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Fas/Fas Ligand-Driven T Cell Apoptosis as a Consequence of Ineffective Thyroid Immunoprivilege in Hashimoto’s Thyroiditis

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Hashimoto’s thyroiditis (HT) is a chronic autoimmune disease resulting from Fas-mediated thyrocyte destruction. Although autocrine/paracrine Fas-Fas ligand (FasL) interaction is responsible for thyrocyte cell death during the active phases of HT, the role of infiltrating T lymphocytes (ITL) in this process is still unknown. Therefore, we investigated the expression and function of Fas and FasL in ITL. All ITL expressed high levels of Fas and CD69, an early marker of T cell activation associated with functional Fas expression in T cells in vivo. In contrast to thyrocytes that were found to produce high levels of FasL, ITL did not express significant amounts of FasL, suggesting that ITL are not directly involved in thyrocyte destruction. The analysis of ITL purified from HT thyroids showed that ITL were massively killed by Fas crosslinking and that a considerable number (24–36%) underwent spontaneous apoptosis within 36 h of culture. Accordingly, in situ TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining revealed that a significant number (10–15%) of ITL in proximity to FasL-producing thyroid follicles were apoptotic. Moreover, virtually all ITL in proximity to thyroid follicles were preapoptotic, as they expressed high levels of GD3 ganglioside, a killer glycolipid responsible for the generation of irreversible apoptotic signals that accumulate in hematopoietic cells shortly after Fas crosslinking. These data demonstrate that ITL are not directly involved in thyrocyte cell death during HT, suggesting that autocrine/paracrine Fas-FasL interaction is a major mechanism in autoimmune thyrocyte destruction.


F as (CD95/Apo-1) is a major component of the newly characterized family of “death receptors” that are able to transduce a potent apoptotic signal upon binding with its ligand (FasL)3 (1–3). Targeted mutation of the gene encoding for Fas is responsible for massive lymphocytosis and generalized lymphadenopathy due to the impairment of activation-induced cell death, a physiological process of lymphocyte deletion responsible for maintenance of peripheral tolerance and prevention of the onset of lymphoproliferative disorders (4, 5). Activated T cells express Fas and are highly susceptible to Fas-induced apoptosis (6, 7). Constitutive expression of FasL in Fas-negative or -insensitive cells, such as normal neurons, Sertoli cells, and epithelial cells from the cornea, may induce apoptotic cell death of neighboring cytotoxic T cells, contributing to the formation of immunoprivileged sites (8, 9).

Hashimoto’s thyroiditis (HT) is a common chronic autoimmune disease characterized by the loss of thyroid epithelial cells that are gradually replaced by mononuclear cell infiltration and diffuse fibrosis (10, 11). FasL is expressed in normal thyrocytes and is further up-regulated during the active phases of HT, suggesting that thyroid epithelium may potentially constitute an immunoprivileged site (12, 13). However, during the autoimmune process, the production of IL-1β by activated macrophages directly induces Fas up-regulation in thyrocytes, breaking thyroid immunoprivilege and priming thyrocytes for FasL-mediated destruction (12, 14).

Two major lytic pathways mediate T cell cytotoxicity. The exocytosis of perforin-containing granules and the expression and release of FasL in proximity to Fas-sensitive targets (15, 16). The interaction of Fas with FasL may constitute a common pathogenic mechanism mediating target destruction in organ-specific autoimmune (14). FasL-producing autoreactive T lymphocytes seem to induce Fas-mediated apoptosis of β cells and oligodendrocytes during the autoimmune process, leading to insulin-dependent diabetes and multiple sclerosis (17, 18). Although it is clear that homocellular Fas-FasL interaction participates in thyrocyte destruction in HT, the role of infiltrating T cells in this destructive process is still unknown (12, 19). Cytotoxic T cells that are potentially able to lyse FasL-producing thyrocytes should be resistant to Fas-induced apoptosis, because the interaction of Fas-sensitive T cells with thyrocytes expressing FasL would result in lymphocyte apoptosis. Moreover, because intrathyroidal T lymphocytes (ITL) do not seem to significantly differ from peripheral blood T cells (PBT) in terms of perforin expression, and thyrocyte destruction in HT seems Fas-mediated, effector cytotoxic ITL should express comparable or higher amounts of FasL than potential thyrocyte targets.
(12). Therefore, we compared FasL expression in HT thyrocytes and ITL and studied Fas sensitivity of ITL and in vivo apoptotic triggering of T cells located in proximity to potential thyrocyte targets during the active phases of HT.

