CLINICAL STUDY

Differential expression of Fas system apoptotic molecules in peripheral lymphocytes from patients with Graves' disease and Hashimoto's thyroiditis

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Abstract

Objective: To examine whether the Fas system apoptotic molecules are differentially expressed in Graves' disease (GD) and Hashimoto's thyroiditis (HT), the two opposite phenotypes of autoimmune thyroid disease (AITD).

Design: The expression of Fas and Fas ligand (FasL) on peripheral CD4 and CD8 lymphocytes, and non-lymphoid immune cells as well as their soluble forms in serum from untreated patients with GD and HT were evaluated.

Methods: Flow cytometry was performed for the study of peripheral immune cells from 70 newly diagnosed patients with AITD (55 with HT and 15 with GD) and 20 controls. ELISA was used for the measurement of soluble Fas (sFas) in serum samples from a subgroup of 35 AITD patients.

Results: An increase in the proportion of CD4 and CD8 cells expressing Fas was found in both GD and HT, albeit with some differences, when compared with controls. Importantly, in GD patients, the intensity of Fas expression on CD4 and CD8 lymphocytes was reduced and sFas levels in serum were simultaneously increased when compared with HT patients and controls.

Conclusions: The Fas system apoptotic molecules appear to be differentially expressed on peripheral lymphocytes in the two opposite phenotypes of AITD.

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Introduction

Autoimmune thyroid disease (AITD) is an organ-specific autoimmune disorder that may manifest as either Hashimoto's thyroiditis (HT) or Graves' disease (GD). Loss of immune tolerance and inappropriate immune reaction against the thyroid gland are implicated in its pathogenesis (1–3).

It has been suggested that activation of the Fas/Fas ligand (FasL) apoptotic pathway by proinflammatory cytokines in HT may play a role in the destruction of thyroid follicular cells leading to hypothyroidism (4–14). In GD, thyroid cells are somehow protected from Fas-mediated apoptosis but they become hyperplastic and hyperfunctioning under the influence of thyroid-stimulating hormone (TSH) receptor-stimulating antibodies, leading to hyperthyroidism (11–13, 15, 16). Fas-mediated apoptosis may also play a role in the deletion of peripheral autoreactive T lymphocytes following immune activation (17–19).

Apart from their cell-surface expression, Fas and FasL molecules are also detected in soluble forms (sFas and

sFasL). sFas is an alternatively spliced form of Fas lacking the receptor transmembrane domain and is thought to inhibit apoptosis by interfering with Fas/FasL interaction (17, 20). Increased sFas has been implicated in the pathogenesis of GD (21–24). On the other hand, sFasL is a proteolytic product of FasL but its physiologic role in apoptosis is less clear (17, 25–27).

Most of the information on the role of apoptosis on AITD is based on experimental animal research and a few clinical studies on thyroid tissues obtained after surgery from patients with GD or HT (28–31). Although very informative, these studies have certain limitations. Patients with GD or HT are usually treated with antithyroid drugs or thyroid hormones before undergoing surgery. However, treatment itself may affect the expression of apoptotic molecules on thyroid cells and infiltrating lymphocytes thus obscuring the real picture. Few studies have examined the expression of apoptotic molecules on peripheral T lymphocytes from patients with AITD and the information is therefore limited.

The aim of this study was to evaluate in a comprehensive manner the expression of Fas and FasL

on peripheral lymphocyte subsets and non-lymphoid immune cells together with their soluble forms in serum of patients with GD and HT in the untreated state and at different clinical stages, in order to clarify whether these molecules are differentially expressed in the opposite phenotypes of AITD.

Materials and methods

Patients

A total of 70 consecutive patients with AITD (age range 12–65 years) participated in the study. All the patients were newly diagnosed and untreated for their condition at the time of blood collection. The patients were divided into two main groups according to the clinical expression of AITD and the stage of thyroid dysfunction. Group 1 consisted of 55 patients with HT of whom 40 were at the stage of subclinical hypothyroidism and 15 in clinical hypothyroidism. Group 2 included 15 patients with GD in hyperthyroidism. For comparison, 20 healthy individuals with normal thyroid function, no goiter and negative for antithyroid autoantibodies were also investigated.

