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Decrease of Intrathyroidal CD161$^+$Vα24$^+$Vβ11$^+$ NKT Cells in Graves' disease

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Abstract. To clarify changes in the intrathyroidal natural killer T (NKT) cell subset, which prevents autoimmunity in patients with Graves' disease (GD), we examined intrathyroidal and peripheral lymphocytes in 11 patients with GD and peripheral lymphocytes in nine healthy volunteers using three-color flow cytometry. The proportion of CD161$^+$ T cell receptor Vα24$^+$Vβ11$^+$ cells, which represent the NKT cell subset, was lower in the thyroid of patients with GD than in the peripheral blood of the same patients and in the peripheral blood of healthy subjects. These results indicate that the proportion of intrathyroidal NKT cells is decreased in patients with GD and that this decrease may contribute to incomplete regulation of autoreactive T cells in GD.

Key words: Graves' disease, NKT cell, Immunoregulation, Autoimmunity, Thyroid

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AUTOIMMUNE thyroid disease (AITD) is an organ-specific autoimmune disease characterized by the breakdown of self-tolerance to thyroid antigens. Two typical AITDs are Hashimoto's disease (HD) and Graves' disease (GD), and with either of these diseases the lymphocytes infiltrate the thyroid of patients [1–4]. We previously reported differences between the proportions of particular lymphocyte subsets in the thyroid and peripheral blood and suggested involvement of these subsets in the pathogenesis of AITD [5–10]. We have also reported the association between the polymorphism of interferon-γ gene and the severity of HD [11].

Natural killer T (NKT) cells are T cells that express the natural killer (NK) cell-associated marker NK1.1 (CD161) [12]. This subset is restricted by CD1d and expresses both a heavily biased, semi-invariant T cell receptor (TCR) and NK cell markers [13]. This TCR consists, in humans, of an invariant TCR Vα24Jα18 chain combined with a variable TCR Vβ11 chain [14]. In humans, only 0.2% of peripheral blood T cells are NKT cells, but these cells help maintain tolerance to self-antigens and can thereby prevent autoimmunity [15]. Studies of autoimmune diseases such as type 1 diabetes, multiple sclerosis, and rheumatoid arthritis have provided evidence that NKT cells are involved in autoimmune regulation [16–19]. In the present study, we examined intrathyroidal and peripheral NKT cells with specific markers in patients with GD and in healthy subjects to clarify the changes in NKT cells associated with GD.

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Materials and Methods

Subjects

Thyroid tissue samples were obtained from 11 patients with GD (eight women and three men; mean

age ± standard deviation (SD), 36.4 ± 15.7 years) without ophthalmopathy. All showed clinical and biochemical features of thyrotoxicosis and underwent surgery while in a controlled euthyroid state in response to treatment with methimazole or propylthiouracil. Peripheral blood samples were obtained from all patients prior to surgery. Peripheral blood samples were also obtained from nine healthy control subjects (seven women and two men; mean age ± SD, 33.9 ± 11.2 years), all of whom were euthyroid and negative for the presence of thyroid autoantibodies. Informed consent was obtained from all patients and control subjects, and the study protocol was approved by the ethics committee of Osaka University.

Isolation of mononuclear cells

Mononuclear cells were isolated as described previously [9]. In brief, peripheral blood was obtained immediately before surgery, and lymphocytes were isolated by density gradient centrifugation with Lymphoprep (density 1.077, Nycomed Pharma AS, Oslo, Norway) at 400 *g* for 30 min at room temperature and then washed twice with phosphate-buffered saline (PBS). Thyroid tissue was obtained during surgery and rinsed in 4°C PBS. Connective tissue was removed, and the thyroid tissue was cut into approximately 30-mm^3 pieces and rinsed in PBS. The pieces were minced finely in a small volume of 4°C PBS to release infiltrating mononuclear cells and resuspended in 10 ml PBS. This mincing process was repeated three times. The supernatant, including intrathyroidal mononuclear cells, was filtered through a 125-μm nylon mesh. Mononuclear cells in the 30-ml minced tissue suspension were separated by density gradient centrifugation at 400 *g* for 30 min at room temperature and washed twice in PBS.