**Materials and Methods**

**Specimens**
Thyroid tissues were obtained at the time of thyroidectomy. The diagnosis was based on clinical criteria and confirmed by appropriate laboratory tests (TSH, T3, T4, ATPO, ATGA, AMCA) and histological findings (follicular epithelial cells showing degenerative change in florid lymphocytic thyroiditis, lymphoid cells, including blasts, germinal center cells, and plasma cells). Tissue from six women patients with HT were examined (age: 38 ± 7.5 yr).

**Immunofluorescence and flow cytometry analysis of thyrocytes and ITL**

Thyroid specimens were digested with collagenase type IV (Sigma, St. Louis, MO) for 2 h at 37°C. The digestion was blocked by cold HBSS without Ca²⁺ and Mg²⁺ and the digest filtered through a 200-μm mesh. Isolated cells, including thyrocytes and lymphocytes, were harvested and centrifuged on Ficoll 1077 gradient to eliminate debris and red and dead cells. For ITL staining, cells were incubated for 30 min at 4°C with 5 μg/ml of purified anti-FasL mAb (NOK-1, IgG1, PharMingen, San Diego, CA) or control IgG1, washed and treated with phycoerythrin (PE)-conjugated donkey anti-mouse IgG (Chemicon, Temecula, CA). After another washing, cells were incubated for 10 min with 6% normal mouse serum and treated with saturating concentrations of FITC-conjugated SK7 mAb (anti-CD3, IgG1) or control FITC IgG1 (Becton Dickinson, San Jose, CA). Alternatively, cells were incubated for 30 min at 4°C with a combination of FITC anti-CD3, and the following PE mAbs: L78 (anti-CD69, IgG1; Becton Dickinson) or anti-Fas mAb (DX2, IgG1; PharMingen), or control IgG1. For thyrocyte staining, cells were incubated for 30 min at 4°C with human serum containing anti-TPO Abs, washed and labeled with FITC-conjugated goat anti-human Ig (Chemicon), washed again, incubated for 10 min with 10% normal human albumin serum and treated with 5 μg/ml NOK-1 or control IgG1, and then washed and labeled with PE-conjugated human and goat IgG-conjugated anti-mouse IgG (Chemicon). Cells were then washed and analyzed by two-color flow cytometry on a FACScan (Becton Dickinson). The percentage of positive cells was electronically determined for each sample by comparing negative and positive fluorescence histograms using a Cell Quest program (Becton Dickinson).

**Immunostaining procedure**

Thyroid fragments (0.5 cm) were snap frozen in isopentane, and kept at −80°C, until used. Serial cryostat thyroid sections (4 μm) and ITL cyto- spins, containing cells treated or untreated for 20 min at 37°C with 50 μM C2-caffeine (Bioulom, Plymouth Meeting, PA), were allowed to equilibrate to room temperature and fixed in pure acetone for 10 min at room temperature. Bound mAbs anti-Fas (DX2), anti-FasL (NOK-1), anti-CD3 (T3, IgG1; Dako, Santa Barbara, CA), anticytokeratin (CK1, IgG1; Dako), and anti-CD3 (R24, IgG3; kindly provided by Dr. L. J. Old, Ludwig Institute, New York, NY) or control IgG were detected by HRP-streptavidin-biotin staining technique (LSAB kit, DAKO). The primary Abs were added to the tissue preparations after incubation for 1 h with albumin human serum. The binding was revealed by aminomethylcarbazole (AEC) colorimetric substrate. Hematoxylin aqueous formula was used as a counterstain. For in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining, CD3- or FasL-labeled serial cryostat thyroid sections (6 μm) and PBL cytospins were fixed with 4% paraformaldehyde for 20 min at room temperature and washed twice in PBS. Sections and cytospins were then permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice and washed twice with PBS. The labeling of 3′-OH fragmented DNA ends (TUNEL) was conducted by an in situ apoptosis detection kit (In Situ Cell Death Detection, AP; Boehringer Mannheim, Indianapolis, IN). Detection of labeled ends was done with an anti-fluorescein Ab, Fab fragment conjugated with alkaline phosphatase. 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Dakopatts, Glostrup, Denmark) was used as colorimetric substrate. Control tissue sections and PBL cytospins were prelabeled with irrelevant isotype-matched mAbs and subjected to identical treatment for TUNEL staining without TdT.

