Expression of monocyte chemoattractant protein-1 mRNA and protein in cultured human thyrocytes

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Abstract Monocytes as well as lymphocytes infiltrate in the stroma of thyroid tissue in autoimmune and destructive thyroiditis. Monocyte chemoattractant protein-1 (MCP-1) is a cytokine that attracts T-lymphocytes as well as monocytes. Using human thyrocytes in primary cultures, we show that expression of MCP-1 mRNA and protein is remarkably stimulated by both interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), and also that interferon-γ (IFN-γ) by itself is a weak stimulant but has a synergistic activity with either IL-1 or TNF-α. The finding indicates that MCP-1 can be produced by thyrocytes themselves, suggesting a possible role of thyrocytes on accumulation of monocytes and T-lymphocytes to the tissue from the blood in autoimmune and destructive thyroiditis.

Key words: Thyrocyte; Monocyte chemoattractant protein-1; Interleukin-1; Tumor necrosis factor-α; Interferon-γ

1. Introduction

The chemokines form a superfamily of small (8–10 kDa), inducible, secreted chemotactic cytokines (for reviews see [1–5]). Chemokines such as interleukin-8 (IL-8) that attract mainly neutrophils are mostly members of the α subfamily, whereas those such as MCP-1 that attract mainly monocytes belong to the β subfamily [1–5]. Although MCP-1 is originally reported to be an attractant for monocytes but not for neutrophils or lymphocytes [6], the cytokine has recently been shown to chemoattract T-lymphocytes in addition to monocytes [7,8]. Based on its in vitro and in vivo functions, the correlation of MCP-1 expression and the pathologies or pathophysiologies have already been studied in several diseases and are still under investigation in a wide variety of immune and inflammatory diseases [1–5,9–14].

The thyroid gland is a main target for autoimmune attack in humans (for a review, see Refs. [15,16]). Hashimoto's thyroiditis and Graves' disease are both autoimmune thyroid diseases. Analysis of the intrathyroidal production of cytokines in such disease has revealed the expression of IFN-γ, TNF-α and β, IL-2, IL-6, IL-8 and IL-10 [17,18]. In addition, human thytocytes themselves have been reported to produce IL-1, IL-6, IL-8 and TGF-β [17–19], although the expression of IL-1 is controversial [18]. Subacute thyroiditis is a relatively acute disease of inflammation possibly caused by certain viral infection [20], and painless thyroiditis is caused by an acute exacerbation of an autoimmune process related to Hashimoto's thyroiditis [20]. Both diseases are known as destructive thyroiditis. Macrophages and lymphocytes are prominent in the infiltrates of destructive thyroiditis as well as autoimmune thyroiditis, and macrophages and dendritic cells can present thyroid antigen to T-lymphocytes in vitro [21]. Thus, cytokines produced by infiltrated mononuclear cells and by thyrocytes themselves play a central role in thyroid autoimmune and inflammatory responses. The initiation of infiltration and further recruitment of mononuclear cells into thyroid stroma, an important step to induce immune and inflammatory responses, should be mediated by some chemoattractant proteins. IL-8 produced by thyrocytes has already been suggested to have a possible role at least in part in accumulation of T-lymphocytes within the gland in autoimmune thyroid disease [18,19], because IL-8 was shown to attract T-lymphocytes [22] in addition to neutrophils [23,24]. However, IL-8 is not a chemoattractant for monocytes and the effect of IL-8 on lymphocytes is still questionable [5]. Thus, it is very important to know whether MCP-1 may be produced by human thyrocytes.

2. Materials and methods

2.1. 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 MCP-1 enzyme immunoassay (EIA) kits were purchased from R&D systems, Inc., MN.

Human thyroglobulin (TG) radioimmunoassay (RIA) kits were obtained from Daiichi RI Lab., Tokyo, Japan. All other chemicals were purchased from Sigma Chemicals Co., St. Louis, MO.

2.2. Cell culture

We obtained two specimens of thyroid tissues resected from two Graves' patients. Each specimen was digested with collagenase as reported elsewhere [25]. 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) or 100-mm dishes (8 ml/dish) and cultured with 5% CO_2 in humidified atmosphere at 37° C. Cells were used for experiment after confluence and the contamination of fibroblast was less than 5%.

2.3. Assay of human MCP-1 and human thyroglobulin (TG) in culture supernatant

Assay of MCP-1 in the culture supernatant of human thyrocytes was performed using human MCP-1 EIA kits according to manufacturer's recommendation. Both the immobilized (coated) monoclonal antibody and enzyme-linked polyclonal antibody raised against rhMCP-1 are specific for human MCP-1 and do not cross-react with 12 factors related to or associated with MCP-1, including

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Abbreviations: MCP, monocyte chemoattractant protein; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; TGF, transforming growth factor; RH, recombinant human; RT-PCR, reverse transcription-polymerase chain reaction

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rhMCP-2, rhMCP-3 and rhIL-8, and also with 86 cytokines, including rhIL-1α, rhIL-1β, rhTNF-α, rhIFN-γ and various recombinant human cytokine soluble receptors. Assay of TG was performed by RIA.

