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$CD4^+$ 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

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SUMMARY: The accumulation of activated $CD4^+$ T cells and antigen (Ag)-dependent cellular interactions between thyrocytes and $CD4^+$ T cells have been determined in thyroid gland from patients with Graves' disease. The Fas/Fas ligand (FasL) interaction between antigen-presenting cells and T cells regulates the apoptosis of the former cells triggered by the latter cells. The inhibition of Fas-mediated apoptosis in thyrocytes could be a underlying mechanism of hyperplasia of thyrocytes in patients with Graves' disease. We investigated the potential role of Fas/FasL interaction between thyrocytes and $CD4^+$ T cells in the induction of Fas-mediated apoptosis of the former cells induced by the latter cells. The presence of only a few specific T cells responsive to a putative autoantigen has hampered the investigation of specific T cell activation toward antigen-presenting cells (APCs). Therefore, we used a superantigen, staphylococcal enterotoxin B (SEB), to examine specific T cell activation toward thyrocytes in vitro since it stimulates a large proportion of T cells with particular Vβ elements. Spontaneous apoptosis of thyrocytes in culture was not found even in the presence of various kinds of cytokines. In contrast, a clear induction of Fas-mediated apoptosis by anti-Fas IgM was determined in interferon-γ (IFN-γ)-stimulated thyrocytes. In addition, a significant cytotoxicity of purified $CD4^+$ T cells toward IFN-γ-stimulated thyrocytes in the presence of SEB was induced, and the addition of anti-HLA-DR and -DQ monoclonal antibodies (mAbs) or blockade of the Fas/FasL interaction reduced this cytotoxicity. FasL expression of $CD4^+$ T cells cocultured with IFN-γ-stimulated thyrocytes in the presence of SEB was clearly induced. Furthermore, the addition of mAbs against CD54 and CD58 inhibited both cytotoxicity and FasL expression of $CD4^+$ T cells. The cytotoxicity of $CD4^+$ T cells toward IFN-γ-stimulated, SEB-pulsed thyrocytes was markedly inhibited when we used thyrocytes cultured with IFN-γ in the presence of thyroid-stimulating hormone (TSH) as target cells. Our results suggest that 1) $CD4^+$ T cells were activated by thyrocytes expressing MHC class II molecules in an SEB-dependent manner and then expressed FasL. 2) These activated $FasL^+$ $CD4^+$ T cells killed thyrocytes by interacting with Fas on thyrocytes and FasL on activated $CD4^+$ T cells. The presence of costimulating molecules such as CD54 and CD58 on thyrocytes was also necessary to generate activated $FasL^+$ $CD4^+$ T cells. 3) Since the actions of thyroid stimulating antibody (TSAb) toward thyrocytes are similar to those of TSH, one goitrogenic activity of TSAb may, in part, be due to the inhibitory effect on Fas-mediated apoptosis of thyrocytes triggered by activated $CD4^+$ T cells. (*Lab Invest 2000, 80:471–484*).

Recent studies have demonstrated that apoptosis is an important process in physiological and pathological cell death (Nagata, 1997; Thompson, 1995). The percentage of apoptotic thyrocytes in situ is increased in Hashimoto's thyroiditis, but decreased in Graves' disease (Giordano et al, 1997; Hammond et al, 1997; Tanimoto et al, 1995), suggesting the importance of apoptotic cell death of thyrocytes in the regulation of functions and numbers of these cells in autoimmune thyroid diseases.

Fas has been identified as the cell surface molecule that induces apoptosis (Itoh et al, 1991; Nagata, 1997; Nagata and Golstein, 1995). Immunohistological studies have revealed Fas expression on thyrocytes in patients with Graves' disease and Hashimoto's thyroiditis (Giordano et al, 1997; Hammond et al, 1997; Tanimoto et al, 1995). We recently reported that Fas is functionally expressed on human thyrocytes in vitro, and Fas-mediated apoptosis of these cells by anti-Fas IgM is significantly suppressed by the addition of TSH (Kawakami et al, 1996) or TSAb containing IgG

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(Kawakami et al, 1997b). In addition, Giordano et al demonstrated that the increase of apoptotic thyrocyte death in Hashimoto's thyroiditis is caused by the augmented Fas-mediated apoptosis of the cells (Giordano et al, 1997). These data strongly suggest that apoptosis of thyrocytes recognized in the histological studies is triggered and regulated through the signal of Fas on thyrocytes.

Active cellular immune responses within the thyroid gland are thought to play an important role in the pathologic process of Graves' disease (Eguchi, et al, 1995; Eguchi et al, 1988; Matsuoka et al, 1996; Nagataki and Eguchi, 1992). Immunohistological examination have shown the expression of MHC class II molecules on thyrocytes in patients with Graves' disease (Eguchi et al, 1995; Nagataki and Eguchi, 1992). In vitro studies have also shown that thyrocytes function as stimulatory APCs to T cells (Eguchi et al, 1995; Eguchi et al, 1988; Matsuoka et al, 1996; Nagataki and Eguchi, 1992), suggesting the presence of antigen (Ag)-dependent active cellular interactions between thyrocytes and T cells in thyroid glands of patients with Graves' disease. Activated $CD4^+$ T cells have been found in thyroid glands of patients with Graves' disease (Eguchi, et al, 1995; Eguchi et al, 1988; Ishikawa et al, 1987; Ishikawa et al, 1993; Matsuoka et al, 1996; Nagataki and Eguchi, 1992). Results of in vitro studies have shown that activated $CD4^+$ T cell clone express Fas ligand (FasL) and kill Fas^+ target cells (Ju et al, 1994; Rouvier et al, 1993; Stalder et al, 1994). In light of previous findings regarding Fas expression on thyrocytes, we speculate that the cytotoxicity of activated $FasL^+CD4^+$ T cells toward Fas^+ thyrocytes in the thyroid gland of Graves' disease is suppressed by TSAb, thus promoting hyperplasia of thyrocytes.

TSH inhibits Fas-mediated apoptosis of thyrocytes in a similar fashion to TSAb (Kawakami et al, 1997b). In the present study, we sought to determine whether the Fas/FasL interaction between thyrocytes and $CD4^+$ T cells developed in a Ag-dependent manner, and whether TSH inhibited this interaction.

Results

Cell Surface Molecule Expression and Anti-Fas-Mediated Apoptosis of Thyrocytes

We initially examined in vitro the expression of cell surface molecules on thyrocytes from patients with Graves' disease as these molecules regulate the cellular interactions between APCs and T cells (Matsuoka et al, 1996; Ozdemirli et al, 1996; Urayama et al, 1997). As shown in Fig. 1, Fas was constitutively expressed on unstimulated thyrocytes. Although HLA-DR expression on unstimulated thyrocytes was negligible, costimulating molecules, such as CD54 and CD58, were expressed on these cells (Fig. 1). Incubation of thyrocytes with interferon-γ (IFN-γ) resulted in a clear induction of expression of HLA-DR (Fig. 1). The expression of CD54 and Fas was also significantly augmented by IFN-γ although the latter did not change the expression of CD58 (Fig. 1). Like HLA-DR, HLA-DQ was expressed only after IFN-γ stimulation (data not shown). As reported previously by our group (Matsuoka et al, 1996), the expression of CD80, CD86, and CD106 was not detected on either unstimulated or IFN-γ-stimulated thyrocytes (data not shown).

We next examined anti-Fas IgM-induced apoptosis of thyrocytes. As we previously described (Kawakami et al, 1996; Kawakami et al, 1997b), anti-Fas-mediated apoptosis was detected only in IFN-γ-stimulated thy-

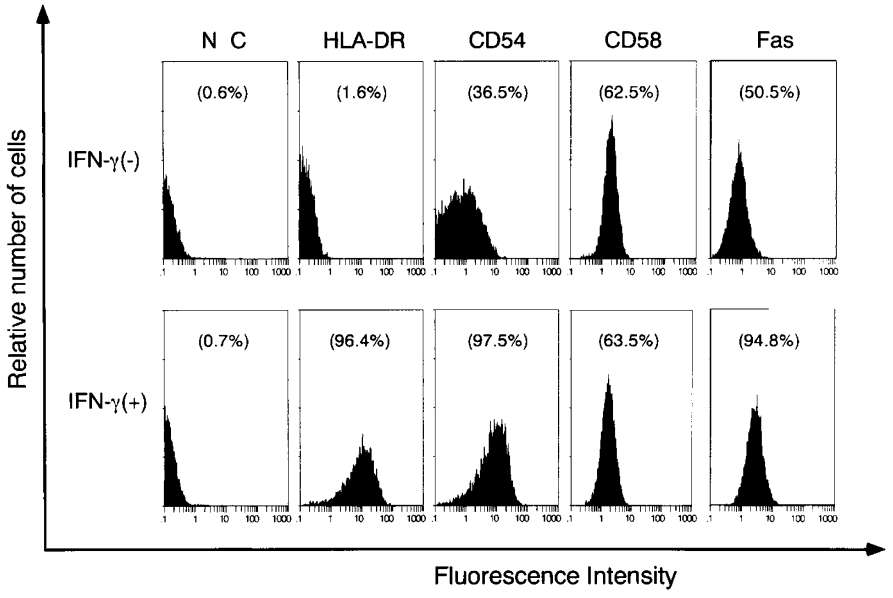

Figure 1.