The diagnosis of GD and HT was based on commonly accepted clinical and laboratory criteria. The patients with HT were classified as having subclinical hypothyroidism if their free thyroxine (FT₄) and total triiodothyronine (TT₃) levels in serum were within normal range in the face of slightly elevated TSH > 5 IU/ml and clinical hypothyroidism if their FT₄ and TT₃ levels were below normal with raised TSH.

Venous blood was obtained from all the patients and controls in the morning (between 0900 and 1000 h). Full blood count was performed and patients with evidence of infection or inflammation as well as patients on immunomodulatory drugs or drugs known to interfere with thyroid function were excluded. All the samples were analyzed for thyroid hormone levels and thyroid autoantibodies. Flow cytometry measurements were performed within 10 min of blood collection.

Sera from a randomly chosen subgroup of 35 patients, 17 with HT in subclinical hypothyroidism (mean age 35.47 ± 13.74 years), 8 with HT in clinical hypothyroidism (mean age 35.67 ± 14.88 years), 10 with GD (32.5 ± 10.67 years) as well as 10 control subjects (30.22 ± 11.18 years), were kept frozen at -40°C for the measurement of sFas and sFasL. Informed consent was obtained from all subjects and the protocol was approved by the hospital ethics committee.

Methods

Measurement of thyroid hormones and thyroid autoantibodies Commercially available kits (DPC, Los Angeles, CA, USA) were used for the measurement of serum TT₃, FT₄, TSH, anti-thyroglobulin (anti-TG), and

anti-thyroid peroxidase (anti-TPO) antibodies with the automated immunoassay analyzer Immunolite 2000 (DPC Ltd, Gwynedd, UK). Measurements were based on solid-phase chemiluminescent immunoassays.

ELISA For the measurement of sFas protein, the sFas(S) sandwich ELISA kit by MBL (Naka-ku Nagoya, Japan) was used. Serum samples were tested at a five-fold dilution and sensitivity of the assay was 0.5 ng/ml. The sFasL levels were evaluated using the sFas ligand ELISA kit (MBL). Serum samples were tested at a twofold dilution and sensitivity of the assay was 0.1 ng/ml. All enzymatic reaction products were determined photometrically at 450 nm.

Each serum sample was tested twice in two different microplate wells and the mean of absorbance was used to report the concentration from standard curves.

Fluorescence-activated cell sorter (FACS) analysis of surface antigens

The following fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP)-conjugated antihuman monoclonal antibodies were used: Leucogate CD 45/CD14, control $\gamma 1/\gamma 2a$ (anti-isotype control MAB used as a negative control), Simultest CD3/CD19, CD3-PE, CD4-FITC, CD4-PerCP, CD8-PE, CD8-FITC, and CD95 (Fas)-PE all purchased from Becton Dickinson (BD Immunocytometry systems, San Jose, CA, USA), and CD178 (Fas ligand)-FITC (Ancell, Immunology research products, Bayport, MN, USA). The lots of the monoclonal antibodies used in our study were the same. The flow cytometer was calibrated prior to performing every single assay.

To detect the expression of the desired surface molecules, 20 μl BD monoclonal antibodies or 80 μl diluted CD-178 FITC were added to 100 μl whole blood in a tube, mixed thoroughly and incubated for 20 min in the dark room at 4°C . About 2 ml $1\times$ FACS lysing solution (Becton Dickinson) was added and the tube was vortexed thoroughly. After 10 min of incubation in the dark room at 4°C , the tubes were centrifuged at 3000 rounds per min (r.p.m.) for 10 min, supernatant was discarded and the cells were washed with $1\times$ PBS with 0.1% azide and centrifuged again at 400 *g* for 10 min. A second wash was performed and after the supernatant was discarded, 0.5 ml PBS was put in the tube and the specimen was analyzed.

