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Production of Granulocyte/Macrophage and Macrophage Colony-Stimulating Factors by Human Thyrocytes in Culture

Kikuo Kasai,*[,1] Nobuyuki Banba,* Satoshi Motohashi,* Harumi Fukuda,* Kenichi Manaka,† Michiko Matsumura,* Yoshio Sekiguchi,* and Yoshiyuki Hattori*

**Department of Endocrinology, Internal Medicine, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan; and †Center of Tissue Culture, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan*

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Monocytes/macrophages can be activated by the colony-stimulating factors (CSFs), granulocyte/macrophage CSF and macrophage CSF, and play a pivotal role in immune and inflammatory responses. We examined whether human thyrocytes can produce these CSFs. Interleukin-1 (IL-1) strongly up-regulated the gene and protein expression of the two CSFs. Interferon-γ stimulated M-CSF expression but inversely suppressed GM-CSF expression in either basal or IL-1-stimulated condition. Thyrocytes prepared from Graves' thyroid tissues produced relatively larger amounts of GM-CSF in response to IL-1 and M-CSF in both basal and IL-1-stimulated conditions when compared to those obtained from normal and adenomatous goiter thyroid tissues. Thyrotropin attenuated M-CSF, but not GM-CSF, production. The present finding indicates that human thyrocytes themselves produce both GM-CSF and M-CSF, and thus may participate in immune and inflammatory responses through these CSFs production.

The thyroid gland is a main target for autoimmune attack in humans (1 and 2). Hashimoto's thyroiditis and Graves' disease are both autoimmune thyroid diseases. Subacute thyroiditis is a relatively acute disease of inflammation possibly caused by certain viral infection (3), and painless thyroiditis is caused by an acute exacerbation of autoimmune process related to Hashimoto's thyroiditis (3). Both diseases generally exhibit reversible thyroid dysfunction associated with destruction and subsequent regeneration of thyroid follicles and thus are called as destructive thyroiditis (3). Analysis of the intrathyroidal production of cytokines in autoimmune thyroid disease has revealed the expression of IFN-γ, TNF-α and β, IL-1α and β, IL-2, IL-6, IL-8, IL-10, IL-12, IL-13 and IL-15 (4,5,6). In addition, human thyrocytes themselves have been reported to produce IL-1, IL-6, IL-8, IL-12, IL-13, IL-15 and transforming growth factor-β (TGF-β) (4-7), although the expression of IL-1 is controversial (5).

In the bone marrow, GM-CSF, M-CSF, and IL-6, either alone or in synergy with other factors, stimulate immature myeloid progenitor cells to proliferate and differentiate (8). Besides activation of neutrophils, GM-CSF differentiates monocytes to macro-phages, activates macrophages to secrete various cytokines and soluble inflammatory mediators such as granulocyte-CSF (G-CSF), M-CSF, IL-1, IL-6 and prostaglandin E, and further stimulates antigen-presenting activities of macrophages as well as dendritic cells (9-11). M-CSF activates and differentiates monocytes to macrophages having trophic or scavenger functions involved in organogenesis and tissue turnover as well as functions involved in immune and inflammatory responses (12-15). Macrophages and lymphocytes are prominent in the infiltrates of the tissue in destructive thyroiditis as well as autoimmune thyroiditis (16). Recently, we have reported that human thyrocytes can produce monocyte chemoattractant protein-1 (MCP-1), a cytokine that attracts monocytes and T-lymphocytes (17). Activation and differentiation of infiltrated monocytes/macrophages in thyroid stroma may be important process to regulate local immune and inflammatory responses. Thus, it is very important to know whether GM-CSF and M-CSF, either alone or both, may be produced by human thyrocytes and if so, the factors to regulate the expression of two CSFs.

[1] Corresponding author. Fax: +81-282-86-4632. E-mail:kkasai@dokkyomed.ac.jp.

Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; TSH, thyrotropin; RT-PCR, reverse transcription-polymerase chain reaction.