Cell surface molecule expression on thyrocytes. Thyrocytes from patients with Graves' disease were incubated with or without rIFN-γ (500 IU/ml) for 72 hours, and the expression of several cell surface molecules was examined as described in the text. *NC*: negative control staining. Numbers in parentheses are the percentages of positive cells. Results are representative of seven similar experiments.

rocytes whose Fas expression was significantly higher than that of unstimulated thyrocytes (Table 1). Apoptotic cell death of IFN-γ-stimulated thyrocytes induced by anti-Fas IgM was also confirmed by Hoechst 33258 dye staining (Fig. 2). When compared with unstimulated thyrocytes, IFN-γ-stimulated thyrocytes show clearly augmented pro-caspase-8 and pro-caspase-3 expression (Fig. 3A). We found no difference in cell surface molecule expression and anti-Fas-mediated apoptosis between Graves' thyrocytes and normal thyrocytes (data not shown), as we previously described(Kawakami et al, 1996; Kawakami et al, 1997b; Matsuoka et al, 1996), including the effect of TSH for Fas-mediated apoptosis of thyrocytes shown later in this manuscript. Therefore, we used thyrocytes from Graves' patients in the following experiments.

CD4$^+$ T Cell-Mediated Cytotoxicity Toward Thyrocytes

We previously reported that thyrocytes are potent APCs against T cells (Eguchi et al, 1988; Matsuoka et al, 1996). Thus, we investigated whether purified CD4$^+$ T cells can be activated by thyrocytes in a staphylococcal enterotoxin B (SEB) dependent manner and then kill the thyrocytes. No cytotoxicity of CD4$^+$ T cells toward unstimulated and IFN-γ-stimulated thyrocytes was noted in the absence of SEB (Fig. 4A). Since SEB was presented to T cells along with MHC class II molecules (Marrack and Kapper, 1990; Seth et al, 1994), only slight cytotoxicity toward unstimulated thyrocytes was demonstrated in the presence of SEB (Fig. 4A, left panel). When IFN-γ-stimulated thyrocytes were used in the experiments, however, a marked cytotoxicity of CD4$^+$ T cells toward thyrocytes was observed (Fig. 4A, right panel). This cytotoxicity increased in a time-dependent manner, and high numbers of CD4$^+$ T cells and concentrations of SEB also augmented the cytotoxicity (Fig. 4). The addition of SEB alone to unstimulated or IFN-γ-stimulated thyrocytes influenced neither Fas expression nor the sensitivity of anti-Fas-mediated apoptosis in these cells (Table 2). In the following experiments, we used an effector to target ratio (E/T ratio) of 10, 1 μg/ml of SEB, and incubated for 18 hours because a significant cytotoxicity of CD4$^+$ T cells toward IFN-γ-stimulated thyrocytes was observed in these experimental conditions (Fig. 4). Giordano et al have recently reported that apoptosis of cultured thyrocytes was induced in fratricide or suicide fashion by interleukin1β (IL-1β) (Giordano et al, 1997). ^{51}Cr-labeled thyrocytes were cocultured with thyrocytes instead of CD4$^+$ T cells in the presence of SEB, then ^{51}Cr release assay was performed. As shown in Table 3, ^{51}Cr release from thyrocytes was small in all thyrocyte combinations in spite of FasL expression on the cells (Fig. 3B). Experiments using Hoechst 33258 dye staining confirmed the data of ^{51}Cr release assays (Fig. 5). These data suggest that ^{51}Cr release from IFN-γ-stimulated thyrocytes cocultured with CD4$^+$ T cells in the presence of SEB is mainly triggered not by thyrocytes themselves but by CD4$^+$ T cells.

Table 1. Anti–Fas–Mediated Apoptosis of Thyrocytes

Thyrocytes	Control IgM	Anti-Fas IgM
Percentage of the cells with hypodiploid DNA		
unstimulated thyrocytes	1.3 ± 0.1%	1.6 ± 0.3%
IFN-γ-stimulated thyrocytes	1.4 ± 0.2%	35.6 ± 2.9%*
^{51}Cr release from thyrocytes		
unstimulated thyrocytes	2.5 ± 0.2%	2.8 ± 0.3%
IFN-γ-stimulated thyrocytes	2.1 ± 0.3%	32.9 ± 3.0%*

Thyrocytes from patients with Graves' disease were incubated with or without rIFN-γ (500 IU/ml) for 72 hours. After incubation, anti-Fas-mediated apoptosis of thyrocytes was examined as described in the text. Data shown are the mean ± SD from five experiments. *$p < 0.01$ vs. unstimulated thyrocytes.

Molecules Involved in CD4$^+$ T Cell-Mediated Cytotoxicity Toward IFN-γ-Stimulated Thyrocytes

Several types of cytokines could be produced during cocultivation of thyrocytes and CD4$^+$ T cells in the presence of SEB, thereby inducing apoptosis of thyrocytes, as IL-1β alone can induce the apoptosis of thyrocytes in culture isolated from nontoxic goiter (Giordano et al, 1997). Thus, we examined whether cytokines themselves were involved in the induction of apoptosis of thyrocytes. As shown in Table 4, all tested cytokines failed to induce apoptosis of thyrocytes. In addition, ^{51}Cr release from thyrocytes did not change following the addition of these cytokines, and Hoechst dye staining did not show apoptosis of these cytokine-treated thyrocytes (data not shown). Therefore, we next examined the cell surface molecules involved in apoptosis of thyrocytes. FasL is a strong candidate molecule for CD4$^+$ T cell-mediated cytotoxicity (Ju et al, 1994; Rouvier et al, 1993; Stalder et al, 1994), and we previously showed the importance of costimulating molecules CD54 and CD58 on thyrocytes for T cell activation (Matsuoka et al, 1996). Thus, we investigated whether blocking the Fas/FasL interaction or using mAbs against CD54, CD58, or HLA influenced CD4$^+$ T cell-mediated cytotoxicity toward thyrocytes. Table 5 shows the profile of blocking experiments. Since SEB is presented to T cells in the complex of MHC class II molecules, the addition of anti-HLA-DR and -DQ mAbs inhibited the cytotoxicity of CD4$^+$ T cells toward IFN-γ-stimulated thyrocytes. Furthermore, the addition of human Fas chimeric protein (hFas-Fc) or anti-FasL mAb blocked the cytotoxicity, indicating that FasL of CD4$^+$ T cells mediates cytotoxicity toward thyrocytes. Anti-tumor necrosis factor α (TNF-α) mAb or anti-IL-1β mAb did not reduce CD4$^+$ T cell-mediated cytotoxicity toward IFN-γ-stimulated thyrocytes (Table 5).

In the next series of experiments, we added several mAbs against costimulating molecules. The expression of CD54 and CD58 was determined on thyrocytes by flow cytometric analysis. The addition of mAb against CD54 and CD58 diminished CD4$^+$ T cell-mediated cytotoxicity toward IFN-γ stimulated thyrocytes (Table 5) although mAb against CD80, CD86, or CD106 did not affect cytotoxicity (data not shown).

