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Thyroid-stimulating hormone induces interleukin-18 gene expression in FRTL-5 cells: immunohistochemical detection of interleukin-18 in autoimmune thyroid disease.

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Thyroid-Stimulating Hormone Induces Interleukin-18 Gene Expression in FRTL-5 Cells: Immunohistochemical Detection of Interleukin-18 in Autoimmune Thyroid Disease

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Interleukin (IL)-18 is a cloned cytokine that was identified originally as a factor having potent interferon (IFN)-γ–inducing activity on Kupffer cells. First, we analyzed IL-18 gene expression by reverse transcription-polymerase chain reaction (RT-PCR) in rat thyroid FRTL-5 cells and human thyroid tissue samples. The expression of IL-18 mRNA in FRTL-5 cells was enhanced by thryoid-stimulating hormone (TSH) in a dose-dependent manner. 8-Bromo-cyclic adenosine monophosphate (cAMP) also increased in IL-18 mRNA levels. Furthermore, TGCT clones that exhibited an increase in intracellular cAMP accumulation showed an increased IL-18 mRNA signal when compared to controls. Taken together, these data suggested that the effect of TSH on IL-18 gene expression was mediated by activating protein kinase A. Treatment of FRTL-5 cells with the antithyroid drug, methimazole (MMI), suppressed this stimulatory action of TSH on IL-18 gene expression. Next, we examined IL-18 expression in human thyroid tissue derived from patients with autoimmune thyroid diseases (ATD). RT-PCR and immunohistology demonstrated that human thyroid follicular cells expressed IL-18. Especially in thyroid tissue from a patient with Hashimoto's thyroiditis, expression was more diffuse and extensive, generally observed in close relation to a lymphocytic infiltrate. Also, IL-18 protein was distributed in the same follicles that express Fas-L and HLA-DR. This study is the first to demonstrate the detection of IL-18 in the thyroid gland. The frequent expression of IL-18 in thyrocytes suggests that IL-18 itself might be a secreted immunomodulator in ATD.

Introduction

CYTOKINES, ESPECIALLY INTERFERON-γ (IFN-γ), are likely to play an important role in autoimmune thyroid disease (ATD; 1). These molecules, produced by both infiltrating cells and thyroid follicular cells, are essential for T and B cell growth and differentiation and they affect thyrocytes directly, inducing expression of major histocompatibility complex (MHC) class II molecules and adhesion molecules (1). Interleukin (IL)-18 was originally cloned by the action of IFN-γ–inducing factor from T_H1 cells in the presence of IL-12 (2). Subsequently, IL-18 has been demonstrated to have a variety of biologic functions (3,4). IL-18 acts to enhance productions of T_H1-type cytokines (IFN-γ, IL-2, granulocyte-macrophage colony-stimulating factor [GM-CSF], tumor necrosis factor [TNF]-α, IL-1β, and CC and CXC chemokines) on nonpolarized T cells and B cells (5,6). IL-18 also stimulates IFN-γ synthesis in natural killer (NK) cells, upregulates perfolin-mediated NK cell activity (7), and enhances Fas-Fas ligand-mediated cytotoxicity by inducing Fas ligand expression (8). In synergy with IL-12, IL-18 induces IFN-γ production in T cells and enhances T_H1 development. Whereas IL-18, without help from IL-12, induces the production of T_H2 cytokine, IL-4 and IL-13 from T cells and NK cells *in vitro* (9), and increases serum immunoglobulin E (IgE), T_H2 cytokines in a mouse model of allergic asthma (10). Therefore, IL-18 should be seen as a unique cytokine that enhances both T_H1- and T_H2-mediated immune responses.

IL-18 was first isolated from Kupffer cells of mice injected with *Propionibacterium acnes* and challenged with bacterial lipopolysaccharide (LPS) to induce toxic shock (2). Subsequently, it has been shown that IL-18 is produced not only by various types of immunocompetent cells but also by nonimmune cells including epidermal keratinocytes (11), osteoblastic stromal cells (12), and intestinal cells (13,14). Moreover, IL-18 secretion is also detectable in the central nervous (15,16) and endocrine systems (17,18).

Because of its ability to induce IFN-γ production and T_H1

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cell activation, the role of IL-18 in autoimmune pathologies is being investigated. A pathogenic role for IL-18 has been suggested by observations in the autoimmune T_H1-dependent insulitis in nonobese diabetic (NOD) mice (18,19), in the development of experimental autoimmune encephalomyelitis (EAE) in the mouse (20) and in clinical studies in patients with Crohn's disease (12,13) and rheumatoid arthritis (21,22). Recently, increased levels of serum IL-18 has been reported in Graves' disease (23).

In view of the potential importance of IL-18 in immune responses in general and autoimmunity in particular, we analyzed the expression of IL-18 in rat thyroid FRTL-5 cells and human thyroid tissues by applying reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry.