**Apoptosis detection of ITL**

For ITL and PBT purification, Ficoll-isolated lymphocytes and thyrocytes were fractionated on a two-step (44 and 46%) Percoll gradient centrifugation. Contaminating B lymphocytes, NK cells, and monocytes were then depleted from cell pellets by negative immunomagnetic selection, by incubating the cells that were previously stained on ice with saturating amounts of 4G7 (anti-CD19), G022 (anti-CD16) and M*-P9 (anti-CD14) mAbs (Becton Dickinson) with sheep anti-mouse IgG-conjugated beads (Dy- nal, Wirral Merseyside, U.K.). This procedure routinely resulted in a >96% pure T cell population.

Cells were then cultured for up to 36 h with 200 ng/ml agonistic anti-Fas mAb (CH-11, IgM; Upstate Biotechnology, Lake Placid, NY) or control IgM. The percentage of T cells undergoing apoptosis was measured by DNA staining and flow cytometry analysis, as previously shown (20). Briefly, the cell pellet was gently resuspended in hypotonic fluorochrome solution (propidium iodide 50 μg/ml Sigma in 0.1% sodium citrate plus 0.1% Triton X-100), and kept overnight at 4°C in the dark until flow cytometry analysis. The percentage of apoptotic cells was determined by evaluating the number of hypodiploid nuclei.

**Results**

**ITL from HT glands are activated and express Fas**

T cells require previous activation to express functional Fas molecules coupled to their apoptotic machinery (6). Therefore, freshly isolated ITL were assessed for expression of Fas and CD69, an early activation Ag rapidly induced upon T cell activation, whose presence in vivo is associated with functional Fas expression in T cells (20). Immunofluorescence staining and flow cytometry analysis showed that ITL from HT glands are Fas⁺ and express high levels of CD69 Ag (Fig. 1A), indicating that these cells are activated and potentially susceptible to Fas-induced apoptosis. As expected, CD69 expression is very low in autologous PBT (Fig. 1A), although both Fas and CD69 expressions are slightly higher in PBT from HT patients than in those from normal controls (not shown).

**FasL expression in ITL is negligible compared with FasL present on HT thyrocytes**

Activated T cells are a potential source of FasL. To determine the potential capacity of ITL to exert a FasL-based cytototoxicity during the autoimmune process, we compared FasL expression of ITL and thyrocytes from HT glands. Fig. 1B shows that FasL, which is not present in PBT, was expressed at low levels by a low number of ITL ranging from 10 to 15%. Moreover, the analysis of TPO⁺ thyroid follicular cells showed that the expression of FasL on HT thyrocytes is very bright, about 3- to 5-fold higher than that found on FasL⁺ ITL (Fig. 1B), suggesting a minor role of FasL producing ITL in thyrocyte destruction during HT.

**ITL are Fas-sensitive and undergo spontaneous apoptosis**

Apoptotic cells are rapidly engulfed and degraded by neighboring phagocytes. To determine the number of ITL receiving a death signal in vivo, we measured the rate of spontaneous apoptosis in freshly purified ITL. Unlike PBT, a substantial number of ITL (24–36%) undergo spontaneous apoptosis after 24–36 h of culture (Fig. 2, A–C). Moreover, collagenase treatment and other purification procedures may lead us to underestimate the percentage of dying cells. Therefore, it is likely that a considerable number of cells have been committed to apoptosis in vivo. Importantly, triggering with agonistic anti-Fas mAb results in rapid and massive ITL apoptosis (Fig. 2A–C), indicating that ITL are extremely sensitive to Fas-induced apoptosis and that the spontaneous apoptosis observed in freshly purified ITL might be due to previous interaction in vivo with FasL producing cells.
T cells located in proximity to FasL-positive HT thyrocytes are apoptotic or preapoptotic

We then investigated whether ITL located in proximity to FasL-producing thyrocytes in HT show an apoptotic phenotype. Immunohistochemistry of serial frozen sections, which were double labeled for CD3 or FasL staining and TUNEL reaction, revealed the presence in HT glands of several apoptotic CD3 cells, whose number was higher than that of apoptotic thyrocytes, although autologous peripheral lymphocytes did not show any TUNEL reactivity (Fig. 3A). Interestingly, lymphocyte apoptosis in HT glands was most evident in areas adjacent to thyroid follicular structures containing abundant reactivity for FasL (Fig. 3A), suggesting the involvement of Fas/FasL interaction in ITL apoptosis.