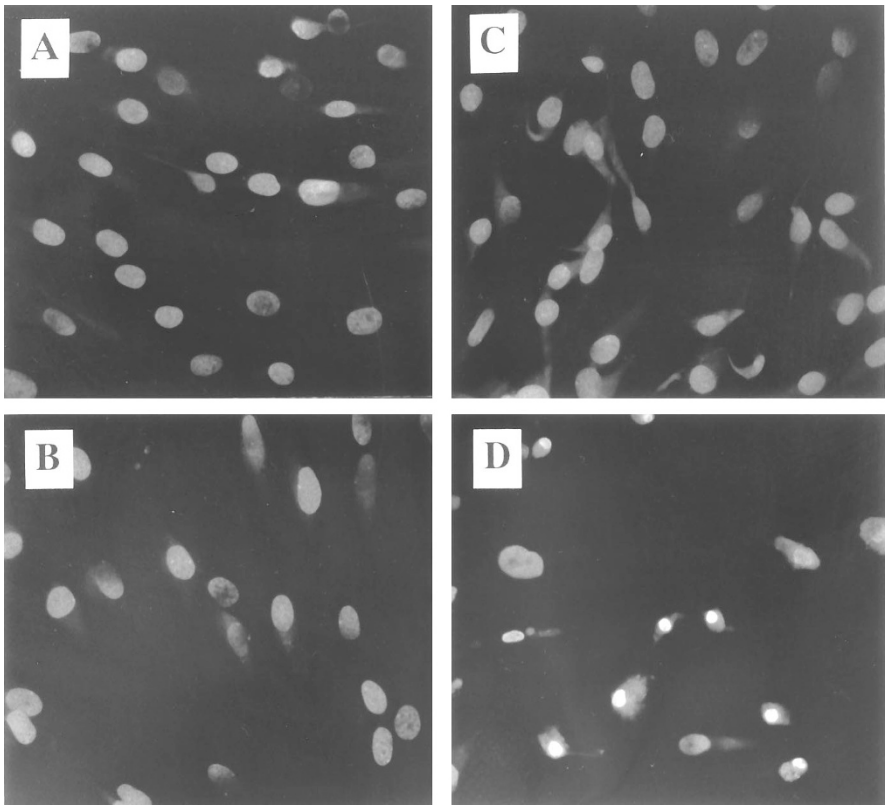

Figure 2.

Anti-Fas IgM-mediated apoptosis of thyrocytes determined by Hoechst 33258 dye staining. Thyrocytes from patients with Graves' disease were incubated with or without rIFN-γ (500 IU/ml) for 72 hours, washed, and incubated with control mouse IgM (1000 ng/ml) or anti-Fas IgM (1000 ng/ml) for an additional 18 hours. After cultivation, apoptosis of cultured thyrocytes was examined by Hoechst 33258 dye staining as described in the text. A: unstimulated thyrocytes with control IgM; B: unstimulated thyrocytes with anti-Fas IgM; C: IFN-γ-stimulated thyrocytes with control IgM; D: IFN-γ-stimulated thyrocytes with anti-Fas IgM. Note that apoptosis of thyrocytes was only observed in D (IFN-γ-stimulated thyrocytes with anti-Fas IgM). Results are representative of six experiments performed.

The inhibition of ^{51}Cr release by the combination of anti-CD54 and anti-CD58 was more prominent than that by hFas-Fc or anti-FasL mAb (Table 5). We examined variable concentrations of hFas-Fc or mAbs (at concentrations of 5 to 20 μg/ml, except for anti-FasL mAb, which was tested at 2.5 to 10 μg/ml), and peak inhibitory effect was noted at concentrations of 10 μg/ml, except for anti-FasL mAb, where peak inhibition was observed at 2.5 to 5 μg/ml (Table 5). These results suggest that CD54 and CD58 expressed on thyrocytes are important costimulating molecules for generating activated $CD4^+$ T cells.

Inhibitory Effect of TSH on $CD4^+$ T Cell-Mediated Cytotoxicity Toward IFN-γ-Stimulated Thyrocytes

We previously reported the inhibitory effects of TSH on Fas-mediated apoptosis of thyrocytes (Kawakami et al, 1996). Thyrocytes were incubated in media containing IFN-γ with or without TSH for 72 hours to examine $CD4^+$ T cell-mediated cytotoxicity toward thyrocytes. Fig. 6 shows the profile of cell surface molecule expression on thyrocytes in the presence or absence of TSH. The addition of TSH significantly inhibited Fas expression on thyrocytes although TSH did not change the expression of HLA-DR, CD54, and CD58 (Fig. 6). Treatment of thyrocytes with TSH reduced the expression of pro-caspase-8 and pro-caspase-3 in IFN-γ-stimulated thyrocytes (Fig. 7). Furthermore, $CD4^+$ T cell-mediated cytotoxicity was significantly suppressed when we used thyrocytes treated with both IFN-γ and TSH as target cells (Fig. 8).

FasL Expression of $CD4^+$ T Cells Cocultured with Thyrocytes

Blocking experiments suggested the importance of FasL in $CD4^+$ T cell-mediated killing of thyrocytes. Therefore, we examined FasL expression of $CD4^+$ T cells cocultured with thyrocytes. $CD4^+$ T cells incubated with thyrocytes in the absence of SEB did not kill thyrocytes, and FasL expression of these cells was virtually absent (Table 6). Although the expression of FasL by $CD4^+$ T cells cocultured with unstimulated thyrocytes with SEB was small, strong FasL expression was demonstrated on $CD4^+$ T cells cocultured with IFN-γ-stimulated thyrocytes in the presence of SEB (Table 6). When mAbs against HLA-DR, HLA-DQ, CD54, and CD58 were added, there was a significant reduction in FasL expression (Table 6), indicating that the reduction of $CD4^+$ T cell-mediated cytotoxicity is due to a decrease in FasL expression by $CD4^+$ T cells. In contrast, FasL expression on $CD4^+$ T cells was not

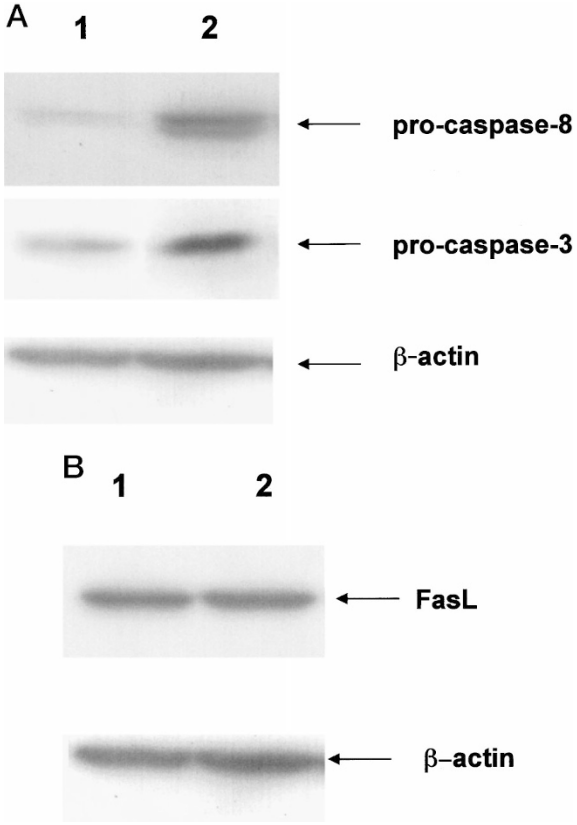

Figure 3.

Expression of pro-caspase-8, pro-caspase-3, and FasL in thyrocytes. Thyrocytes from patients with Graves' disease were incubated with or without rIFN-γ (500 IU/ml) for 72 hours, and the expression of pro-caspase-8/-3 (A) and FasL (B) was examined by Western blotting as described in the text. Lane 1: unstimulated thyrocytes; Lane 2: IFN-γ-stimulated thyrocytes. Note that the expression of pro-caspase-8/-3 was clearly increased in IFN-γ-stimulated thyrocytes. FasL expression in thyrocytes was not changed by IFN-γ stimulation. Results are representative of five experiments performed.

affected by treatment of thyrocytes with TSH (Table 6). Since TSH did not change the expression of HLA-DR, CD54, and CD58 (Fig. 6), the inhibitory effect of TSH on CD4$^+$ T cell-mediated cytotoxicity was due not to a reduction of FasL expression on CD4$^+$ T cells but to a reduction of Fas-mediated apoptosis of thyrocytes.

Discussion

Graves' disease is an organ-specific autoimmune disorder characterized by immune responses against a number of thyroid-specific antigens such as TSH receptor (Eguchi et al, 1995; Nagataki and Eguchi, 1992). Previous studies have revealed the aberrant expression of MHC class II molecules on thyrocytes in patients with Graves' disease (Eguchi et al, 1995; Nagataki and Eguchi, 1992), suggesting that Ag-dependent cellular interactions between thyrocytes and CD4$^+$ T cells are involved in the autoimmune process in this disease. Thyrocytes function as stimulatory APCs to T cells in vitro (Eguchi, et al, 1995; Eguchi et al, 1988; Matsuoka et al, 1996; Nagataki and Eguchi, 1992), and Fas$^+$ Ag-presenting B cells are killed by Ag-specific CD4$^+$ FasL$^+$ T cell clone (Ozdemirli et al, 1996). Although Fas is expressed in situ on thyrocytes in patients with Graves' disease, apoptosis of these cells is reported to be suppressed, as determined by in situ end-labeling of fragmented DNA (Hammond et al, 1997; Tanimoto et al, 1995), indicating that humoral factors such as TSAb inhibit Fas/FasL interactions between thyrocytes and activated CD4$^+$ T cells.