Materials and Methods

Cell culture

FRTL-5 cells, a continuous rat thyroid follicular cell line, were obtained from Interthyr Research Foundation (Baltimore, MD). The TG7 rat cell lines used in this study were created by permanently transfecting wild-type FRTL-5 cells with thyroglobulin promoter-cholera toxin A1 subunits (24). TG4 is an FRTL-5 clone that is transfected with the neomycin resistance (pMAM neo) gene only and served as a control (24). The cells were grown in 100-mm diameter dishes in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and a six-hormone mixture containing insulin (1 μg/mL), hydrocortisone (10^{-6} M), transferrin (5 μg/mL), glycyl-L-histidyl-L-lysine (10 ng/mL), somatostatin (10 ng/mL), and TSH (1 mU/mL; 6H medium), as described previously (24). Passage was every 7 days; fresh medium was added every 2 or 3 days, and then the cells were maintained in 3H medium (no TSH, no hydrocortisone, no insulin) containing 0.2% calf serum for 7 days before use in individual experiments. It has been reported that the cells can be maintained in culture medium without TSH for at least 10 days and remain viable.

Materials

Bovine TSH, other hormones, and chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. Anti-human IL-18 monoclonal antibody (clone: 25-2G) was purchased from MBL (Nagoya, Japan). Monoclonal anti-human HLA-DR α-chain antibody (clone: TAL.1B5) was purchased from DAKO (DAKO, Carpinteria, CA) and anti-FasL antibody (Q20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

RNA and cDNA preparation

Total RNA was extracted from FRTL-5 cells and thyroid whole tissue by homogenization using TRIzol® (GIBCO, Painsly, UK), according to the manufacturer's protocol. The samples obtained were quantified by absorbance at 260 nm. cDNA was synthesized using an A-murine leukemia virus transcriptase (AMV-RT) in a 20-μL reaction containing 1.6 μg oligo-p$(dT)_{15}$ primer, 50 units RNase inhibitor, 1 mM each of dNTPs, 2 μL 10 × reaction buffer, 5 mM $MgCl_2$, 20 units AMV-RT (all from Boehringer Mannheim, Mannheim, Germany) and 2 μg of total RNA. Reactions were incubated at 25°C for 10 minutes, 42°C for 60 minutes, 99°C for 55 minutes, and finally cooled to 4°C.

RT-PCR

IL-18 cDNA amplification were performed using Expand™ High Fidelity PCR System (Boehringer Mannheim) in 25 μL of final volume containing one fifth of RT products, 10 pmol of sense and antisense primers, and 1.75 units of *Taq* DNA polymerase. The primers used for the amplifications were a rat-specific 3′ primer (5′-AGTGAACATTA CCGCTTTATCCC-3′) and a 5′ primer (5′-ACTGTACAACCGC-AGTAATACGGG-3′) as previous reported (17). Human specific primers were 5′-GCTTGAATCTAAATTA TCAGTC and 3′-GAAGATTCAAATTGCATCTTAT (25). The amplification was performed in 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute; the last extension step at 72°C was prolonged for 5 minute. β-Actin cDNA amplifications were performed using one twenty-fifth of RT products in 20 cycles. The sequences of the primer pairs for β-actin were as follows; a 5′ primer (5′-ACCCACACTGTGCCCATGTA-3′) and a 3′ primer (5′-CGGAACCGCTCATTGC C-3′). To measure PCR products semiquantitatively, 5 μL of cDNA were serially diluted twofold and amplified by using 10–20 cycles for β-actin and 20–30 cycles for IL-18 under the same conditions described above. The linear range of amplification was obtained for representative of at least three separate experiments. Loading dye (1.5 μL) and 10 μL TE buffer (10 mM Tris-HCl, pH 7.4, 1.0 mM ethylenediaminetetraacetic acid [EDTA]) were added to each 5-μL PCR product and loaded onto 1% agarose gels (GIBCO, Grand Island, NY), 1-kb ladder DNA was run in parallel as a molecular size marker. The expected sizes of PCR-amplified products, based on the known mRNA sequences and primer sequences, were rat IL-18, 437 bp; human IL-18,342 bp; β-actin, 289 bp. Gel were stained with ethidium bromide to reveal DNA bands under UV illumination, and images were acquired using Adobe® Photoshop program (Adobe Systems Incorporated, San Jose, CA), and processed using NIH Image for Densitometric Analysis (26). Normalization was performed using the reference gene β-actin as internal control. The amplified PCR products were excised from the gel and subcloned into TA cloning vector (Invitrogen Co., Carlsbad, CA). Inserts were sequenced by automatic DNA sequencer (Perkin-Elmer Cetus, Norwalk, CT).