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MATERIALS AND METHODS

Materials. Recombinant human (rh) IL-1α (2×10^7 U/mg protein) was generously supplied by Otsuka Pharmaceuticals Co.,Tokushima, Japan. rhIFN-γ was supplied by Shionogi Pharmaceuticals Co., Osaka, Japan. rhTNF-α (3.5×10^7 U/mg protein) was purchased from Funakoshi, Tokyo, Japan. Human GM-CSF enzyme-linked immunosorbent assay (ELISA) kit and M-CSF ELISA kit were purchased from R&D systems, Inc., MN. Other chemicals were purchased from Sigma Chemical Co., ST. Louis, MO.

Cell culture. We obtained five specimens which consisted of two Graves' thyroid tissues, two adenomatous goiter tissues and one normal thyroid tissue. Each specimen was digested with collagenase as reported elsewhere (18). Cells (open thyroid follicles) in Ham F-12 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS) were seeded into 48-well plates (0.2 ml/well), 60-mm dishes (2 ml/dish), 100-mm dishes (8 ml/dish) or 8-well chamber slides (0.4 ml/well) and cultured with 5% CO_2 in humidified atmosphere at 37° C. Cells were used for experiment after confluence and the contamination of fibroblasts was less than 5 %.

Assay of human GM-CSF and human M-CSF in culture supernatant. Assay of GM-CSF in the culture supernatant of human thyrocytes was performed using human GM-CSF ELISA kits according to manufacture's recommendation. The immobilized (coated) monoclonal antibody and enzyme-linked polyclonal antibodies are specific for human GM-CSF. Assay of M-CSF in the culture supernatant was performed using human M-CSF ELISA kits according to manufacture's recommendation. The immobilized (coated) monoclonal antibody and enzyme-linked polyclonal antibodies raised against rhM-CSF-158, comprising the amino-terminal 158 amino acid residues of the extracellular domain of native human M-CSF, are specific for human M-CSF. Both rhM-CSF-221, comprising the amino-terminal 221 amino acid residues and naturally secreted human M-CSF have a lesser (about $\frac{1}{3}$ - $\frac{1}{2}$) immunoreactivity with these antibodies. When either rhM-CSF-221 or naturally secreted human M-CSF is used as standards, the standard curve is, however, parallel to the one using rhM-CSF-158 as standards (by manufacture's report). Since it has been reported that major fraction of secreted M-CSF consists of higher molecular weight forms (19), the values of M-CSF in the culture supernatant assayed in this kit may probably be underestimated.

Analysis of mRNA levels for GM-CSF and M-CSF by RT-PCR. RNA was extracted from thyrocytes in a 60-mm or 100-mm dish using a modified guanidinium isothiocyanate method (ISOGEN; Takara, Japan). RT-PCR was performed using standard methods. Briefly, the first strand cDNA was synthesized using random primers and M-MLV reverse transcriptase (Promega, Madison, WI), followed by PCR amplification using synthetic gene primers specific for human GM-CSF and human M-CSF deduced from the cDNA sequences reported previously (20, 21), and for human thyroglobulin (TG), a thyroid specific protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reported previously (18). Primers used were: GM-CSF forward 27-mer, 5′-TGCTGCTGAGATGAATGAAACAGTAGA-3′; GM-CSF reverse 26-mer, 5′-CCAAGATGACCATCCTGAGTTTCTAG-3′; M-CSF forward 21-mer, 5′-GGTGTCGGAGTACTGTAGCCA-3′; M-CSF reverse 22-mer, 5′-GGACCCAATTAGTGCCCATTGC-3′; TG forward 21-mer, 5′-TGCCCTGGCAATGGAGACAAA-3′ TG reverse 21-mer, 5′-ACACGGGCTGACCTTTCTTAC-3′ GAPDH forward 26-mer, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′; GAPDH reverse 24-mer, 5′-CATGTGGGCCATGAGGTCCACCAC-3′. PCR amplification was performed for 30 cycles using a DNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) as reported previously (17, 18). PCR products were electro-phoresed on a 1.5% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence.