Using FACScan (Becton Dickinson) and the appropriate software (Cell Quest, Becton Dickinson), the population of lymphocytes was identified from forward and side scatter characteristics on dot plot profiles and analyzed for fluorescence intensity using defined gates. For the detection of CD8 cells that express Fas and FasL, at least 20 000 lymphocytes were gated and two-color

Figure 1 Characteristic dot plots of Fas and FasL expression on peripheral blood CD4 lymphocytes in (A) a healthy individual, a patient with (B) subclinical HT, (C) clinical HT, and (D) GD. The x-axis represents FasL expression and fluorescence and the y-axis represents Fas expression and fluorescence on gated CD4 cells. The CD4+ Fas+ lymphocytes are depicted at the upper left quadrant of each plot.

flow cytometry was performed, while for Fas/FasL expression on CD4 cells at least 8000 CD4 lymphocytes were gated and three-color flow cytometry was performed. The population of monocytes and polymorphonuclear cells (PMNC) was identified from CD14 and CD45 expression and forward-side scatter characteristics. Percentage of CD4 or CD8 cells expressing Fas

was calculated from two-parameter dot plots displaying FITC fluorescence on the x-axis and PE fluorescence on the y-axis (Figs 1 and 2). Negative control fluorescence was set at the 99.5% percentile of the negative control. Histogram plots in the form of one-parameter files were also used to show expression of specific membrane-bound molecules when compared with the negative

Figure 2 Characteristic dot plots of Fas expression on peripheral blood CD8 lymphocytes in (A) a healthy individual, a patient with (B) subclinical HT, (C) clinical HT, and (D) GD. The x-axis represents CD8 expression and fluorescence on gated lymphocytes population and the y-axis represents Fas expression and fluorescence on gated lymphocytes. CD8+ Fas+ lymphocytes are depicted at the upper right quadrant of each plot.

controls. Data collected are reported as either percentage of positive cells or mean fluorescence intensity (MFI) values.

Statistical analysis

Probability values <5% ($P<0.05$) were considered statistically significant. The normality of distribution of different values was evaluated using the Kolmogorov–Smirnov test. To compare the means among different groups of patients we used ANOVA test followed by -Scheffe’s test. Correlations between different parameters -were detected using the Pearson’s correlation coefficient. Data are presented as mean values $\pm$ s.d.

Results

Clinical and laboratory characteristics

The demographic, clinical, and laboratory characteristics of the patient groups and controls are shown in Table 1. As expected, patients with GD had increased thyroid hormone levels with suppressed TSH and patients with HT had increased TSH levels with either normal (subclinical HT) or low (clinical HT) thyroid hormone levels in serum. Antithyroid antibodies were positive in all patients with AITD and negative in controls. There were no differences in the mean age between the different groups of patients and controls. Also no differences were seen in the number of white blood cells (WBC) or WBC subgroups (lymphocytes, monocytes, and PMNC) among the groups studied.

Proportions of T-cell subsets

The percentage of T cells among the population of lymphocytes did not differ between the groups of patients and the control group (subclinical HT: $72.5 \pm$

5.8% , clinical HT: $75.5 \pm 4.8\%$, GD: $72.0 \pm 7.1\%$, and controls: $71.5 \pm 8.1\%$). There was also no difference in the percentage of B cells between the patients with AITD and the group of healthy subjects (subclinical HT: $12.1 \pm 2.6\%$, clinical HT: $12.0 \pm 3.4\%$, GD: $12.8 \pm 3.9\%$, and controls: $10.9 \pm 3.9\%$).

The percentage of T cells expressing CD8 was similar in the patient subgroups and the controls (subclinical HT: $29.4 \pm 5.2\%$, clinical HT: $30.2 \pm 8.8\%$, GD: $32.1 \pm 5.7\%$, and controls: $33.8 \pm 8.3\%$) as was the percentage of T cells expressing CD4 (subclinical HT: $61.3 \pm 7.6\%$, clinical HT: $62.4 \pm 9.0\%$, GD: $62.5 \pm 4.4\%$, and controls: $58.3 \pm 8.8\%$).

Fas and FasL expression on T-cell subsets

The proportion of CD4 lymphocytes expressing Fas was higher in patients with subclinical HT and those with GD when compared with controls ($P<0.05$), but the difference in the patients with clinical HT did not reach statistical significance. There was no difference in the proportion of CD4 cells expressing Fas between the different subgroups with AITD.

