Abstract

We previously reported that serum interleukin-12 (IL-12) levels were significantly increased in patients with hyperthyroid Graves’ disease and in normal subjects after administration of thyroid hormone. In the present study, we investigated which cells produce IL-12 and the interactions between IL-12 and thyroid hormones, using a hyperthyroid mouse model. Thyroid hormones induced IL-12 production, and IL-12 was mainly produced by dendritic cells outside the thyroid glands in a hyperthyroid state.

European Journal of Endocrinology 141 625–629

Introduction

Interleukin-12 (IL-12) plays a crucial role in the initiation and regulation of immune responses in various diseases, such as infectious diseases and autoimmune diseases (1, 2). We have previously reported that serum IL-12 levels were significantly increased in hyperthyroid Graves’ disease (3), and Hidaka et al. (4) have reported that serum IL-12 levels were significantly increased in hyperthyroid Graves’ disease and hyperthyroid Hashimoto’s thyroiditis. Although IL-12 was originally isolated from B-cells, it is secreted by a variety of antigen-presenting cells, including B-cells and B-lymphoblastoid cells, monocytes and macrophages, dendritic cells (DCs), Langerhans cells, neutrophils, and keratinocytes (1, 5–10). Recently, Gorak et al. (11) have demonstrated that DCs are the major source of IL-12 following protozoal infection.

In the present study, we investigated which cells produce IL-12 and the interactions between IL-12 and thyroid hormones, using a hyperthyroid mouse model.

Materials and methods

Treatment of animals

Male C57BL/6 mice obtained from Clea Japan Inc. (Tokyo, Japan) were housed, five per cage, under controlled temperature and illumination. Food and water were freely available. All mice received humane care in compliance with our Institution’s guidelines. Total thyroidectomy was performed on 10-week-old mice to induce a hypothyroid state as previously described (12). A hyperthyroid state was induced in some mice by administering 2 μg/ml L-thyroxine (T₄, Wako Pure Chemical Industries Ltd, Tokyo, Japan) in their drinking water for 4 weeks after total thyroidectomy (13). Splenectomy was performed on some mice and a proportion of these received 2 μg/ml T₄ in their drinking water for 4 weeks after splenectomy. We estimate the total T₄ administered per mouse as 10 μg/day. The following six groups were formed: a thyroid control (sham operation of thyroid alone) group; a thyroidectomy group; a thyroidectomy+T₄ (TT) group; a splenectomy group; and a splenectomy + T₄ (ST) group.

At various intervals, approximately 100 μl blood were obtained from the tail vein. Sera were separated by centrifugation and stored at −20°C until analyzed in the same assay for each point.
cells by rosetting with antibody-coated sheep erythrocytes. Pure populations of DCs were obtained by treating Fc receptor-negative cells with a mixture of anti-Thy-1.2 (clone 5a-8, Cedarlane Laboratories Ltd, Hornby, Ontario, Canada), anti-Lyt-1.2 (clone G16, Cedarlane), anti-CD45R (clone RA-3–3A1/6.1, TIB146; ATCC, Rockville, MD, USA) plus low-toxic complement (Cedarlane). The purity of DCs was more than 90% as confirmed by flow cytometry using monoclonal antibody (hamster anti-mouse CD11c, N418, Pharmingen, San Diego, CA, USA).

The number of cells/ml of culture and the days of culture were determined by preliminary experiments (data not shown). Whole spleen cells ($5 \times 10^6$), SACs ($1 \times 10^5$), and spleen DCs ($5 \times 10^4$) from mice in the thyroid control group and the TT group were cultured in serum-free CG-medium (Serotec Ltd, Oxford, Oxon, UK) with 20 µg/ml gentamycin sulfate in 24-well culture plates. At the end of culturing, the supernatants were collected, centrifuged, and filtered. One-half of the aliquots of all samples was stored at $-20^\circ$C until assay at the end of the experiment. Each experiment was carried out ten separate times.

**Assay of IL-12 and IL-10**

IL-12 levels in sera and in cell culture supernatants were measured by an ELISA using an Intertest-12X Total Mouse IL-12 ELISA kit (Genzyme Co., Cambridge, MA, USA). In this assay, microtiter plates pre-coated with anti-mouse IL-12 p40 antibody were used to extract mouse IL-12 from samples and standards. After washing the wells to remove the unbound fraction, an anti-mouse IL-12 p40 biotinylated antibody, which binds to mouse IL-12, was added. The wells were washed again to remove the unbound fraction. A horseradish peroxidase-conjugated streptavidin reagent was added, which binds to the biotin in the immune complex on the plate. The wells were washed and a substrate was added to initiate a peroxidase-catalyzed color change that was stopped by 1 mol/l H$_2$SO$_4$. The absorbance of each well was measured at 450 nm.

IL-10 levels in sera and in cell culture supernatants were measured by an ELISA using a BioSource Cyto-screen Mouse IL-10 Ultrasensitive kit (BioSource International Inc., Camarillo, CA, USA). In this assay, an antibody specific for mouse IL-10 was first immobilized onto the microtiter plates. Plates and standards were added and incubated, followed, after washing the wells, by a biotinylated antibody specific for mouse IL-10, then a horseradish peroxidase-conjugated streptavidin reagent was added and plates incubated. Finally wells were washed and a substrate solution was added. The intensity of this colored product was measured at 450 nm.