In situ *reverse transcription*

The human thyroid tissue samples were immersed and fixed overnight in 10% neutral buffered formalin. Tissues were dehydrated in alcohol, embedded in paraffin, and sectioned at less than 5-mm thicknes. Histologic examination of each tissue preparation was performed with hematoxylin and eosin. For the molecular analysis, three tissue sections were mounted side by side on a microscopic slide coated with 3-aminopropyl-triethoxysialine. Prior to experimentation the slides were deparaffinized with xylene and dehydrated with alcohol. The deparaffinized sections were treated with protein kinase K (DAKO) for 10 minutes at room temperature. The digestion was stopped with RNase-free water, followed by dehydration. In two of three sections on each microscope slide, genomic DNA was digested at 37°C overnight, using 1 U/μL RNase-free DNase (Promega, Madi-

son, WI) suspended in a diethyl pyrocarbonate (DEPC)-treated solution composed of 5 mM $MgSO_4$ and 105 mM sodium acetate. The third section did not receive DNase treatment and was used as a positive control. The DNase was removed with a 1-mL rinse in DEPC-water followed by a 1-mL rinse in 100% ethanol.

cDNA was generated using an AMV-RT transcriptase in 20-μL reaction containing 1.6 μg oligo-p$(dT)_{15}$ primer, 50 units RNase inhibitor, 1 mM each of dNTPs, 2 μL 10 $\times$ reaction buffer, 5 mM $MgCl_2$, 20 units AMT-RT. Reactions were incubated at 25°C for 10 minutes, 42°C for 60 minutes, 99°C for 5 minutes, and finally cooled to 4°C. One of the DNase-treated sections was chosen for the RT reaction while the other served as a negative control.

In situ *PCR*

To initiate PCR, 10-μL aliquots of PCR mixture composed of 1.5 mM $MgCl_2$, 200 μM dNTP, 0.05 U/μL *Taq* DNA polymerase, and 1 μM sense and antisense primer for rat or human IL-18 were added to each of three sections and coverslipped. To detect the amplified PCR products *in situ*, we added 10 μM digoxigenin-labeled 11-dUTP (dig-dUTP; Boehringer Mannheim), which was directly incorporated into the PCR products, to the PCR mixture. The amplification was performed in 20 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, the last extension step at 72°C was prolonged for 5 minutes. After PCR, the slides were immersed in chloroform, and dehydrated in 100% ethanol. The amplified PCR products were subsequently detected colorimetrically by incubation at 37°C for 10 minutes with antidigoxigenin-alkaline peroxidase antibody. Then, slides were washed in Tris-buffered saline (TBS) twice for 1 minute and in distilled water for 1 minute. Sections were incubated with Fast Red solution (DAKO) for 5 minutes, followed by counterstaining with Mayer's hematoxylin for 5 minutes. The counterstain produces a dark blue for the nuclei, whereas the signal is pink.

Immunohistochemistry

In this study, we examined IL-18 expression in 15 cases of Graves' disease and 14 cases of Hashimoto's thyroiditis. The normal thyroid tissues were obtained from the sections, which were resected for the surgery of thyroid cancer. Formalin-fixed sections were deparaffinized and incubated with 3% hydrogen peroxidase in methanol for 30 minutes to block endogenous peroxidase activity. Then, the specimens were incubated with anti-human IL-18 monoclonal antibody (clone: 25-2G; MBL), monoclonal anti-human HLA-DR, α-chain antibody (clone: TAL.1B5; DAKO), and anti-FasL antibody (Q20; Santa Cruz Biotechnology) overnight, rinsed in phosphate-buffered saline (PBS) and subsequently incubated with EnVision$^+$ (DAKO) for 30 minutes. The reactions were visualized with 3, 3'-diaminobenzidine tetrahydrochloride for 2–3 minutes, which resulted in a brown color.

Results

Effect of TSH and MMI on IL-18 mRNA expression in rat thyroid FRTL-5 cells

TSH is a prime regulator for the function and proliferation of thyroid follicular cells. Therefore, we first evaluated the effect of TSH on IL-18 gene expression in FRTL-5 cells (Fig. 1A). TSH stimulated IL-18 mRNA expression at 10 μU/mL–1 mU/mL in a dose-dependent manner. The antithyroid drug, methimazole (MMI), suppressed the TSH-induced stimulation of IL-18 mRNA levels (Fig. 1A). This effect of MMI was concentration-dependent (Fig. 1B). A detectable inhibition of IL-18 mRNA levels was evident at 0.1 mmol/L MMI. MMI alone had no effect on the IL-18 mRNA expression.

Effect of 8-bromo-cAMP on IL-18 mRNA expression

The TSH effects on the proliferation and differentiation of thyroid cells are mainly mediated by cAMP (27,28). To determine if the stimulatory effect of TSH on IL-18 mRNA was mimicked by cAMP, precultured cells were exposed to 0.5 mM of 8-bromo-cAMP (8 Br-cAMP), a cAMP analogue that directly increased the intracellular cAMP in the thyroid cells. 8-Br-cAMP increased IL-18 mRNA (Fig. 2A). MMI suppressed 8-Br-cAMP–induced IL-18 mRNA expression (Fig. 2A).

We also studied the effect of long-term cAMP elevation on IL-18 mRNA expression by using the transfected FRTL-5 cell lines (24) (Fig. 2B). The phenotype produced in the TG7 was characterized by markedly (10-fold) increased basal levels of intracellular cAMP, increased adenylyl cyclase activity, and increased cell proliferation (24). Expectedly, TG7 showed an increased IL-18 mRNA signal compared with controls (TG4). MMI also could reduce the levels of IL-18 mRNA of TG7.