CD8 lymphocytes from all patient groups and controls expressed Fas at a higher proportion than CD4 cells. The proportion of Fas-expressing CD8 lymphocytes was also higher in patients with clinical and subclinical HT when compared with controls ($P<0.05$), while a non-significant increase was found in patients with GD (Table 2).

There was a positive correlation between age and the proportion of CD4 lymphocytes expressing Fas, in patients with both clinical (Pearson’s correlation: 0.675 , $P<0.001$, $r^2=0.366$) and subclinical HT (Pearson’s correlation: 0.605 , $P<0.05$, $r^2=0.455$), but not in patients with GD or healthy controls. No correlation was found between the proportion of Fas-expressing CD4 or CD8 cells and thyroid hormone levels or antithyroid antibodies.

Table 1 Clinical data of patients with AITD and healthy subjects.

Parameter	Hashimoto’s thyroiditis		Graves’ disease	Healthy subjects
	Subclinical	Clinical		
Age (years)	34.9 ± 14.9	37 ± 18.4	35.8 ± 14.0	37.4 ± 15.3
N	40	15	15	20
Sex	M=7, F=33	M=2, F=13	M=2, F=13	M=3, F=17
TT ₃ (ng/ml)	1.18 ± 0.23	0.917 ± 0.24	4.24 ± 1.94	1.1 ± 0.21
FT ₄ (ng/dl)	0.96 ± 0.14	0.59 ± 0.09	4.91 ± 0.12	1.04 ± 0.15
TSH (IU/ml)	6.86 ± 2.81	35.76 ± 31.47	<0.03	1.4 ± 0.6
Anti-TG (IU/ml)	182.7 ± 189.6	694.5 ± 1227.2	598 ± 914.5	22.2 ± 9.2
Anti-TPO (IU/ml)	1312.7 ± 1360.2	1501.2 ± 1187.7	943.3 ± 847.1	12.8 ± 5.6
WBC (cells/mm ³)	5870 ± 870	6100 ± 1320	5720 ± 1120	6500 ± 1180
Neutrophils	3250 ± 570	3240 ± 1060	2980 ± 630	3550 ± 570
Lymphocytes	2210 ± 440	2330 ± 400	2180 ± 620	2490 ± 700
Monocytes	280 ± 98	310 ± 70	340 ± 150	320 ± 70

AITD, autoimmune thyroid disease; TT₃, tri-iodothyronine; FT₄, free thyroxine; TSH, thyroid-stimulating hormone; anti-TG, antithyroglobulin autoantibodies; anti-TPO, antithyroid peroxidase autoantibodies; WBC, white blood cells. Values are given as mean $\pm$ s.d.

Table 2 Proportion of CD4 and CD8 peripheral lymphocytes expressing Fas and respective MFI values in healthy subjects and patients with AITD.

Parameter	Hashimoto's thyroiditis		Graves' disease	Healthy subjects
	Subclinical	Clinical		
N	40	15	15	20
Fas+CD4+ cells (%)	61.4 ± 12.1*	61.1 ± 15.1	64.1 ± 14.2*	49.9 ± 7.8
Fas+CD8+ cells (%)	74.4 ± 14.4†	77.4 ± 16.6†	67.2 ± 10.7	59.8 ± 14
Fas MFI on CD4 cells	124.7 ± 47.6	117.7 ± 46.3	89.9 ± 12.5‡	117.5 ± 15.3
Fas MFI on CD8 cells	103.6 ± 30.6	108.7 ± 43.9	62.5 ± 13.4§	102.2 ± 33.7

AITD, autoimmune thyroid disease; MFI, mean fluorescence intensity. Values are given as mean ± s.d. ANOVA test followed by Scheffe's test was used to compare means. * $P < 0.05$ compared with healthy subjects. † $P < 0.05$ compared with healthy subjects. ‡ $P < 0.05$ compared with healthy subjects, subclinical HT, and clinical HT. § $P < 0.05$ compared with healthy subjects, subclinical HT, and clinical HT.