As previously described (Kawakami et al, 1996; Kawakami et al, 1997b), Fas was constitutively expressed on unstimulated thyrocytes. Anti-Fas-mediated apoptosis of thyrocytes was only detected on IFN-γ-stimulated thyrocytes whose Fas expression was significantly increased compared with unstimulated thyrocytes.

Caspase-8 and caspase-3 are major effector molecules acting downstream of Fas (Nagata, 1997). The expression of both pro-caspase-8 and pro-caspase-3, pro-enzymes converted into active forms upon signaling through Fas, in cultured thyrocytes was also markedly augmented by IFN-γ, which may explain why Fas-mediated apoptosis is efficiently induced in IFN-γ-stimulated thyrocytes. Similar experimental results have also recently been seen in cultured synovial cells isolated from osteoarthritis (OA) patients (Kobayashi et al, 1999). In that study, unstimulated OA synovial cells were resistant to Fas-mediated apoptosis in spite of Fas expression. However, stimulation of OA synovial cells with TNF-α augmented the expression of pro-caspase-8 and pro-caspase-3 and sensitized the cells to Fas-mediated apoptosis.

The aim of the present study in vitro was to determine whether Ag-dependently activated CD4$^+$ T cells kill thyrocytes via Fas/FasL interaction. To emphasize the effect of cellular interaction, we used an SEB to investigate the cellular interaction between CD4$^+$ T cells and thyrocytes since it stimulates a large proportion of T cells expressing particular Vβ elements. When SEB is presented to CD4$^+$ T cells, MHC class II molecules on APCs are essential because these molecules are thought to form a trimolecular complex with SEB and a T cell receptor (Marrack and Kapper, 1990; Seth et al, 1994). Since both HLA-DR and HLA-DQ expression by unstimulated thyrocytes was negligible, only a small level of cytotoxicity of CD4$^+$ T cells was detected after cocultivation with unstimulated thyrocytes in the presence of SEB. In contrast, significant CD4$^+$ T cell-mediated cytotoxicity toward IFN-γ-stimulated thyrocytes, in which HLA-DR and -DQ expression was markedly induced, was found in the presence of SEB. The fact that the addition of anti-HLA-DR and -DQ mAbs diminished cytotoxicity and that no cytotoxic activity of CD4$^+$ T cells was demonstrated in the absence of SEB suggests that induction of cytotoxicity of CD4$^+$ T cells toward SEB-pulsed, IFN-γ-stimulated thyrocytes was SEB-dependent.

In the present study, FasL expression of CD4$^+$ T cells cocultured with IFN-γ-stimulated thyrocytes in the presence of SEB was significantly induced, and blocking the Fas/FasL interaction reduced the cytotoxicity. In addition, treatment of thyrocytes with IFN-γ, with or without SEB, in the absence of CD4$^+$ T

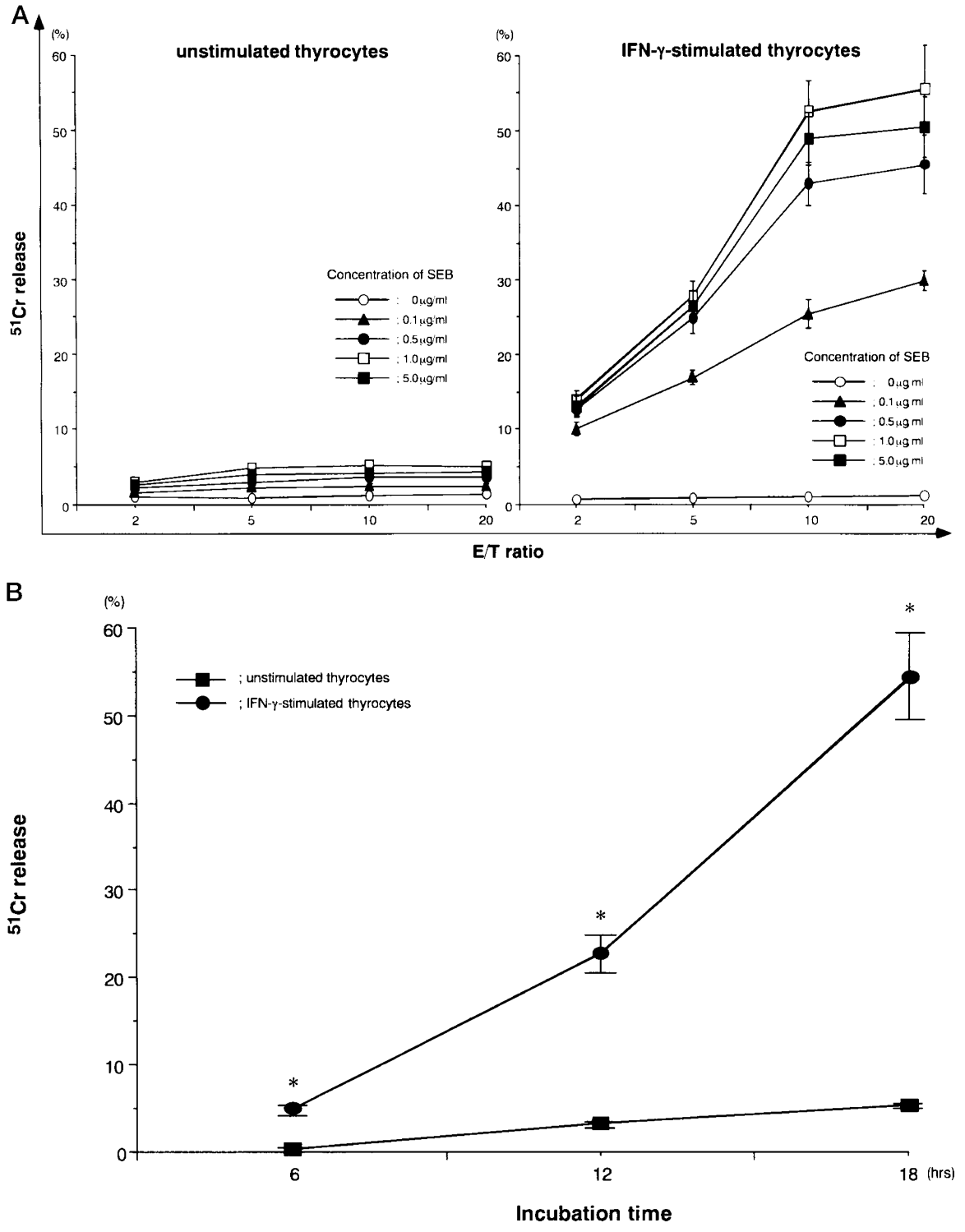

Figure 4.

Cytotoxic activity of $CD4^+$ T cells against SEB-pulsed thyrocytes. *A*: Thyrocytes from patients with Graves' disease were cultivated with or without rIFN-γ (500 IU/ml) for 72 hours. After cultivation, the cells were labeled with ^{51}Cr and further incubated with different numbers of $CD4^+$ T cells in the presence of varying concentrations of SEB for 18 hours. *B*: Time kinetic study. Thyrocytes from patients with Graves' disease were cultivated with or without rIFN-γ (500 IU/ml) for 72 hours, labeled with ^{51}Cr, and further incubated with $CD4^+$ T cells at an E/T ratio of 10 in the presence of 1 μg/ml of SEB for indicated time intervals. Results shown are the mean±SD from five experiments. *; $p < 0.01$, vs. unstimulated thyrocytes.

cells affects neither Fas expression nor sensitivity of anti-Fas-mediated apoptosis. This suggests that FasL expression on activated $CD4^+$ T cells is induced by SEB-specific cellular interactions between thyrocytes and $CD4^+$ T cells, with apoptosis of thyrocytes being mediated via Fas/FasL pathways. FasL was also expressed in cultured thyrocytes; however, it did not efficiently induce apoptotic cell death of thyrocytes in

Table 2. No Significant Effect of SEB on Fas Expression and Anti-Fas-Mediated Apoptosis of Thyrocytes

Treatment of thyrocytes	SEB	Fas expression on thyrocytes	Anti-Fas-mediated apoptosis determined by flowcytometer
(−)	(−)	52.5 ± 4.0%	2.4 ± 0.3%
		NS	NS
	(+)	51.9 ± 3.5%	2.5 ± 0.3%
IFN-γ	(−)	95.0 ± 5.0%	36.9 ± 3.0%
		NS	NS
	(+)	96.0 ± 5.5%	37.8 ± 4.0%

Thyrocytes from patients with Graves' disease were cultured with or without rIFN-γ (500 IU/ml) for 72 hours, further incubated in the presence or absence of SEB (1 μg/ml) for 18 hours. After incubation, Fas expression and anti-Fas-mediated apoptosis of thyrocytes were examined as described in the text. Data shown are the mean ± SD from five experiments. NS, not significantly different in the absence or presence of SEB.