Analysis of cell surface markers

Surface markers were analyzed by three-color flow cytometry with a combination of monoclonal antibodies. These antibodies included fluorescein isothiocyanate (FITC)-anti-CD161 (Becton Dickinson, Mountain View, CA, USA) and PE-anti-Vα24 and biotin-anti-Vβ11 antibodies (Immunotech, Beckman Coulter Group, Marseille, France). Aliquots (100 μl) of mononuclear cells were incubated with 20 μl of appropriate antibody for 15 min at 4°C. For staining of Vβ11, cells were incubated again with 20 μl of PerCP-streptavidin (Becton Dickinson) for 15 min at 4°C. Cells were then lysed and fixed with lysing reagent (FACS Lysing Solution, Becton Dickinson). After incubation for 10 min at 4°C in the dark, cells were washed twice and resuspended in 500 μl PBS and analyzed with a FACSCalibur flow cytometer (Becton Dickinson). Lymphocytes were analyzed by gating a lymphocyte area in a dot-plot of linear forward light scatter versus linear side-angle light scatter.

Assay of thyroid function and autoantibody levels

The serum concentration of free T4 (FT4) was measured with a radioimmunoassay kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The normal range of serum FT4 is 1.0–1.6 ng/dl (12.9–20.6 pmol/L). The serum concentration of free T3 (FT3) was measured with a radioimmunoassay kit (Japan Kodak Diagnostic Co., Ltd., Tokyo, Japan). The normal range of serum FT3 is 2.4–4.6 pg/ml (3.8–7.2 pmol/L). The serum TSH concentration was also measured with a radioimmunoassay kit (Daiichi Radioisotope Laboratories Ltd., Tokyo, Japan). The normal range of serum TSH is 0.6–5.4 μU/ml. Anti-thyrotropin receptor antibodies (TgAb) and McAb were measured with a particle agglutination kit (Fujirebio Inc., Tokyo, Japan). A reciprocal titer of >1 : 100 was considered positive. Serum TRAb was measured with a radioreceptor assay (Cosmic Co., Tokyo, Japan); results are expressed as percent inhibition of binding of labeled TSH. The normal value is less than 10%.

Statistical analysis

Paired *t*-test was used to analyze differences in the proportions of lymphocyte subsets in thyroid tissue and peripheral blood of individual patients. Student's *t*-test was used to analyze differences in the proportions of lymphocyte subsets in peripheral blood of GD patients and healthy subjects. Probability (P) values of less than 0.05 were considered significant. The JMP6 software package (SAS Institute Japan, Tokyo, Japan) was used for all statistical analyses.

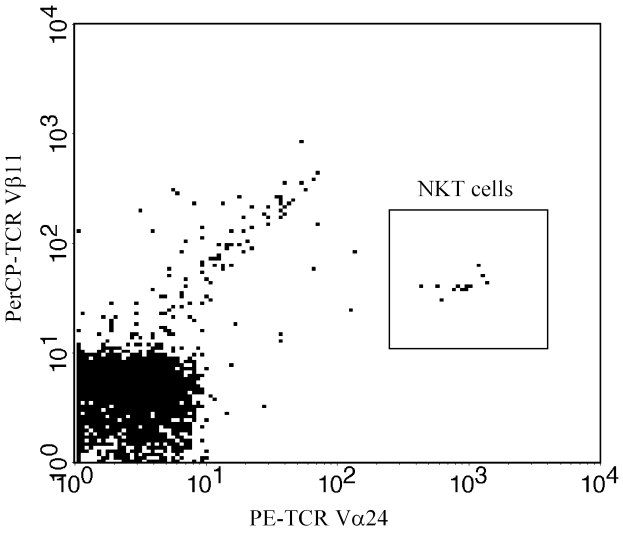

Fig. 1. Typical flow cytometric plot of intrathyroidal $V\alpha24^+V\beta11^+$ cells in the $CD161^+$ cell subset.