**Statistical analysis**

Data are expressed as mean ± standard deviation (S.D.). Statistical analysis of the data was performed using a paired t-test or Wilcoxon test, and an unpaired t-test or Mann–Whitney test. $P < 0.05$ was considered to indicate significance.

**Results**

**Serum IL-12 levels**

The serum IL-12 levels in the thyroid control, thyropectomy, SC and splenectomy groups did not change during the experimental period. The levels in the TT group were significantly increased to $1808 \pm 212$ pg/ml after 2 weeks of T$_4$ administration following thyropectomy, compared with the sham-operated group ($1338 \pm 128$ pg/ml), and continued to be high for an additional 2 weeks. The levels in the ST group were also significantly increased to $1901 \pm 293$ pg/ml after 4 weeks of T$_4$ administration following splenectomy, compared with the SC group ($1045 \pm 206$ pg/ml) (Figs 1 and 2).

**Serum IL-10 levels**

The serum IL-10 levels in all groups did not differ during the experimental period (data not shown).
**Spleen weights**

In the TT group, the spleen weight and the spleen/body weight ratio were increased, compared with the thyroid control group at the time of sacrifice. On the other hand, in the thyroidectomy group, the spleen weight and the spleen/body weight ratio were similar to those in the thyroid control group (Table 1).

**IL-12 production by spleen DCs**

We preliminarily studied the production of IL-12 by various spleen cells from normal mice and from hyperthyroid mice. The cells were cultured in RPMI-1640 medium with 10% fetal calf serum (FCS) or with 10% resin-treated FCS that contained no thyroid hormones. We also studied the production by these cells cultured in serum-free CG-medium with or without *Staphylococcus aureus* Cowan I strain (Pansorbin, Calbiochem, La Jolla, CA, USA), and with various concentrations of T4 (none, vehicle, $10^{-9}$ mol/l, $10^{-6}$ mol/l). As the IL-12 levels under various culture conditions were not significantly different, we decided to culture the cells in CG-medium without Pansorbin and without T4. The IL-12 levels in culture supernatants of whole spleen cells from mice in the thyroid control and TT groups were gradually increased during the culture period, with no significant difference between the two groups (Fig. 3). On the other hand, IL-12 production by SACs in the TT group after 2 and 4 days was significantly higher (1362 ± 100 and 2328 ± 221 pg/ml respectively) than that in the thyroid control group (813 ± 116 and 1172 ± 57 pg/ml respectively) (Fig. 4). Moreover, the IL-12 production by DCs in the TT group after 2 and 4 days was also significantly higher (1466 ± 240 and 1837 ± 444 pg/ml respectively) than that in the thyroid control group (862 ± 35 and 1016 ± 63 pg/ml respectively) (Fig. 5).

**IL-10 production by spleen cells**

The IL-10 levels in culture supernatants of spleen whole cells, SACs, and spleen DCs from hyperthyroid mice were similar to those from control mice (data not shown).

**Discussion**

IL-12 has three secreted forms: heterodimeric p70 (p40+p35), homodimeric p40, and monomeric p40. Among these three forms, p40 is the most abundantly produced in hyperthyroid mice.
to occur outside the thyroid gland.

mice after thyroidectomy, IL-12 production was found in the present study. IL-12 production was increased in hyperthyroid autoimmune thyroiditis prone rats. Since in the present study IL-12 production was increased in hyperthyroid mice. On the other hand, the spleen weight and spleen/body weight ratio were increased in hyperthyroid mice. Therefore, we investigated IL-12 production in hyperthyroid mice. IL-12 production was found to occur outside the thyroid gland.

We next evaluated the distribution of IL-12-producing cells. IL-12 was also produced in the mice after splenectomy, but it is produced by various antigen-presenting cells inside the spleen. Therefore, we investigated IL-12 production in various spleen cell populations. Thyroid hormone stimulates gene expression of several growth factors, increases erythropoiesis, red blood cell mass, and plasma volume mediated by elevated erythropoietin responding to increased oxygen requirements, and increases in lymphocytes. In the preliminary study IL-12 production was increased in hyperthyroid mice after thyroidectomy. IL-12 production was found to occur outside the thyroid gland.

We investigated IL-10 levels in sera and in culture supernatants of whole spleen cells, SACs, and spleen DCs from the mice. The IL-10 levels in hyperthyroid mice did not differ from those in control mice. As indicated in the present and previous studies, thyroid hormones are not thought to affect IL-10 production. In other words, IL-12 synthesis might not be suppressed by IL-10 in a hyperthyroid state and thyroid hormones alone might be a self-perpetuating factor in a hyperthyroid state via increased IL-12.

In conclusion, thyroid hormones might induce IL-12 production by DCs and IL-12 might not be dependent on immunological conditions in a hyperthyroid state. However, as DCs are important antigen-presenting cells in the initiation of immune responses, further investigations of various functions of DCs in thyroid glands and in systemic organs in autoimmune thyroid disease appear to be necessary.

Acknowledgements
The authors are grateful to Dr S M F Akbar (School of Medicine, Ehime University) for his critical advice.

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Received 18 May 1999
Accepted 18 August 1999