Table 3. No Significant Apoptosis of Thyrocytes in Fractricide or Suicide Fashion

Effector thyrocytes	Target thyrocytes	^{51}Cr release (%)
unstimulated	unstimulated	7.2 ± 0.6
unstimulated	IFN-γ-stimulated	7.5 ± 0.7
IFN-γ-stimulated	unstimulated	6.9 ± 0.6
IFN-γ-stimulated	IFN-γ-stimulated	7.7 ± 0.6

Target thyrocytes were labeled with ^{51}Cr, washed, and further incubated with effector thyrocytes for 18 hours in the presence of 1 μg/ml of SEB. After incubation, ^{51}Cr release from target thyrocytes was examined as described in the text. Data are mean ± SD from four experiments.

fractricide or suicide fashion. The function of FasL on CD4$^+$ T cells and thyrocytes could differ. Nevertheless, considerable cytotoxicity remains even after hFas-Fc or anti-FasL mAb is added to the culture.

The addition of IL-1β to thyrocytes from nontoxic goiter was reported to induce the apoptosis of the cells (Giordano et al, 1997), although we could not reproduce this phenomenon using thyrocytes from Graves' patients or thyrocytes isolated from normal thyroid tissue adjacent to thyroid carcinoma. We previously showed the increase of thyrocyte proliferation by IL-1β without the induction of apoptosis (Kawabe et al, 1989). Therefore, the different action of IL-1β on thyrocytes in our study and in Giordano et al (Giordano et al, 1997) may be due to the differences in the subjects used or in culture conditions. Since cytokines including IL-1β and TNF-α did not induce apoptosis of thyrocytes in the present study, other molecules such as TIA-1 (Tian et al, 1991), granzyme B (Heusel et al, 1994), or tumor necrosis factor-related ligand (TRAIL) (Pitti et al, 1996) may be responsible for the remaining cytotoxicity of activated CD4$^+$ T cells toward thyrocytes.

Recent studies have shown that interference with either CD28/CD80, CD86 (Azuma et al, 1993; Freeman et al, 1993), CD2/CD58, LFA-1/CD54, or VLA-4/CD106 (Bohmig et al, 1994; Damle et al, 1992) results in T cell inactivation toward APCs. We also reported previously that CD54 and CD58 on thyrocytes are indispensable costimulating molecules in activating T cells efficiently in an Ag-specific manner (Matsuoka et al, 1996). In the present study, the addition of mAbs against CD54 and CD58 reduced both cytotoxicity and FasL expression of CD4$^+$ T cells. These data strongly suggest that costimulating molecules expressed on thyrocytes have an important role in the generation of activated FasL$^+$ CD4$^+$ T cells with cytotoxic activity. Furthermore, the inhibition of ^{51}Cr release from thyrocytes by the combination of anti-CD54 mAb and anti-CD58 mAb was more significant than that by hFas-Fc or anti-FasL mAb. These data also support the idea that CD4$^+$ T cells express not only FasL but also other molecules such as TIA-1, granzyme B, or TRAIL in response to SEB-pulsed thyrocytes.

The inhibitory effect of TSH for Fas-mediated apoptosis may reflect the in vivo action of TSH on the growth of thyroid gland since TSH can stimulate thyroid growth in vivo. In vitro studies, however, failed to demonstrate the same results in cultured human thyrocytes (Dumont, 1971; Valente et al, 1983; Wadeleux and Winard, 1981; Westermark et al, 1979). The addition of cyclic adenosine monophosphate (cAMP) also suppressed Fas-mediated apoptosis of thyrocytes, and both TSH and TSAb containing IgG increased cAMP level in thyrocytes (Kawakami et al, 1996; Kawakami et al, 1997b), suggesting that TSH and TSAb act on thyrocytes in a similar manner to functional Fas expression on thyrocytes. In the present study, TSH significantly inhibited both Fas-mediated apoptosis of thyrocytes and CD4$^+$ T cell-mediated cytotoxicity toward thyrocytes. In contrast, TSH did not affect HLA-DR and costimulating molecule expression on thyrocytes. Furthermore, FasL expression on CD4$^+$ T cells cocultured with thyrocytes was not suppressed by the use of TSH-treated thyrocytes. Treatment of thyrocytes with TSH clearly suppressed the expression of Fas, pro-caspase-8, and pro-caspase-3 in IFN-γ-stimulated thyrocytes. These data indicate that the addition of TSH to thyrocytes affects not the activation of CD4$^+$ T cells but the effector function of CD4$^+$ T cells through down-regulation of the Fas signaling pathway in thyrocytes. Alternatively, phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase C (PKC), both activated

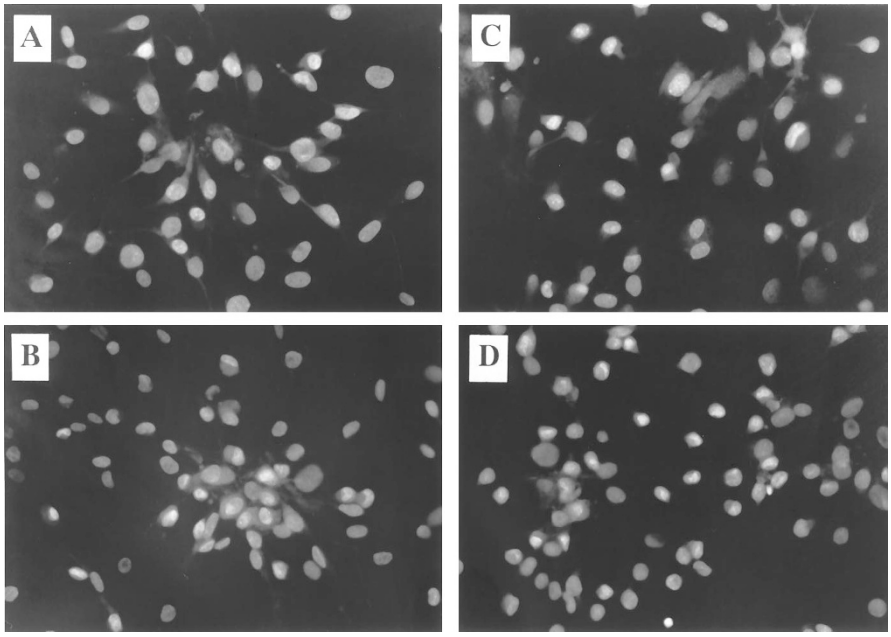

Figure 5.
No significant apoptosis of thyrocytes in fractricide or suicide fashion examined by Hoechst 33258 dye staining. A: unstimulated thyrocytes cocultured with unstimulated thyrocytes in the presence of SEB; B: unstimulated thyrocytes cocultured with IFN-γ-stimulated thyrocytes in the presence of SEB; C: IFN-γ-stimulated thyrocytes cocultured with unstimulated thyrocytes in the presence of SEB; D: IFN-γ-stimulated thyrocytes cocultured with IFN-γ-stimulated thyrocytes in the presence of SEB. Note that apoptotic cell death of thyrocytes was not observed in A-D. Results are representative of five experiments performed.

Table 4. No Significant Effect of Cytokines on Thyrocyte Apoptosis

Treatment of thyrocytes	Percentage of the cells with hypodiploid DNA (%)	
(−)	2.0 ± 0.2	NS
IL-1β	2.1 ± 0.3	
IL-2	1.9 ± 0.3	
TNF-α	2.4 ± 0.2	
IL-6	2.2 ± 0.2	
G-CSF	2.1 ± 0.3	
GM-CSF	2.2 ± 0.3	

Thyrocytes from patients with Graves' disease were incubated with rIFN-γ (500 IU/ml) for 72 hours, further incubated in various cytokines for 18 hours. After incubation, apoptosis of thyrocytes was examined as described in the text. Data shown are the mean ± SD from four experiments. NS, not significantly different in each stimulation.

by TSH (De Groot et al, 1996), may also play an important role in the inhibition of Fas-mediated apoptosis of thyrocytes. One recent report suggested that both PI 3-kinase and PKC act as inhibitors for apoptosis (Lan and Wong, 1999).