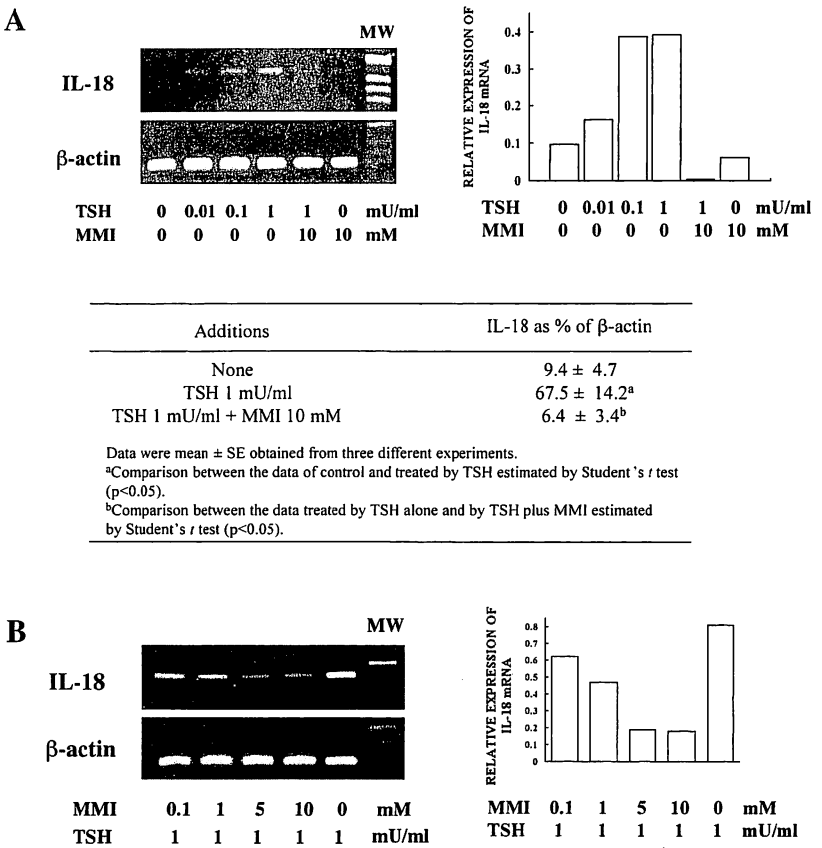
Effects of protein kinase C activator and a calcium ionophore on IL-18 mRNA expression in FRTL-5 cells

In addition to activating adenylyl cyclase, TSH can liberate diacylclycerol from membrane lipids to activate protein kinase C, and can liberate inositol phosphates to increase intracellular Ca^{++} concentrations at higher doses than required to raise cAMP levels (27). We examined the effect of tissue plasminogen activator (TPA), a potent protein kinase C activator, on IL-18 gene expression. TPA had no effect on IL-18 mRNA expression by the treatment of 4–24 hours (Fig. 2C). The calcium ionophore A23187, which increases intracellular levels of ionized calcium, has been used to explore the actions of the latter pathway. A23187 stimulated IL-18 mRNA expression at low concentrations (0.1–1 μM), whereas cells treated with 10 μM of A23187 did not show IL-18 mRNA (Fig. 2C).

It has been demonstrated that serum contained in the cultured medium include many growth factors and hormones and these substances influence cell function and growth. Thus, we examined the effect of bovine calf serum on IL-18 gene expression. Cells treated with low concentrations (1%–2%) of calf serum showed IL-18 mRNA expression. However, high concentrations of calf serum did not stimulate the expression of IL-18 mRNA (Fig. 2D).

Detection of IL-18 gene expression in human thyroid tissue samples using RT-PCR and in situ RT-PCR

Second, we examined the expression of human IL-18 mRNA in human thyroid diseases using the RT-PCR method (Fig. 3). Human thyroid tissue samples, normal thyroid tissue (N), Graves' disease (G), Hashimoto's thyroiditis (H), and tumor (papillary thyroid carcinoma) (T) constitutively



Additions	IL-18 as % of β-actin
None	9.4 ± 4.7
TSH 1 mU/ml	67.5 ± 14.2[a]
TSH 1 mU/ml + MMI 10 mM	6.4 ± 3.4[b]

Data were mean ± SE obtained from three different experiments.
[a]Comparison between the data of control and treated by TSH estimated by Student's *t* test ($p<0.05$).
[b]Comparison between the data treated by TSH alone and by TSH plus MMI estimated by Student's *t* test ($p<0.05$).