Immunocytochemistry. Human thyrocytes were cultured on chamber slides (Nunc, Naperville, IL) in Ham F-12 medium with 10% FBS. After confluence, the thyrocytes were incubated in the optimal condition to produce each CSF; IL-1 (20 ng/ml) for GM-CSF staining or IL-1 (20 ng/ml)/IFN-γ (250 U/ml) for M-CSF staining. After a 12-h incubation, the cells were gently washed three times with PBS, fixed with cold acetone, and stored at − 80° C. After the specimens were restored at room temperature, inactivation of internal peroxidase activity was performed in a solution of methanol and concentrated hydrogen peroxide (9 : 1) for 30 min, followed by washing with the washing buffer (20 mM Tris, 150 mM NaCl, 0.01% Tween 20, 10 mM EDTA, pH 7.5), and then were treated for 30 min with blocking buffer (10 % normal rabbit serum for GM-CSF or 10 % normal goat serum for M-CSF in washing buffer). Specimens for GM-CSF and M-CSF were respectively reacted with primary antibody [monoclonal anti-human GM-CSF antibody, (Genzyme, Cambridge, MA); 10 μg/ ml, and with polyclonal anti-human M-CSF rabbit antibodies, (Genzyme, Cambridge, MA); 10 μg/ ml in blocking buffer], and as negative controls, were respectively reacted with mouse immunoglobulin-G (IgG) [(Cappel, West Chester, PA); 10 μg/ ml] and with rabbit IgG [(Cappel, West Chester, PA); 10 μg/ml in blocking buffer] for 2 h, followed by thorough washing. Then, biotinylated-anti-mouse IgG goat serum [Histofine SAB-PO(M) kit, Nichirei, Japan] for GM-CSF and biotinylated-anti-rabbit IgG goat serum [Histofine SAB-PO(M) kit, Nichirei, Japan] for M-CSF were respectively placed on the specimens for 10 min, followed by washing. Peroxidase-conjugated streptoavidine [Histofine SAB-PO(M) kit, Nichirei, Japan] was then placed on these specimens for 5 min, followed

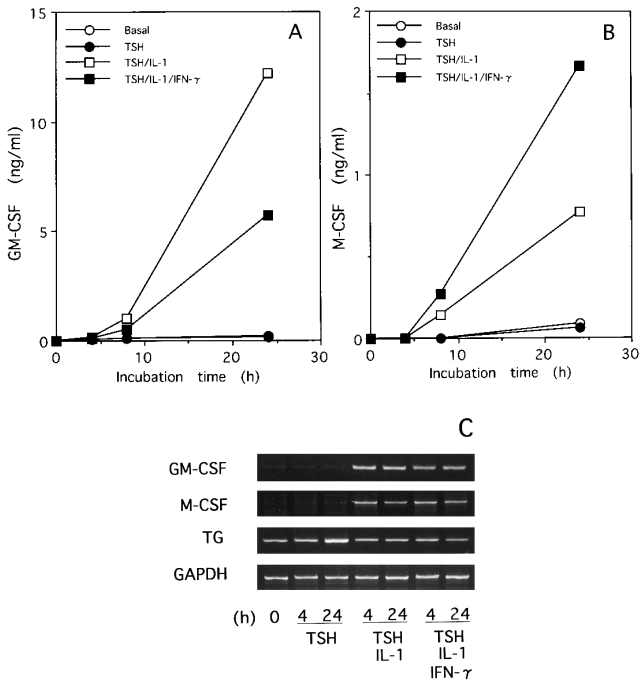

FIG. 1. Time-course of changes in the amounts of GM-CSF (A) and M-CSF (B) secreted by human thyrocytes, and the gene expression for GM-CSF, M-CSF and TG in the cells (C). Human thyrocytes in 100-mm dishes were cultured in 8 ml of Ham F-12 medium containing 10 % FBS. The cells were incubated in the medium alone (basal), or in the medium supplemented with TSH (20 mU/ml), TSH+ IL-1 (20 ng/ml) or TSH+ IL-1/IFN-γ (20 ng/ml+100 U/ml). The amounts of GM-CSF and M-CSF were respectively measured in the culture supernatant at the indicated times. The data represented are a mean value of two dishes. RNA was extracted at the indicated times after the stimulation. The abundance of the respective mRNA for GM-CSF, M-CSF, TG or GAPDH as reference was evaluated by RT-PCR.