In summary, we showed in the present study that apoptosis of thyrocytes could be induced by activated $CD4^+$ T cells via Fas/FasL interaction. The accumulation of activated $CD4^+$ T cells in thyroid gland from patients with Graves' disease has been demonstrated (Ishikawa et al, 1987; Ishikawa et al, 1993), and restricted T cell receptor gene usage was recently demonstrated in thyroidal infiltrate from Graves' patients (Davies et al, 1991a; 1991b). These data suggest that Ag-specific T cell activation is present in thyroid glands from Graves' patients. Although the activation of $CD4^+$ T cells by SEB may be an artificial system, Fas-mediated cytotoxicity toward thyrocytes by $CD4^+$ T cells was only induced when cultured with thyrocytes that express MHC class II molecules in the presence of SEB. We speculate that $CD4^+$ T cells in thyroid gland from Graves' patients are activated by MHC class II molecule-expressed thyrocytes with such putative antigens as the TSH receptor. TSH inhibits $CD4^+$ T cell-mediated cytotoxicity toward thyrocytes, and TSAb found in Graves' disease could also suppress the cytotoxicity of $CD4^+$ T cells in a similar fashion. This action of TSAb may explain both the decrease of apoptotic cell death of thyrocytes in Graves' disease even in the presence of activated $CD4^+$ T cells and the acceleration of thyroid gland growth in patients with Graves' disease.

Materials and Methods

Preparation of Thyrocytes

Thyroid tissue samples were obtained at the time of subtotal thyroidectomy from 10 patients with Graves' disease. In some experiments, normal thyroid tissues adjacent to thyroid carcinomas were also obtained from another group of patients. The experimental protocol was approved by the Hospital Human Ethics Review Committee and a signed consent was obtained from each patient. Isolation of thyrocytes was performed according to the method described previously by our laboratory (Eguchi et al, 1988; Matsuoka et al, 1996). In brief, thyroid tissues from Graves' patients or normal thyroid tissues were digested with

Table 5. Blocking of Cytotoxicity of CD4$^+$T Cells Toward IFN-γ-Stimulated Thyrocytes

Treatment of thyrocytes with mAb/hFas-Fc	Concentration of mAb/hFas-Fc (μg/ml)				
	0	2.5	5	10	20
(−)	56.5 ± 4.6				
control IgG		NT	56.9 ± 4.9	57.2 ± 5.1	58.0 ± 5.0
anti-HLA-ABC		NT	54.9 ± 4.6	55.0 ± 5.1	57.0 ± 3.9
anti-HLA-DR and -DQ		NT	18.8 ± 1.1	12.7 ± 1.0	12.2 ± 0.9
hFas-Fc		NT	35.9 ± 2.1	27.1 ± 1.5	27.9 ± 1.9
anti-FasL		26.9 ± 1.7	25.5 ± 1.9	24.9 ± 1.5	NT
anti-CD54		NT	33.7 ± 1.7	28.5 ± 1.7	28.0 ± 1.4
anti-CD58		NT	39.9 ± 2.0	33.7 ± 2.2	33.0 ± 1.5
anti-CD54 and -CD58		NT	18.1 ± 1.0	11.9 ± 1.2	10.9 ± 1.3
anti-TNF-α		NT	55.9 ± 4.2	52.9 ± 3.9	54.0 ± 2.9
anti-IL-1β		NT	54.3 ± 3.2	54.0 ± 4.2	54.7 ± 2.7

Thyrocytes from patients with Graves' disease were cultured with rIFN-γ (500 IU/ml) for 72 hours. After incubation, the cells were labeled with ^{51}Cr and further incubated with CD4$^+$T cells (E/T ratio of 10) in the presence of 1 μg/ml of SEB with or without various mAb/hFas-Fc for 18 hours. After incubation, cytotoxicity of CD4$^+$T cells toward IFN-γ-stimulated thyrocytes was examined as described in the text. Results are the mean ± SD of four experiments. NT, not tested.

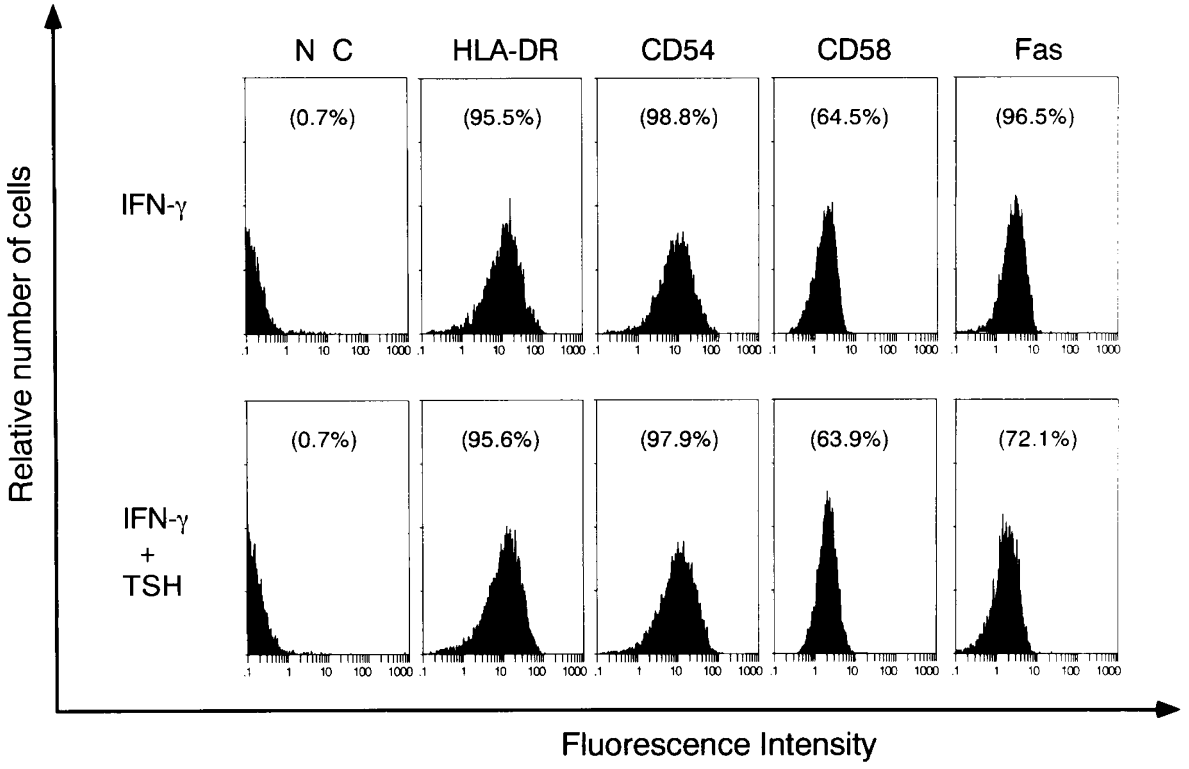

Figure 6.
Effect of TSH on cell surface molecule expression on thyrocytes. Thyrocytes from patients with Graves' disease were incubated with rIFN-γ (500 IU/ml) in the presence or absence of 10 mIU/ml of TSH for 72 hours. Numbers in parentheses are the percentages of positive cells. *NC*, negative control staining. Results are representative of six similar experiments.

collagenase (Sigma Chemical Co., St. Louis, Missouri) and dispase (Godo Shusei Co., Tokyo) in Hanks' Balanced Salt Solution. To eliminate non-adherent cells, the dispersed cells were cultured for 18 hours in RPMI 1640 supplemented with 10% FBS (Gibco Laboratories, Grand Island, New York) and then extensively washed with PBS containing 2% FBS. To obtain pure thyrocyte preparations, we cultured the cells for an additional 7 days in RPMI 1640 supplemented with 10% FBS. The resultant thyrocyte preparations were less than 1% reactive with mAbs CD3 (Coulter Immunology, Hialeah, Florida), CD68 (DAKO JAPAN Co., Kyoto, Japan), CD20 (Coulter Immunology), and anti-human von Willebrand factor (Immunotech SA, Marseille, France) which define an antigen on all mature T cells, monocytes/macrophages, pan-B cells and on

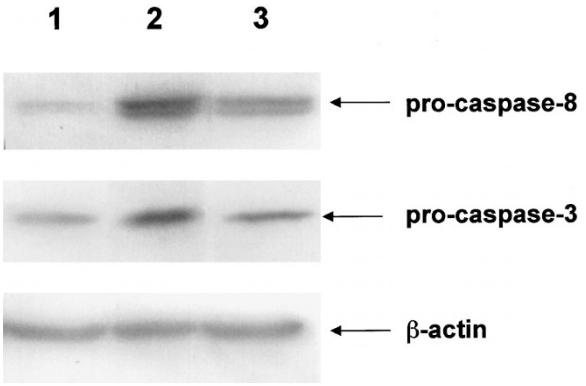

Figure 7.
Inhibitory effect of TSH on the expression of pro-caspase-8 and pro-caspase-3 in IFN-γ-stimulated thyrocytes. Thyrocytes from patients with Graves' disease were incubated with rIFN-γ (500 IU/ml) for 72 hours in the presence or absence of 10 mIU/ml of TSH. After cultivation, the expression of pro-caspase-8 and pro-caspase-3 in thyrocytes was examined by Western blotting as described in the text. Lane 1: unstimulated thyrocytes; Lane 2: IFN-γ-stimulated thyrocytes; Lane 3: thyrocytes cultured with IFN-γ and TSH. Note that the expression of pro-caspase-8 and pro-caspase-3 was augmented by IFN-γ; however, their expression was reduced by TSH. Results are representative of data from five experiments.

vascular endothelial cells. Moreover, thyrocyte preparations were more than 99% reactive with anti-thyroglobulin antibody by an avidin-biotin immunoperoxidase technique.