FIG. 1. Effect of thyrotropin (TSH) and methimazole (MMI) on interleukin-18 (IL-18) mRNA expression in rat thyroid FRTL-5 cells. Cells were maintained for 7 days in 3H medium plus 0.2% calf serum as described in Materials and Methods before being challenged with TSH and MMI for 24 hours. Total RNA was extracted, and reverse transcription-polymerase chain reaction (RT-PCR) was performed. Bands analyzed by densitometry are shown right. Results are expressed as the IL-18/β-actin ratio of mRNA expression. Similar results were observed in three independent experiments. TSH stimulated IL-18 mRNA expression at 10 μU/mL–1 mU/mL in a dose-dependent manner; 10 mM of MMI abolished this TSH action (**A**). The table shows the significant differences between groups. The MMI effects were concentration-dependent. A remarkable decrease in TSH-induced IL-18 mRNA was measured at 0.1 mM MMI. MMI alone did not affect the IL-18 mRNA expression (**B**).

expressed IL-18 mRNA (Fig. 3A). There was no specific difference of IL-18 gene expression between these samples. This methodologic approach did not allow us to discriminate which cells actually produce IL-18. Therefore, we tried to detect the precise localization of IL-18 mRNA in thyroid glands, using the methods of *in situ* RT-PCR (Fig. 3B). IL-18 mRNA was detected exclusively in the thyroid follicular cells, consistent with the data obtained by conventional RT-PCR. Moreover, the thyrocytes accompanied with ATD demonstrated higher IL-18 mRNA compared to the normal thyroid gland. Intensive signal of IL-18 was detected in thyroid tissue from a patient with Hashimoto's thyroiditis.

Immunohistochemical staining for human IL-18 in paraffin-embedded sections from ATDs

Applying immunohistochemistry, IL-18 was detected in a small scattered groups of follicular cells (Fig. 4). In thyroid tissue from a patient with Hashimoto's thyroiditis, expression was more diffuse and extensive, generally observed in close relation to a lymphocytic infiltrate. Of note, the increased staining was detected in the cytoplasm in hyperplastic and metaplastic cells, which formed small follicles. The expression of IL-18 was least frequently in Graves' disease. The frequency of the findings in Graves' disease correlated with the degree of focal thyroiditis in the glands. Moreover, endothelial cells also showed cytoplasmic IL-18 expression (data not shown).

IL-18 affects the immune system by inducing IFN-γ secretion by T, NK, or B cells and augmenting Fas ligand-mediated NK cell cytotoxic activity (8,29,30). Then, we evaluated the localization of HLA-DR, which could be induced by IFN-γ, and FasL in thyroid tissues using respective monoclonal antibodies (Fig. 4). Graves' thyroid tissue stained weakly for HLA-DR and FasL, localized in the lesions adjacent to lymphocytic infiltrates (Fig. 4). In contrast, thyrocytes from Hashimoto's thyroiditis sections stained strongly positive for HLA-DR and FasL, exhibiting a membranous and a cytoplasmic pattern of staining, respectively (Fig. 4). The intensity and distribution of positive staining of HLA-DR and FasL were even higher in follicles from areas adjacent to lymphocytic infiltrates. IL-18 protein was