In contrast to Fas, FasL was not expressed on CD4 or CD8 lymphocytes in patients with AITD or controls.

Intensity of Fas expression on T-cell subsets

MFI of Fas reflects the number of Fas molecules expressed per cell. Importantly, the MFI of Fas on CD4 lymphocytes from patients with GD was significantly decreased in comparison with not only controls but also patients with HT in both forms ($P < 0.05$). No difference in MFI of Fas on CD4 cells was observed between patients with either form of HT and controls. Likewise, the MFI of Fas on CD8 lymphocytes was lower in patients with GD when compared with controls or patients with HT ($P < 0.05$; Table 2). No correlation between intensity of Fas expression on CD4 or CD8 lymphocytes and age was found in any of the groups studied.

Fas and FasL expression on non-lymphoid immune cells

The evaluation of Fas and FasL expression on monocytes and PMNC showed that both cell types express Fas with monocytes showing a higher intensity of expression. Interestingly, a great proportion of monocytes and PMNC also expressed FasL with much lower intensity of expression than Fas (data not shown). However, no statistical difference was found in the proportion of monocytes expressing Fas (subclinical HT: $96.8 \pm 2.8\%$, clinical HT: $94.5 \pm 6.8\%$, GD: $96.7 \pm 2.6\%$, and controls: $93.0 \pm 6.4\%$) or FasL (subclinical HT: $84.9 \pm 15.6\%$, clinical HT: $86.7 \pm 8.4\%$, GD: $88.4 \pm 10.6\%$, and controls: $84.9 \pm 10.55\%$) or the intensity of Fas and FasL expression on monocytes among the groups studied. Also, no difference was found in the proportion of PMNC expressing Fas (subclinical HT: $97.4 \pm 2.1\%$, clinical HT: $96.6 \pm 2.6\%$, GD: $98.0 \pm 1.1\%$ and controls: $95.5 \pm 3.1\%$) or FasL (subclinical HT: $89.4 \pm 9.1\%$, clinical HT: $93.4 \pm 5.3\%$, GD: $91.5 \pm 7.3\%$ and controls: $90.2 \pm 7.8\%$) or in Fas and FasL intensity of expression on PMNC among the different groups studied (data not shown).

sFas and sFasL levels in patients with AITD

There was a non-significant decrease in serum sFas of patients with subclinical HT relative to controls (2.37 ± 0.66 vs 2.8 ± 0.75 ng/ml) whereas in patients with clinical HT, sFas levels (3.25 ± 1.15 ng/ml) were not different from those in the control group. On the other hand, in patients with GD, sFas levels (4.47 ± 1.0 ng/ml) were significantly higher relative to controls or patients with HT ($P < 0.05$) (Fig. 3). Importantly, in these patients, sFas levels were inversely correlated with MFI of Fas on CD4 lymphocytes (Pearson's correlation: -0.633 , $P = 0.05$, $r^2 = 0.401$).

No correlation was found between sFas and levels of thyroid hormones or antithyroid antibodies. Also, no correlation was found between sFas levels and the proportion of Fas-expressing CD4 and CD8 lymphocytes in all groups of patients and controls in whom sFas was measured.

The measurements of sFasL were below the sensitivity level of the assay (< 0.1 ng/ml) in both controls and in all but three patients with AITD (two with subclinical hypothyroidism and one with GD).

Figure 3 Box diagrams of sFas serum levels in healthy subjects and groups of untreated AITD patients. Boxes show mean, s.d., minimum and maximum values.

Discussion

In this study, we evaluated the expression of the apoptotic molecules Fas and FasL on peripheral immune cells together with their soluble forms in serum from patients with GD and HT in order to assess whether these molecules are differentially expressed in the two opposite phenotypes of AITD. From the analysis of the data, the following points have emerged.

First, expression of Fas was detected in a greater proportion of CD4 and CD8 lymphocytes in patients with AITD regardless of its clinical manifestation, albeit with some minor deviations, when compared with controls. By contrast, FasL was not expressed on these lymphocyte subsets in either the patients or the controls. The significance of this finding is not obvious but may reflect an induction of the Fas-mediated apoptotic machinery on lymphocytes in AITD. However, the lack of FasL expression by the same cells may prevent their elimination by apoptotic death. In this regard, a previous study also reported an increased proportion of Fas-expressing CD4 cells in untreated GD while expression of FasL was not detected (32).