Accumulation of GD3 ganglioside is an early event in Fas-induced apoptosis that generates irreversible apoptotic signals in hematopoietic cells (21, 22). To determine the rate of Fas-triggered preapoptotic ITL in HT glands, we evaluated the number of GD3 positive cells among ITL by immunohistochemistry of frozen serial sections. Resting peripheral T cells express negligible amounts of GD3, as shown by anti-GD3 staining of cytospins containing PBL from HT patients, unless their apoptotic program has been triggered (Fig. 3B). By contrast, a strong immunoreactivity for GD3 ganglioside colocalized in serial sections with the CD3+ cells, located in proximity to thyroid follicles (Fig. 3C), suggesting that a considerable number of T cells exposed to FasL produced by HT thyrocytes had been committed to Fas-induced apoptosis.

Discussion

Although the pathogenic mechanisms determining thyrocyte destruction in HT have been extensively investigated, it is still not clear how this process occurs (10, 11). The direct killing of thyrocytes by cytotoxic T cells has been classically regarded as the major pathogenic mechanism in HT. This concept was raised after observing that a large number of T cells contribute to the lymphocytic infiltration observed in HT (11). Moreover, several studies of animal models have shown that T cells are required for the development of both spontaneous and immunization-induced experimental autoimmune thyroiditis (10, 23). However, although T cells are likely to play a major role in the initiation and amplification of the autoimmune response against thyroid cells, there is not evidence of a direct involvement of cytotoxic effector T cells in autoimmune thyrocyte destruction (10).
The results of this study show that a substantial number of ITL present in HT thyroids have been committed to apoptosis, probably through the interaction with FasL-producing thyrocytes. ITL are extremely sensitive to Fas-induced apoptosis. About 10–15% of ITL in proximity to thyroid follicular structures are apoptotic and show a clear TUNEL reactivity. Moreover, a considerable number of freshly purified ITL undergo spontaneous apoptosis, probably because the majority of lymphocytes located in close proximity to thyrocytes have been committed to Fas-induced apoptosis, as they express high levels of GD3 ganglioside, a killer glycolipid induced by Fas crosslinking in Fas-sensitive cells, whose accumulation is required for execution of apoptosis in hematopoietic cells (21, 22). Although ITL may potentially die through autocrine or paracrine Fas-FasL interactions (12). Thyrocyte suicide might not be the only mechanism of thyrocyte depletion, as activated T cells are a possible source of FasL and may participate in thyrocyte apoptosis through other cytotoxic mechanisms such as those mediated by TNF-α or the combined action of granzymes and perforin. Theoretically, it is possible that before dying, preapoptotic GD3 ITL might kill neighboring thyrocytes. However, only a few ITL express FasL, possibly because ITL are extremely sensitive to Fas-induced apoptosis, and FasL ITL are likely to die following autocrine Fas-FasL interaction, whereas TNF-α produced by ITL in HT seems to up-regulate adhesion molecules and increase inflammation, rather than induce thyrocyte apoptosis (10). Moreover, ITL from autoimmune glands do not show any perforin-mediated cytotoxic activity when analyzed ex vivo unless repeatedly stimulated in vitro (10). Therefore, it is unlikely that ITL are directly involved in thyrocyte execution during HT.

Other mechanisms of thyrocyte depletion not involving apoptosis induction as a major cytotoxic function have been suggested in HT, including complement-mediated injury of Ab-coated thyrocyte targets (10, 11). However, the common finding of thyrocyte apoptosis in HT glands (12, 19, 24) suggests a prevalent role for homocellular Fas-FasL interaction among thyrocytes in the destructive phases of autoimmune hypothyroidism.

**FIGURE 3.** Analysis of in situ apoptosis and GD3 ganglioside accumulation. A, DNA breaks were detected by TUNEL and BCIP (black) staining on PBL cytospins and autologous serial thyroid sections labeled with anti-CD3 or anti-FasL mAb and revealed by AEC (red). Upper panels show labeling with AEC anti-cytokeratin (CK), and TUNEL reaction in the absence of TdT. Arrowheads indicate apoptotic cells. B, PBL cytospins of cells untreated (Control) or treated with 50 μM of ceramide (Ceramide) were labeled with anti-GD3 mAb and revealed by AEC. C, Thyroid sections from HT patients were labeled with anti-CD3, or anti-GD3, or anti-cytokeratin mAb, and revealed by AEC. A representative experiment from one HT patient of five examined is shown.
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References