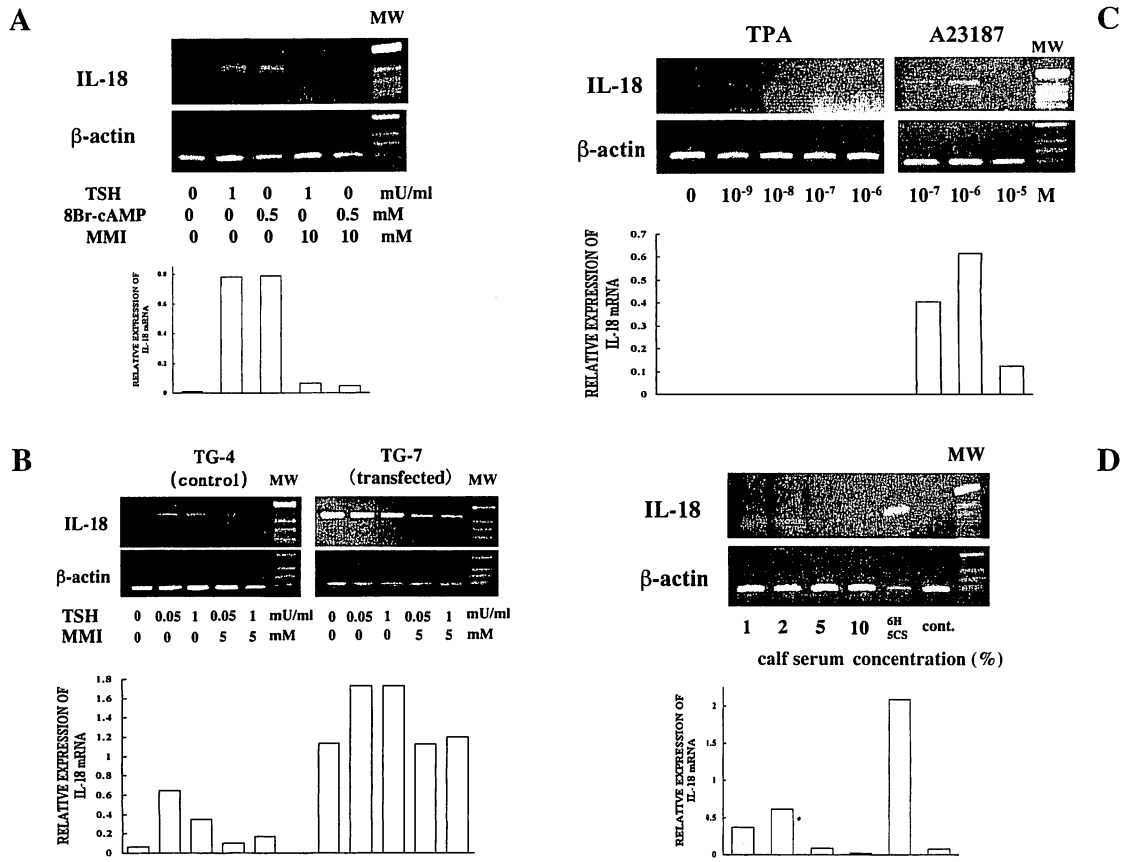


FIG. 2. Regulation of interleukin-18 (IL-18) mRNA expression in FRTL-5 cells by protein kinase A pathway not by protein kinase C pathway. Cells were maintained for 7 days in 3 H medium plus 0.2% calf serum before the addition of methimazole (MMI) in the presence of absence of reagents. After 24 hours, total RNA was extracted and reverse transcription-polymerase chain reaction (RT-PCR) was performed. Bands analyzed by densitometry are shown in the lower panel. Results are expressed as the IL-18/β-actin ratio of mRNA expression. Similar results were observed in three independent experiments. 8-Bromo-cAMP (8-Br-cAMP) mimicked the stimulatory effect of thyrotropin (TSH) on IL-18 mRNA. MMI also suppressed 8-Br-cAMP–induced IL-18 mRNA expression (**A**). The TGCT-transfected clones (TG-7), which exhibited an increase in intracellular cAMP accumulation over nontransfected FRTL-5 cells (TG-4) as reported previously (24), showed an increased IL-18 mRNA signal when compared to controls. MMI could reduce the levels of IL-18 mRNA of TG-7 (**B**). The expression of IL-18 mRNA of cells treated with TPA was not detected. A23187 stimulated IL-18 mRNA expression at low concentrations (0–0.1 μM), however, cells treated with 10 μM of A23187 did not demonstrate IL-18 mRNA (**C**). Cells treated with low concentrations (1%–2%) of calf serum demonstrated IL-18 mRNA expression. However, high concentrations of calf serum did not stimulate the expression of IL-18 mRNA (**D**).

distributed in the same lesions that express HLA-DR and FasL (Fig. 4). There was a close correspondence of the increase in IL-18 protein and increased the intense expression of HLA-DR and FasL.

Discussion

In this study, we first revealed that thyroid follicular cells could produce IL-18, which is thought to play an important roles in induction of IFN-γ and FasL, and suggested that IL-18 production in thyrocytes was under the control of TSH.

First, using rat FRTL-5 cell line, we examined the effects of various hormones and reagents including the main thyroid regulator, TSH, on the expression of IL-18 mRNA. We found that TSH stimulated the expression of IL-18 in FRTL-5 cells. TSH has been shown to augment antigen-specific spleen plaque-forming cell responses in mice (31–33), and enhance the IFN-γ–induced expression of HLA-DR on cultured human thyrocytes (34–36). Moreover, TSH has been shown to enhance cytokine responses of hematopoietic cells (37) to increase the cytotoxic activity of NK cells (38), and to serve as a costimulatory factor for mitogen or IL-2–induced T-cell proliferation (38). The present data suggest the possibility that those regulations of immune responses by TSH might be in part mediated by IL-18 production.

The effects of TSH on the proliferation and differentiation of thyroid cells are mainly mediated by cAMP (27,28). To determine whether the stimulatory effect of TSH on IL-18 mRNA was mimicked by cAMP, precultured cells were exposed to 0.5 mM of 8-Br-cMAP.8-Br-cAMP also increased IL-18 mRNA. Furthermore, TG7 cells that have increased basal levels of intracellular cAMP showed an increased IL-18

mRNA signal compared with controls (TG4). These findings suggest that the effect of TSH on IL-18 gene expression was mediated by activating protein kinase A.

In addition to activating adenylyl cyclase, TSH can liberate diacylglycerol (DAG) from membrane lipids to activate protein kinase C, and can liberate inositol phosphate to increase intracellular Ca^{++} concentrations (28). A potent protein kinase C activator, TPA had no effect on the expression of IL-18 mRNA, whereas the calcium ionophore A23187 induced slight levels of IL-18 at low concentrations. Therefore, cAMP protein kinase A pathway, rather than protein kinase C pathway, regulates the IL-18 gene expression in rat thyroid FRTL-5 cells.