Purification of CD4$^+$ T Cells

A sample of heparinized peripheral blood was obtained from consenting healthy donors. Peripheral blood mononuclear cells were isolated by Ficoll-Conray density gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, New Jersey) and incubated for 2 hours at 37° C on Petri dishes to eliminate the monocyte-rich fraction. Non-adherent cells were further incubated with anti-CD4-coated magnetic beads (Dynabeads M450; DYNAL, Oslo, Norway) at 4° C for 30 minutes, and then sorted by a magnetic particle concentrator (Dynal MPC, DYNAL). The sorted cells were detached from magnetic beads by DETACH-a-BEAD (DYNAL). The purity of the isolation technique was determined by a flow cytometer (EPICS-PROFILE-II, Coulter Immunology) using an anti-CD4 mAb (MBL, Nagoya, Japan); more than 99% of the cell preparations were CD4 positive.

Expression of HLA-DR, HLA-DQ, CD54, CD58, and Fas on Thyrocytes

We examined the expression of the above molecules on cultured thyrocytes as described previously (Matsuoka et al, 1996). Briefly, thyrocytes (3×10^5) were cultured in 6-well tissue culture clusters (Costar, Cambridge, Massachusetts) in RPMI 1640 supplemented with 2% BSA (Sigma) with or without recombinant IFN-γ (rIFN-γ, 500 IU/ml, Shionogi Pharmaceutical Co., Osaka) for 72 hours. After incubation, the expression of HLA-DR, HLA-DQ, CD54, CD58, and Fas on thyrocytes was determined by an indirect immunofluorescence method using an anti-HLA-DR mAb (MBL), anti-HLA-DQ mAb (MBL), anti-CD54 mAb (MBL), anti-CD58 mAb (MBL), or anti-Fas mAb (MBL) and phycoerythrin (PE)-conjugated anti-mouse IgG (MBL) as the second reagent. In brief, cultured thyrocytes were detached from the plate by the addition of 0.265 mM EDTA. Thyrocyte preparations were washed twice with PBS containing 1% FBS. Thyrocytes were then incubated with the primary mAb for 30 minutes on ice, washed three times with PBS, and resuspended in PE-conjugated anti-mouse IgG. After incubation for 30 minutes on ice, the expression of cell surface molecule on thyrocytes was determined by a flow cytometer (EPICS-PROFILE-II). In some experiments, bovine TSH (Sigma) was added to the culture and the expression of cell surface molecules was also examined. As a control, the cells were stained with isotype-matched control mouse IgG in place of the specific primary mAb.

Expression of Pro-Caspase-8, Pro-Caspase-3, and FasL in Cultured Thyrocytes

We examined the expression of pro-caspase-8, pro-caspase-3, and FasL in cultured thyrocytes by Western blotting as previously described (Kawakami et al, 1997a). In brief, treated thyrocytes were collected and lysed by the addition of lysis buffer (50 mM Tris buffer, pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml of aprotinin, 1% NP-40, 0.5% sodium deoxycholate) for 20 minutes at 4° C. Insoluble material was removed by centrifugation at 13,000 rpm for 30 minutes at 4° C. The supernatant was collected and the protein concentration was determined by the Bio-Rad (Melville, New York) protein assay kit. An identical amount of protein for each lysate (20 μg/ml) was subjected to 15% SDS-PAGE. Proteins were transferred to a nitrocellulose filter, and the filter was blocked for 1.5 hours using 5% non-fat dried milk in PBS containing 0.1% Tween 20 (PBS-T), washed with PBS-T, and incubated at room temperature for 1 hour in the presence of each antibody (pro-caspase-8: mouse monoclonal, MBL; pro-caspase-3: mouse monoclonal, Transduction Laboratories, Lexington, Kentucky; FasL: mouse monoclonal, Transduction Laboratories). The filter was washed with PBS-T and incubated with 1:1000 dilution of sheep anti-mouse IgG coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham) was used for detection. We used mAb toward β-actin for an internal control protein in Western blotting.

Cytotoxic Activity of CD4$^+$ T Cells Toward SEB-Pulsed Thyrocytes

Cytotoxic activity of CD4$^+$ T cells toward SEB-pulsed thyrocytes was determined by using a ^{51}Cr release assay. In brief, thyrocytes were labeled with ^{51}Cr sodium chromate (Amersham). Then 5×10^3 labeled thyrocytes in each well (Costar 3799, Cambridge, MA; total volume = 200 μl) were mixed with CD4$^+$ T cells

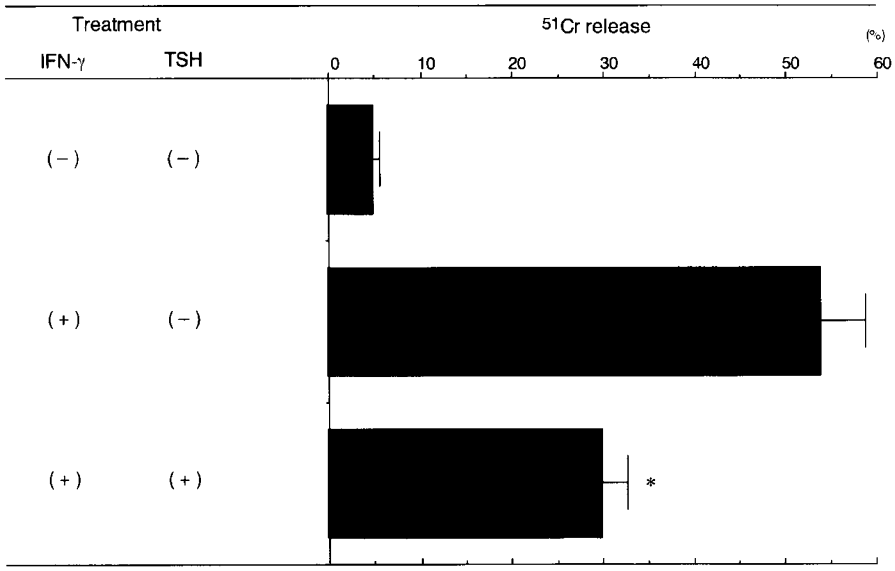

Figure 8.
Inhibitory effects of TSH on $CD4^+$ T cell-mediated cytotoxicity toward IFN-γ-stimulated thyrocytes. Thyrocytes from patients with Graves' disease were incubated with rIFN-γ (500 IU/ml) for 72 hours in the presence or absence of 10 mIU/ml of TSH. After cultivation, the cells were labeled with ^{51}Cr, and further incubated with $CD4^+$ T cells (E/T ratio of 10) for 18 hours in the presence of 1 μg/ml of SEB. Results shown are the mean$\pm$SD of five experiments. *; $p < 0.01$, compared with data in the absence of TSH.