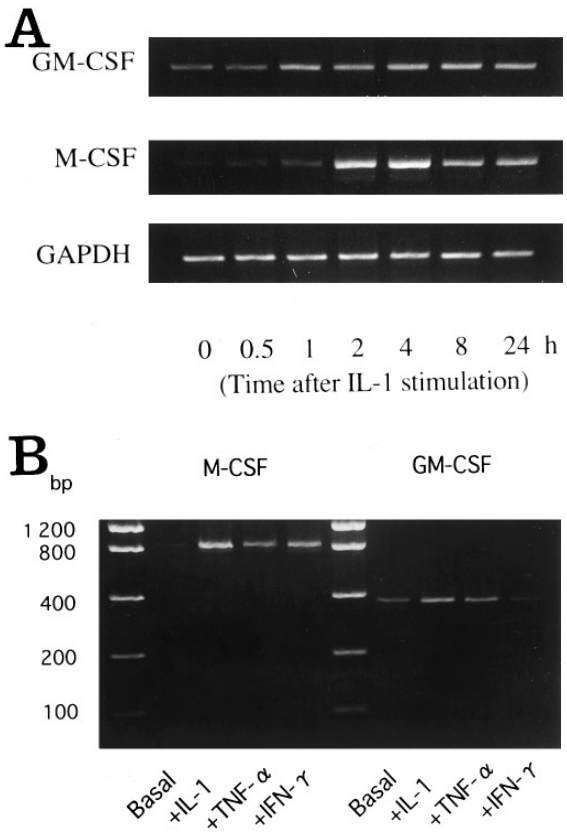

FIG. 2. Changes in mRNA levels for GM-CSF and M-CSF in human thyrocytes. Time-course of changes in the abundance of GM-CSF and M-CSF mRNAs in IL-1-stimulated thyrocytes (A). Expression of GM-CSF and M-CSF mRNAs in thyrocytes 4 h after stimulation with IL-1, TNF-α or IFN-γ (B). Thyrocytes were cultured in 60 mm-dish in 2 ml of Ham F-12 medium containing 10 % FBS. RNA was extracted at the indicated times after IL-1 (20 ng/ml) stimulation, or 4 h after stimulation with IL-1 (20 ng/ml), TNF-α (20 ng/ml) or IFN-γ (100 U/ml). The abundance of the respective mRNA for GM-CSF, M-CSF or GAPDH as reference was evaluated by RT-PCR.

by washing. Finally TrueBlue Peroxidase substrate (KPL, Geithersburg, MD) was applied on the specimens and color was developed.

Statistical analysis. Values are expressed as a mean ± standard deviation (SD) of 3 individual wells or a mean of 2 individual dishes.