MMI is used as an effective therapeutic agent in autoimmune Graves' disease, by inhibiting the formation of thyroid hormones (39). MMI reduces the level of thyroid autoantibodies (40), suggesting an immunosuppressive action on lymphocytes and antigen-presenting cells and/or direct thyroid action to reduce antigen signaling (41,42). Moreover, recent papers described other effects on the thyroid cells by MMI, including *in vitro* reduction of major histocompatibility complex (MHC) class I antigen as well as reductions of hsp-72, prostaglandin E_2, IL-1, IL-6 expression by thyrocytes (42–44). Thus, our data indicated the novel immunosuppressive effect of MMI that suppressed TSH-induced IL-18 production.

Next, we observed that the constitutive expresiosn of IL-18 mRNA in human thyroid glands using conventional RT-PCR methods (Fig. 3A). Moreover, we found that IL-18 mRNA was specifically expressed in the thyroid follicular cells by *in situ* RT-PCR (Fig. 3B). To limited numbers of human thyroid samples, there was the tendency that higher levels of IL-18 mRNA were detected in T_H1-dominant type disease, Hashimoto's thyroiditis (Fig. 3B).

Finally, by applying an immunohistochemical study, we have shown here that IL-18 is upregulated in Hashimoto's thyroiditis, and we provided evidence that IL-18 expression clearly differentiates both Hashimoto's thyroiditis and Graves' disease from the normal thyroid tissue (Fig. 4). We also showed that the extent and density of IL-18 staining were markedly increased in close relation with lymphocytic infiltrates as described in previous papers (14,20). Recent papers have indicated IFN-γ upregulates IL-18 gene expression (45,46). Therefore, the cytokine, especially IFN-γ, which are produced by the lymphocytic infiltrate may induce IL-18 production in the thyroid cells. Therefore, these interactions between the lymphocytes and thyroid follicular cells via the cytokines may perpetuate the local immune responses.

Furthermore, we detected the simultaneous expressions of IL-18, FasL, and HLA-DR on thyroid follicular cells (Fig. 4). Because IL-18 regulates IFN-γ production and the subsequent expression of MHC class I and II antigens by the epithelial cells and induces T_H1 cell proliferation, it is conceivable that IL-18 may contribute to the local immune response in Hashimoto's thyroiditis by inducing HLA-DR and FasL in thyroid follicular cells and surrounding immunocompetent cells, and promoting the expansion of T_H1-primed intrathyroidal lymphocytes.

Recently, Miyauchi et al. (23) showed increased levels of serum IL-18 in Graves' disease. Moreover, our results *in vitro* also proposed the possibility that TSH receptor antibody (TRAb) could also stimulate IL-18 expression in thyroid follicular cells. Since relatively low levels of IL-12 gene expression have been detected in the thyroids of Graves' disease (47), a crucial role for IL-18 in the thyroid of Graves' disease may be due to promote T_H2 responses. However, the expression of IL-18 was weak and less frequent in Graves' disease than in Hashimoto's thyroiditis (Fig. 4). The frequency of the findings in Graves' disease correlated with the degree of focal thyroiditis in the glands (Fig. 4). Because the patients examined in the present study were treated with antithyroid drugs, resulting in a euthyroid state at the time of operation, this treatment may have influenced IL-18 expression in our materials by inhibiting TSH- or TRAb-induced IL-18 production.

In conclusion, the data presented in this paper indicate that TSH stimulates IL-18 mRNA expression in rat thyroid cells, and constitutive and frequent expression on human thyroid follicular cells, indicating that IL-18 upregulation is an immunologic feature of ATD and suggesting that IL-18 may play a role in promoting the local immune response.

FIG. 3. Detection of interleukin-18 (IL-18) gene expression in human thyroid tissue samples using reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* RT-PCR. Total RNA was extracted from human thyroid whole tissue by homogenization using TRIzol® (GIBCO, Painsly, UK), according to the manufacturer's protocol and RT-PCR was performed. Human thyroid tissue samples constitutively expressed IL-18 mRNA (**A**). N, normal thyroid tissue; G, Graves' disease; H, Hashimoto's thyroiditis; T, tumor, papillary thyroid carcinoma. The precise distribution of the IL-18 in the thyroid gland was explored by detecting the corresponding PCR-amplified cDNAs by *in situ* RT-PCR (**B**). IL-18 mRNA was detected exclusively in the thyroid follicular cells, whereas absent or a weak signal for IL-18 mRNA was detected in connective tissues and infiltrating lymphocytes in the thyroid gland. Moreover, the thyrocytes accompanied with autoimmune thyroid disease (ATD) demonstrated higher IL-18 mRNA compared to the normal thyroid follicular cells (**B**). Original magnification, 400×.

FIG. 4. Immunohistochemical staining for interleukin-18 (IL-18), HLA-DR, and FasL in paraffin-embedded sections from autoimmune thyroid disease (ATD). Using anti-human IL-18 monoclonal antibody, monoclonal anti-human HLA-DR α-chain antibody and anti-FasL antibody, thyroid follicular cells in a patient with Hashimoto's thyroiditis revealed diffuse and extensive staining generally observed within or adjacent to lymphocytic infiltrates. The expression of IL-18 was least frequently in Graves' disease. The frequency of the findings in Graves' disease correlated with the degree of focal thyroiditis in the glands. IL-18 protein was distributed in the same follicles that express HLA-DR and FasL. There was a close correspondence of the increase in IL-18 protein and increased the intense expression of HLA-DR and FasL. Original magnification, 400×.

