland NG, et al: Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 356:314-317, 1992

25. Zhou T, Bluethmann H, Zhang J, et al: Defective maintenance of T cell tolerance to a superantigen in MRL-lpr/lpr mice. J Exp Med 176:1063-1072, 1992

26. Zhang HG, Liu D, Heike Y, et al: Induction of specific T-cell tolerance by adenovirus-transfected, Fas ligand-producing antigen presenting cells. Nature Biotech 16:1045-1049, 1998

27. Arscott PL, Knapp J, Rymaszewski M, et al: Fas (APO-1, CD95)-mediated apoptosis in thyroid cells is regulated by a labile protein inbitor. Endocrinology 138:5019-5027, 1997

28 Takeda K, Ohara E, Kaneda T, et al: Increased serum soluble Fas ligand in hyperthyroid Graves' disease. Rinsho Byori Jpn J Clin Pathol 47:961-965, 1999