Table 6. FasL Expression of $CD4^+$ T Cells Cocultured with Thyrocytes

Treatment of thyrocytes			FasL expression of $CD4^+$ T cells (%)
IFN-γ	SEB	mAb/TSH	
(−)	(−)	(−)	2.8 ± 0.3
(−)	(−)	control IgG	2.9 ± 0.2
(−)	(+)	(−)	4.4 ± 0.8
(−)	(+)	control IgG	4.4 ± 0.9
(+)	(−)	(−)	3.1 ± 0.3
(+)	(−)	control IgG	3.0 ± 0.3
(+)	(+)	(−)	28.2 ± 3.0*
(+)	(+)	control IgG	28.0 ± 2.7*
(+)	(+)	anti-HLA-DR and -DQ	9.0 ± 0.8**
(+)	(+)	anti-CD54 and -CD58	6.8 ± 0.5**
(+)	(+)	TSH	27.4 ± 2.5**

Thyrocytes were incubated with or without rIFN-γ (500 IU/ml) for 72 hours. After incubation, the cells were incubated with purified $CD4^+$ T cells at the E/T ratio of 10 for 18 hours in the presence or absence of 1 μg/ml of SEB with or without various mAb (10 μg/ml) or TSH (10 mIU/ml). FasL expression of $CD4^+$ T cells was examined as described in the text. *$p < 0.01$ vs. IFN-γ-stimulated thyrocytes in the absence of SEB. **$p < 0.01$ vs. IFN-γ-stimulated thyrocytes in the presence of SEB with control IgG or in the absence of mAb. Results shown are the mean ± SD from four experiments.

at different E/T ratios in RPMI 1640 supplemented with 5% FBS in the presence of different concentrations of SEB (Sigma). After incubation for the indicated time intervals, the plates were centrifuged, and 100 μl-aliquots of the supernatants were assayed for radioactivity using a gamma counter. The spontaneous release of ^{51}Cr was determined by incubating thyrocytes with the medium alone, whereas the maximum release was determined by adding Triton X-100 to a final concentration of 1%. In some experiments, variable concentrations of hFas-Fc (kindly provided by Dr. Shigekazu Nagata, Osaka Bioscience Institute and Department of Genetics, Osaka University Medical School), neutralizing anti-FasL mAb (clone 4H9, MBL; this mAb is different from anti-FasL mAb used in Western blotting), anti-CD54 mAb (MBL), anti-CD58 mAb (MBL), anti-HLA-DR mAb (MBL), anti-HLA-DQ mAb(MBL), anti-HLA-ABC mAb (MBL), anti-TNF-α mAb (R&D Systems, Minneapolis, Minnesota), or anti-IL-1β mAb (R&D Systems) was added to the culture (2.5 to 10 μg/ml for anti-FasL mAb. The others were examined from 5 to 20 μg/ml). The percentage of specific lysis was calculated as follow: Lysis (%) = [(experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(maximum ^{51}Cr release - spontaneous ^{51}Cr release)] $\times$100.

Detection of Apoptosis of Thyrocytes

Various cytokines can be produced during cocultivation of thyrocytes and $CD4^+$ T cells, which may induce apoptosis of thyrocytes. To investigate the direct effect of cytokines on apoptosis, thyrocytes were incubated with or without rIFN-γ (500 IU/ml) for 72 hours, washed, and then cultured in the presence or absence of rIL-1β (10 IU/ml, Otsuka Pharmaceuticals Co., Tokushima, Japan), rIL-2 (200 IU/ml, Takeda Pharmaceuticals Co., Tokyo), rTNF-α (200 IU/ml, Genzyme, Cambridge, Massachusetts), rIL-6 (100 ng/ml, Genzyme), rIL-10 (20 ng/ml, Genzyme), r granulocyte colony-stimulating factor (rG-CSF, 500 pM, Kirin Co., Tokyo), or r granulocyte macrophage colony-stimulating factor (rGM-CSF, 500 pM, Kirin) for 18 hours. After incubation, thyrocytes were detached from the plate, fixed with 70% ethanol, treated with RNase (100 μg/ml, Sigma), and then stained with

propidium iodide (100 μg/ml, Sigma) for 30 minutes on ice. The stained cells were analyzed using a flow cytometer (EPICS-PROFILE-II). Apoptosis was quantified by flow cytometric determination of the proportion of cells with hypodiploid DNA (Nicoletti et al, 1991). The cytotoxic activity of cytokines against thyrocytes was also examined by a ^{51}Cr release assay. Briefly, thyrocytes in microtiter plates (5×10^3/well) were incubated for 72 hours with or without rIFN-γ (500 IU/ml), then labeled with ^{51}Cr sodium chromate (Amersham). After labeling, cytokines listed above were added to the culture (total volume = 200 μl) and further incubated for 18 hours. After incubation, the plates were centrifuged, and the percentage of specific lysis was calculated as described above. Apoptotic cell death of thyrocytes was further confirmed by Hoechst 33258 dye staining as previously described (Kawakami et al, 1997a). In brief, treated thyrocytes were fixed with 2% glutaraldehyde solution (Wako Pure Chemical Industries, Osaka, Japan) for 10 minutes and stained with 0.2 mM Hoechst 33258 (Wako) to visualize DNA. Cells were examined under a fluorescence microscope (AHB-LB, Olympus, Tokyo) to determine the number of cells with chromatin condensation and/or nuclear fragmentation.

Cultured thyrocytes were also examined for anti-Fas IgM-mediated apoptosis. For this purpose, thyrocytes were cultured in RPMI 1640 containing 2% BSA in the presence or absence of rIFN-γ (500 IU/ml) for 72 hours, then treated with either anti-Fas IgM (1000 ng/ml, MBL) or control mouse IgM (1000 ng/ml, Seikagaku Co., Tokyo) for 18 hours as we previously described (Kawakami et al, 1996; Kawakami et al, 1997b). After cultivation, thyrocytes were detached from the plate and the percentage of cells with hypodiploid DNA was determined by a flow cytometer. ^{51}Cr release from anti-Fas IgM-treated thyrocytes was also examined. Thyrocytes treated with or without rIFN-γ (500 IU/ml) in microtiter plate (5×10^3/well) for 72 hours were labeled with ^{51}Cr, washed, and then cultured for 18 hours in the presence of either control mouse IgM (1000 ng/ml) or anti-Fas IgM (1000 ng/ml). After incubation, cytotoxic activity was examined as described above. Confirmation of anti-Fas IgM-mediated apoptosis of thyrocytes was also investigated by Hoechst 33258 dye staining as described above.

Examination of Thyrocyte Apoptosis in Fratricide or Suicide Fashion

We examined the above mechanism by ^{51}Cr release assay and Hoechst 33258 dye staining. Thyrocytes (unstimulated or IFN-γ-stimulated) were labeled with ^{51}Cr, then 5×10^3 labeled thyrocytes in each well (total volume = 200 μl) were mixed with 5×10^3 thyrocytes (unstimulated or IFN-γ-stimulated) instead of CD4$^+$ T cells in RPMI 1640 supplemented with 5% FBS in the presence of 1 μg/ml of SEB (Sigma). After incubation for 18 hours, the plates were centrifuged, and the cytotoxicity of thyrocytes toward thyrocytes (fratricide or suicide fashion) was calculated as described above. Thyrocyte apoptosis in fratricide or suicide fashion was also examined by Hoechst 33258 dye staining. In brief, thyrocytes (3×10^5 of unstimulated or IFN-γ-stimulated thyrocytes in 6-well tissue culture clusters) were cocultured with 3×10^5 of unstimulated or IFN-γ-stimulated thyrocytes for 18 hours in the presence of 1 μg/ml of SEB. After incubation, Hoechst 33258 dye staining was performed.

FasL Expression of CD4$^+$ T Cells Cocultured with SEB-Pulsed Thyrocytes

We examined FasL expression of CD4$^+$ T cells cocultured with SEB-pulsed thyrocytes by flow cytometry. Briefly, incubated CD4$^+$ T cells with SEB-pulsed thyrocytes were initially reacted with PE-conjugated anti-CD4 mAb (MBL). After incubation, cells were permeabilized with digitonin, as described previously (Anderson et al, 1990; Kawakami et al, 1996), because the polyclonal anti-FasL antibody used in the detection of FasL by flow cytometer recognized the cytoplasmic portion of FasL (Kawakami et al, 1997a; Hakuno et al, 1996, corresponding to amino acids; 41–55). After confirming the adequacy of permeabilization by trypan blue uptake, permeabilized cells were incubated with anti-FasL Ab for 30 minutes on ice. After washing three times with PBS, cells were further incubated for 30 minutes on ice with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (MBL). After incubation, CD4$^+$ FasL$^+$ cells were examined by flow cytometry (EPICS-PROFILE-II). Rabbit IgG (Seikagaku Co.) and PE-conjugated mouse IgG1 (MBL) were used as negative control. To check the adequacy of permeabilization on flow cytometric analysis, we used anti-α-tubulin mAb (CedarLane Laboratories Ltd., Hornby, Ontario, Canada) as previously described (Anderson et al, 1990). Almost all cells were stained with anti-α-tubulin mAb.

Statistical Analysis

Data were expressed as mean$\pm$SD. Statistical analysis was performed using Student's *t* test. *P* value less than 0.05 was considered significant.

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