Interestingly, there was a positive correlation between proportion of Fas-expressing CD4 cells and age in HT but not in GD patients. Such a trend was also found in the control group but the correlation was not statistically significant, probably due to the small number of subjects. Similar findings were reported by Maruoka *et al.* in euthyroid patients with AITD and healthy subjects (32). It appears that more cells express Fas with age and this may become more prominent in HT, a condition associated with a higher degree of apoptosis in relation to GD.

Although a greater number of CD4 and CD8 cells were expressing Fas in patients with AITD, the intensity of Fas expression per cell was reduced in patients with GD as opposed to those with HT and the controls. On the other hand, sFas levels in serum were higher in GD patients and tended to be lower in subclinical HT patients than in patients with clinical HT and controls. Furthermore, the reduced number of membrane Fas molecules per CD4 cell was associated with increased sFas in GD patients. These novel findings indicate that the expression of Fas on peripheral lymphocytes and the concentration of its soluble form in serum differ in GD and HT patients. In addition, the expression of the full-length transmembrane Fas and its soluble form may be inversely regulated in patients with GD and possibly HT.

With regard to sFas, previous studies have also reported increased sFas levels in patients with Graves' hyperthyroidism whereas those with GD in remission and euthyroid HT had decreased sFas levels (21–24, 33). It has been suggested that the increased sFas in GD may protect Fas-expressing cells from apoptosis (20–23). Interestingly, however, a recent study revealed that sFas is increased in hyperthyroidism, independent of the underlying cause of thyroid disease (24). Even

though we and others found no correlation of sFas with FT₄ in GD patients, it is possible that hyperthyroidism itself may also influence the production of sFas (23, 34).

Another point of interest that emerged from our study is that non-lymphoid immune cells, including monocytes and PMNC, express the apoptotic molecules Fas and FasL in high proportions. However, the expression of these molecules was not different between patients with AITD and controls. Whether non-lymphoid immune cells participate in the peripheral deletion of activated lymphocytes or in the apoptosis of Fas-expressing thyroid cells in AITD remains a speculation. Interestingly, however, a recent study showed that the proportion and intensity of FasL expression in intrathyroidal monocytes/dendritic cells was higher than CD4 T cells. The authors suggested that these cells may participate in the apoptosis of thyrocytes in patients with HT (35).

Our study has certain limitations. The information we obtained is based on the evaluation of peripheral immune cells and not the thyroid cells or intrathyroidal lymphocytes that are actually involved in the pathogenesis of AITD. However, it is difficult to obtain intrathyroidal cells for direct *in vivo* examination. Previous studies have examined thyroid tissues obtained after surgery from already treated patients with AITD. As mentioned above, the pretreatment of these patients may obscure the real picture.

The importance of our study lies in the fact that it captures, *in vivo*, the expression of apoptotic molecules on peripheral immune cells and simultaneously their soluble forms in serum from untreated patients with AITD and at different clinical stages of their disease in the case of HT. Still, the cross-sectional design of our study limits our ability to distinguish whether the alterations described above preceded or followed the development of AITD. This question would be better answered by a longitudinal study. Perhaps, examining these parameters on thyroid cells obtained by fine needle aspiration from patients with AITD would be a better approach but this has also methodological and ethical constraints.

Taking into consideration the above limitations, the inverse relation of the intact transmembrane and the alternatively spliced soluble form of Fas may have a role in the increase of sFas observed in GD. Whether increased sFas can prevent Fas/FasL interaction and protect thyroid cells from apoptosis in GD remains speculative. On the other hand, the intensity of Fas expression on peripheral lymphocytes in HT remained high and the release of sFas low. In accordance with what was previously described by Giordano *et al.* about the differential expression of Fas molecules on thyrocytes and intrathyroidal lymphocytes in the two opposite phenotypes of AITD (30), it appears that apoptotic molecules might also be differentially regulated in the periphery of patients with GD versus HT.

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