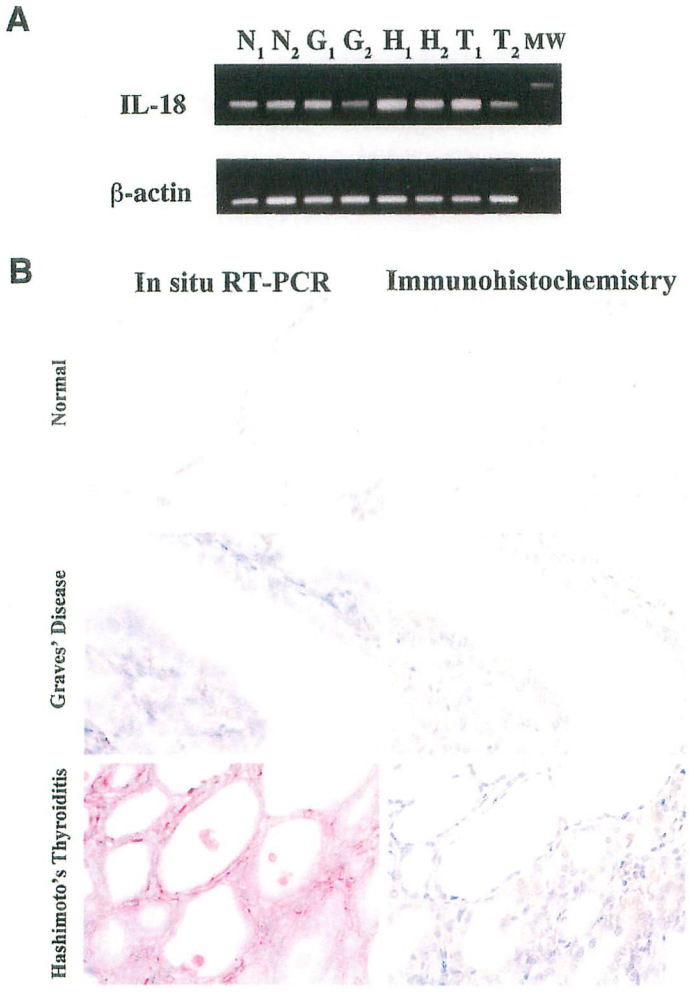

A
N1 N2 G1 G2 H1 H2 T1 T2 MW
IL-18
β-actin
B
In situ RT-PCR
Immunohistochemistry
Normal
Graves' Disease
Hashimoto's Thyroiditis

FIG. 3.

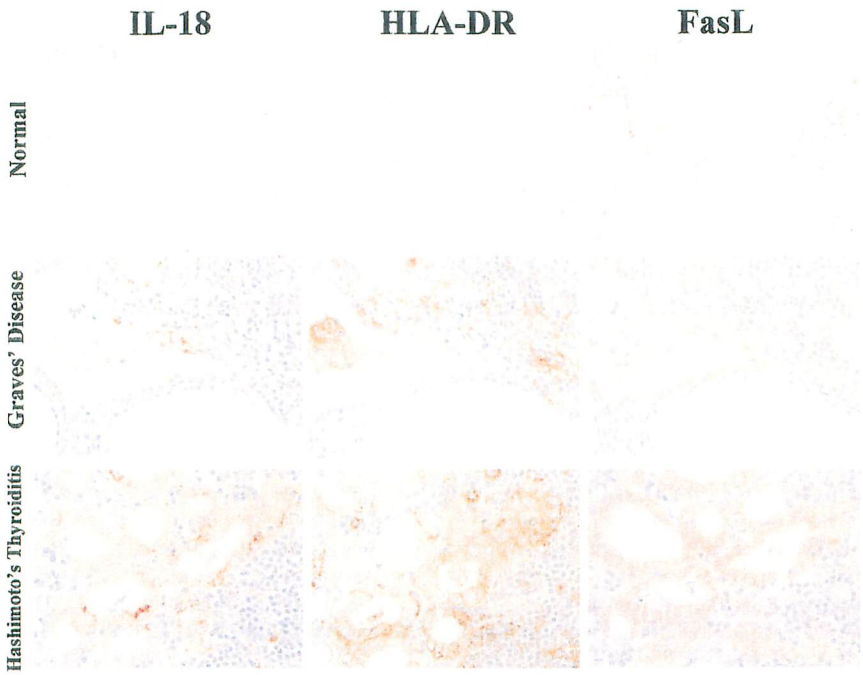

IL-18
HLA-DR
FasL
Normal
Graves' Disease
Hashimoto's Thyroiditis

FIG. 4.

Acknowledgments

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