RESULTS

Gene and Protein Expression of M-CSF and GM-CSF in Human Thyrocytes

Thyrocytes obtained from Graves' thyroid tissue gradually secreted small amounts of GM-CSF and M-CSF in basal condition during a 24-h incubation. Thyrotropin (TSH; 20 mU/ml) by itself at least did not stimulate the gene and protein expression of either GM-CSF or M-CSF by the cells (Fig. 1A-C). In contrast, IL-1 (20 ng/ml) remarkably stimulated the gene and protein expression of both CSFs. IFN-γ (100 U/ml) attenuated IL-1-induced GM-CSF production but inversely potentiated IL-1-induced M-CSF production. TSH clearly stimulated the gene expression of TG, a thyroid specific protein, after a 24-h incubation, but this TSH-induced TG mRNA expression was completely blocked by IL-1 in the presence or absence of IFN-γ (Fig.1C). Next, we studied the time-course changes in abundance of both GM-CSF and M-CSF mRNAs in thyrocytes stimulated IL-1 in the absence of TSH. An increase in GM-CSF mRNA levels became evident as early as 1 h after IL-1 stimulation, peaked at 4 - 8 h and its level appeared to be relatively sustained up to 24 h. In M-CSF mRNA levels, an increase was evident 1 h after IL-1 stimulation, peaked at 2 - 4 h and then declined to 24 h (Fig. 2A). In comparison with IL-1 stimulation, TNF-α (20 ng/ml) had a weak effet on the gene expression of both CSFs (Fig. 2B). IFN-γ (100 U/ml) by itself stimulated M-CSF gene expression but inversely inhibited GM-CSF gene expression. PCR reactions resulted in the amplification of a single product of the predicted size for M-CSF (812 bp) or GM-CSF (370 bp).

Immunocytochemical Staining for M-CSF and GM-CSF

In immunocytochemical study, human thyrocytes were stimulated in optimal condition for GM-CSF or M-CSF production. IL-1-stimulated cells were positively stained by anti-human GM-CSF antibody (Fig. 3A) and IL-1/IFN-γ-stimulated cells were positively stained by anti-human GM-CSF antibody (Fig. 3C) when compared with the respective negative control (Fig. 3B and 3D). In comparison with GM-CSF which was positively

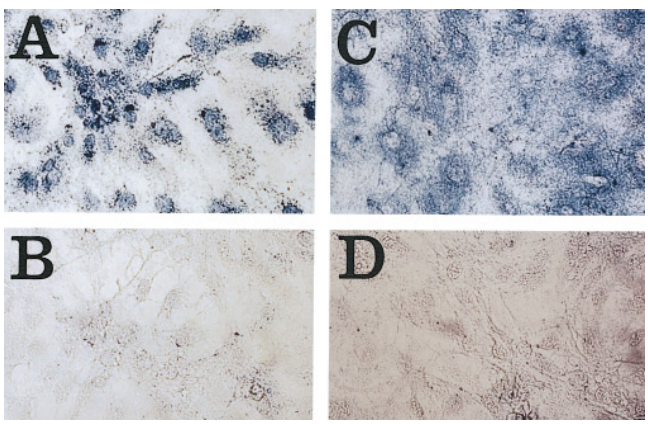

FIG. 3. Immunocytochemical staining with anti-human GM-CSF or anti-human M-CSF antibody of human thyrocytes. Human thyrocytes were cultured with IL-1 (20 ng/ml) for GM-CSF or IL-1 (20 ng/ml) + IFN-γ (250 U/ml) for M-CSF in Ham F-12 medium containing 10 % FBS. After a 12-h culture, the cells were fixed with cold acetone and then immunocytochemical staining was performed using anti-human GM-CSF (A) or anti-human M-CSF antibody (C). The negative controls for GM-CSF and M-CSF are respectively shown in (B) and (D).