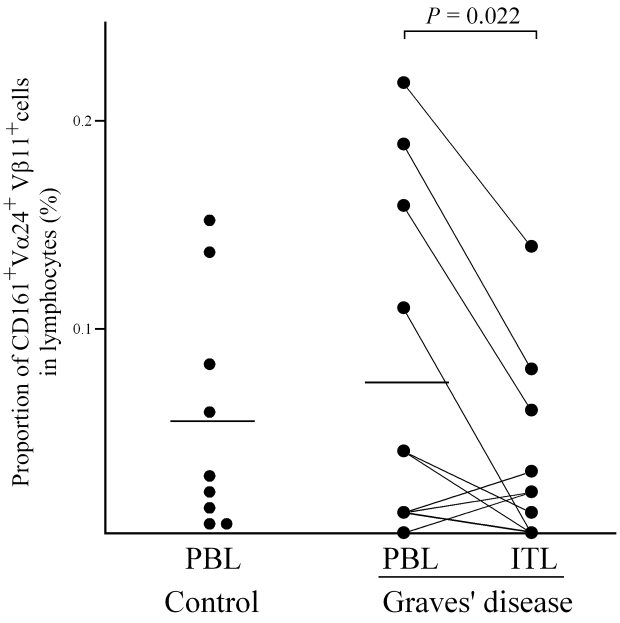

Fig. 2. Proportion of $CD161^+V\alpha24^+V\beta11^+$ cells among peripheral and intrathyroidal lymphocytes in patients with Graves' disease and among peripheral lymphocytes in healthy subjects. PBL, peripheral blood lymphocytes; ITL, intrathyroidal lymphocytes.

Results

Peripheral and intrathyroidal NKT cell subsets

The population of $V\alpha24^+V\beta11^+$ cells in the $CD161^+$ lymphocyte subset was very small but clearly detectable as shown in Fig. 1. The proportion of $CD161^+V\alpha24^+V\beta11^+$ NKT cells among lymphocytes was significantly lower in the thyroid than in the peripheral blood of GD patients (n = 11, $P = 0.022$) (Fig. 2). This proportion did not differ significantly between peripheral blood of GD patients and peripheral blood of healthy control subjects (Fig. 2). Although these are small samples, none of the values of laboratory tests examined were significantly correlated with the proportions of NKT cells in either thyroid or peripheral blood.

Discussion

To clarify the pathogenesis of GD it is important to compare the immune environment between patients and healthy subjects, but this is very difficult to achieve due to the small number of lymphocytes in the thyroid tissue from healthy subjects who are negative for thyroid-specific autoantibodies. Hence we attempted to compare intrathyroidal and peripheral lymphocyte subsets to clarify the thyroid-specific changes in the immune system. In the present study we showed that the number of $CD161^+V\alpha24^+V\beta11^+$ cells (NKT cells) is decreased in the thyroid of GD patients. It is known that NKT cells regulate autoimmune disease in a manner similar to regulatory T cells [15], which are also decreased in the thyroid of GD patients [10]. We suggest that a decrease in the number of intrathyroidal NKT cells may allow an autoimmune response in the thyroid of patients with GD, contributing to the pathogenesis of GD. Unfortunately, there are only a very small number of intrathyroidal NKT cells to examine their function.

Because activation of NKT cells leads to upregulation of CD154 (CD40 ligand) [20], our present data suggest that CD154 expression may be suppressed in GD patients. This is supported by our previous report that CD154 intensity on T cells is decreased in patients with GD [21]. It has been reported that the CD154-CD40 interaction contributes to negative regulation of T cell autoreactivity and that a defect in this interaction can lead to autoimmunity [22]. Therefore, reduced CD154 expression due to a decreased number of NKT cells may also contribute to elevated T cell autoreactivity and the pathogenesis of GD.

In humans, approximately 40% to 60% of invariant NKT cells are $CD4^+$ cells [12]. We previously reported that intrathyroidal $CD4^+$ T cells strongly express Fas

and that intrathyroidal $CD4^+CD8^+$ macrophage/dendritic cells express Fas ligand in patients with AITD [9]. Therefore, the number of NKT cells in the thyroid might be reduced by Fas-mediated apoptosis caused by intrathyroidal $FasL^+CD4^+CD8^+$ macrophage/dendritic cells.

In conclusion, the proportion of intrathyroidal NKT cells is decreased in patients with GD. This decrease may cause inappropriate activation of autoreactive T cells, contributing to the pathogenesis of GD. Although it is important to clarify the function of intrathyroidal NKT cells, in the present study it was impossible to obtain sufficient amounts of NKT cells to fully examine their functions.

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