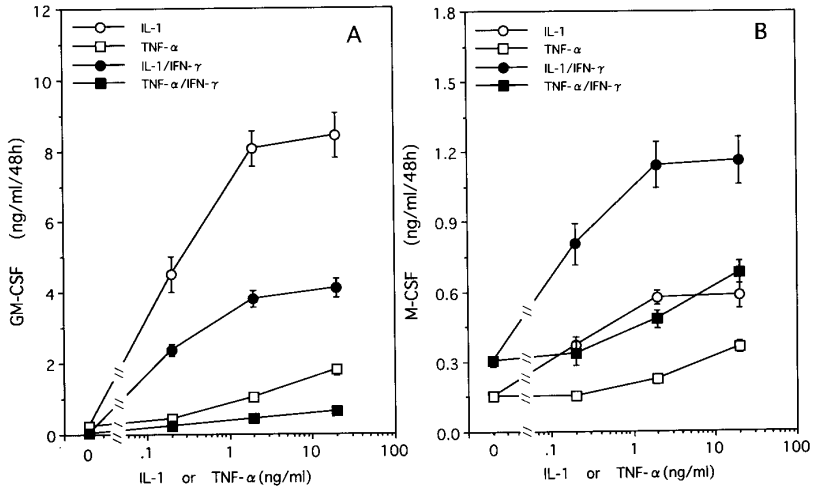

FIG. 4. Effects of IL-1 and TNF-α on the secretion of GM-CSF (A) and M-CSF (B) by thyrocytes in the presence or absence of IFN-γ. Thyrocytes in 48-well plates were stimulated with graded doses of IL-1 or TNF-α in the presence or absence of IFN-γ (100 U/ml) for 48 hours. The data represented are a mean ± SD of three wells.

stained especially around the nuclei, M-CSF appeared to be positively stained overall the cells.

Effects of Various Cytokines on the Production of Both CSFs

As shown in Figs. 4A and 4B, either IL-1 or to a lesser extent TNF-α dose-dependently stimulated both GM-CSF and M-CSF production by the cells during a 48 h-incubation. IFN-γ (100 U/ml) clearly attenuated GM-CSF production but inversely exhibited a potential effect on M-CSF production induced by either IL-1 or TNF-α.

Comparison of CSFs Production by Cultured Thyrocytes Prepared from Various Thyroid Tissues in the Presence or Absence TSH

The amounts of M-CSF produced by Graves' thyrocytes in basal, IL-1 or IL-1/IFN-γ-stimulated condition seemed to be larger when compared to those by normal or adenomatous goiter thyrocytes (Table 1). Also, IL-1-induced GM-CSF production by Graves' thyrocytes seemed to be greater than that in normal or adenomatous goiter thyrocytes. TSH attenuated M-CSF, but not GM-CSF, production in either basal or cytokine-stimulated condition in all specimens tested.

DISCUSSION

The gene and protein expression of both CSFs, GM-CSF and M-CSF, in human thyrocytes and its regulation by various cytokines and other factors have not been reported previously. The expression of both CSFs were up-regulated by either IL-1 or TNF-α. IFN-γ, however, differentially regulated the expression of the two CSFs. Namely, IFN-γ up-regulated M-CSF gene and protein expression but inversely down-regulated GM-CSF expression in basal as well as IL-1 or TNF-α-stimulated conditions. IFN-γ has been already reported to enhance M-CSF gene and protein expression in human synovial fibroblasts, human arterial smooth muscle cells and mouse mesangial cells (22-24) but inversely to down-regulate IL-1 or TNF-α-induced GM-CSF expression in human monocytes, rheumatoid arthritis synoviocytes, thymic epithelial cells and articular chondrocytes (25-27) and in murine vascular endothelial cells possibly by destabilizing the mRNA (28). Accordingly, the present results in human thyrocytes are compatible with these reports. Also in human thyrocytes, similar reciprocal control by IFN-γ have been observed on the expression of two chemokines, IL-8 and MCP-1 (6,17).

In immunocytochemical study, human thyrocytes stimulated by IL-1 or IL-1/IFN-γ were positively stained with anti-human GM-CSF or anti-human M-CSF antibody (Fig. 3). GM-CSF appeared to be dominantly stained around nuclei, but M-CSF seemed to be stained over all the cells. It is known that M-CSF consists of several forms. Major form can be secreted but minor one is expressed on plasma membranes (14,19). Thus, positive staining over all the cells might be mainly attributed to M-CSF expressed on plasma membranes. This finding strongly indicates that human thyrocytes themselves can produce both GM-CSF and M-CSF. This assumption is further supported by the facts that TSH-induced TG, a thyroid specific protein, gene expression in the cells was completely blocked by the addition of IL-1 (Fig. 1C) and also that IL-1-induced as well as basal M-CSF production by the cells was clearly attenuated by the addition of TSH (Table 1).

TABLE 1

Comparison of M-CSF and GM-CSF Production by Human Thyrocytes Prepared from Various Thyroid Tissues and Effects of TSH on Production of Both CSFs

	Normal	Adenom. G.	Adenom. G.	Graves	Graves
M-CSF (ng/ml/48h)					
Basal	0.084±0.008	0.081±0.003	0.076±0.001	0.165±0.016	0.182±0.014
+TSH	0.071±0.010	0.065±0.015	0.066±0.006	0.133±0.012	0.132±0.006
IL-1	0.428±0.002	0.483±0.066	0.353±0.024	0.743±0.021	0.683±0.012
+TSH	0.315±0.022	0.356±0.020	0.229±0.022	0.592±0.045	0.472±0.014
IL-1/IFN-γ	0.814±0.122	0.856±0.045	0.721±0.009	1.182±0.067	1.236±0.046
+TSH	0.560±0.008	0.545±0.070	0.499±0.012	0.896±0.021	0.770±0.020
GM-CSF (ng/ml/48h)					
Basal	0.209±0.028	0.220±0.018	0.206±0.019	0.229±0.010	0.256±0.020
+TSH	0.218±0.022	0.208±0.014	0.200±0.022	0.240±0.023	0.264±0.022
IL-1	5.030±0.246	5.904±0.883	4.654±0.374	7.976±0.111	8.297±0.375
+TSH	4.885±0.294	5.768±0.429	4.253±0.364	7.452±0.233	8.442±0.272
IL-1/IFN-γ	3.768±0.325	4.149±0.284	3.892±0.425	5.884±0.236	5.434±0.361
+TSH	3.671±0.269	4.747±0.386	3.608±0.122	5.356±0.486	5.312±0.205

Human thyrocytes prepared from one normal, two adenomatous goiter (Adenom. G.) and two Graves' (Graves) thyroid tissues were cultured in 48-well plates. The respective cells were incubated in 0.2 ml fresh medium for 48 h with or without IL-1 (20 ng/ml) or IL-1/IFN-γ (20 ng/ml/100 U/ml) in the presence or absence of TSH (20 mU/ml) after confluence. The corrected values by cell numbers (ng/ml/48h/5×10^4 cells) are represented as a mean ± SD of three wells.

In comparison with thyrocytes obtained from normal and adenomatous goiter thyroid tissues, those prepared from Graves' thyroid tissues produced relatively larger amounts of M-CSF in basal, IL-1 and IL-1/IFN-γ-stimulated conditions and GM-CSF in IL-1-stimulated condition. These results suggest that Graves' thyrocytes might have been already activated by cytokine(s) like IL-1, TNF-α or IFN-γ, and/or sensitized to them *in vivo*, because macrophages and lymphocytes are prominent in the infiltrate of the tissue in both autoimmune and destructive thyroiditis (16) and such cytokines are expressed in autoimmune thyroid tissues (4,5). In addition, we have recently reported that human thyrocytes themselves produce MCP-1, a specific chemoattractant for monocytes/macrophages and T-lymphocytes (17). Both GM-CSF and M-CSF can activate monocytes/macrophages to produce several cytokines like IL-1 and TNF-α and soluble inflammatory mediators, and also activate monocytes/macrophages as well as dendritic cells to up-regulate the antigen presenting capacity (9-15). Activated T-lymphocytes produce IFN-γ. This would constitute an amplification loop of immune and inflammatory responses through a paracrine way.

The present study demonstrates that expression of GM-CSF and M-CSF in cultured human thyrocytes is remarkably up-regulated by IL-1 and to a lesser extent by TNF-α, that IFN-γ differentially regulates the expression of these CSFs, and further that TSH attenuates M-CSF, but not GM-CSF, production. Production by human thyrocytes of GM-CSF and M-CSF provides a possible mechanism by which thyrocytes themselves may participate in the processes of thyroid autoimmune and inflammatory disease.

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