2.4. Analysis of mRNA levels for MCP-1 by RT-PCR

RNA was extracted from thyrocytes in a 60-mm or 100-mm dish using a modified guanidinium isothiocyanate method (RNAzol; Cinna/Biotex, Houston, TX). 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 MCP-1 deduced from the cDNA sequences reported previously [26], and for human thyroglobulin (TG) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reported previously [25]. Primers used were: MCP-1 forward 25-mer, **5′-TCGCTCAGCCAGATGCAATCAATGC-3′**: MCP-1 reverse 24-mer, **5′-CCCAGGGGTAGAACTGTGGTTCAA-3′**; TG forward 21-mer, **5′-CTGCGGAGGGAGAGTTTATGC-3′**; TG reverse 21-mer, **5′-AGGTCGTGCTGGCTGAAGTAG-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 [25]. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. PCR reactions resulted in the amplification of a single product of the predicted size for MCP-1 (479 bp) and TG (613 bp) [data not shown].

2.5. Statistical analysis

Values are expressed as a mean ± standard deviation (S.D.) of 3 wells or a mean of 2 dishes. Statistical analysis was performed by unpaired *t*-test and two-way analysis of variance (ANOVA). A *P*-value less than 0.05 was considered significant.

3. Results

Unstimulated thyrocytes gradually secreted small amounts of MCP-1 during a 24-h incubation. Thyroid stimulating hormone (TSH; 20 mU/ml) did not increase but appeared to inhibit slightly the amounts of MCP-1 secreted by the cells. IL-1 (20 ng/ml) remarkably stimulated MCP-1 secretion by over 10-fold. IFN-γ (100 U/ml) potentiated IL-1-induced MCP-1 secretion throughout the period (Fig. 1A). TSH clearly stimulated the expression of TG, a thyroid-specific protein, mRNA after a 24-h incubation, but IL-1 with or without IFN-γ blocked this TSH-induced TG mRNA expression. The secretion of TG induced by TSH was higher by about 5-fold after a 48-h incubation when compared with that in basal condition. TSH-induced secretion of TG was again completely blocked by IL-1 in the presence or absence of IFN-γ (Fig. 1B). Next, we studied the time course changes of MCP-1 mRNA expression in IL-1-stimulated thyrocytes. An increase in abundance in MCP-1 mRNA became evident as early as 1 h after IL-1 (20 ng/ml) stimulation when compared with the basal level, peaked at 2–4 h and then declined gradually to 24 h (Fig. 2A). TNF-α (20 ng/ml) also strongly stimulated MCP-1 gene expression as similarly as IL-1, whereas IFN-γ (100 U/ml) only slightly did so (Fig. 2B). As shown in Fig. 3A,B, both IL-1 and TNF-α dose-dependently stimulated MCP-1 production by the cells during a 48-h incubation. IFN-γ (100 U/ml) by itself slightly stimulated its production but exhibited a synergistic activity with either IL-1 or TNF-α.

Actinomycin D (1 μg/ml) almost completely inhibited the expression MCP-1 mRNA and protein in both IL-1-stimulated and unstimulated cells. Cycloheximide (10 μg/ml) also inhibited MCP-1 secretion while the gene expression was clearly augmented in basal condition but slightly inhibited in

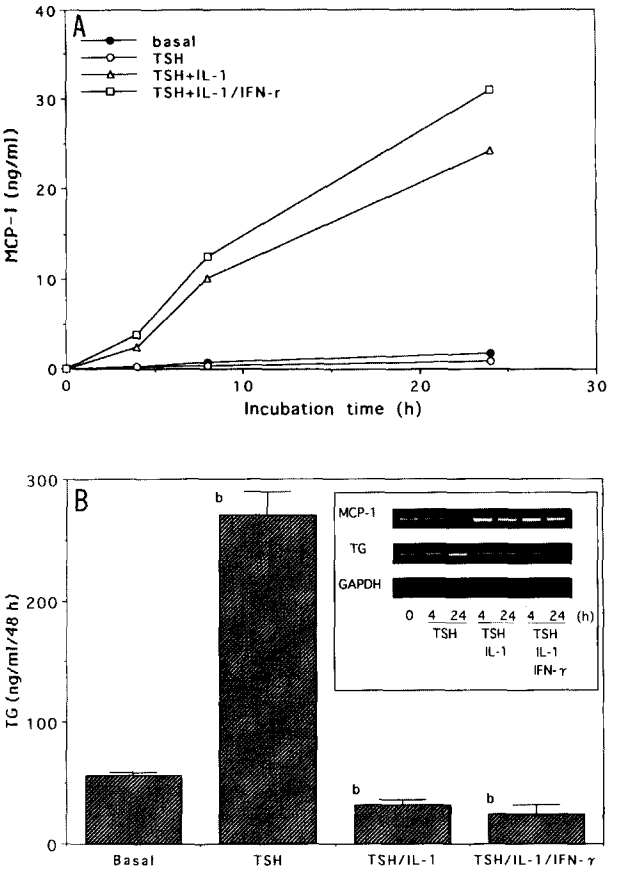

Fig. 1. Time course changes in the amounts of MCP-1 in the culture supernatant of human thyrocytes (A), and changes in the amounts of TG and the expression of MCP-1 and TG mRNAs in the cells (B). Human thyrocytes were incubated in the medium alone (basal), or in the medium supplemented with TSH (20 mU/ml), TSH+IL-1 (20 ng/ml) or TSH+IL-1/IFN-γ (20 ng/ml+ 100 U/ml). At the indicated times, MCP-1 secreted in the culture supernatant was measured and expression of mRNAs for TG and MCP-1 was analyzed in the cells cultured in 100 mm-dish. After 48 h incubation, TG secreted by the cells in the culture supernatant of 48-well plate was measured. The data represented are a mean value of two dishes or a mean value ± S.D. of three wells.

IL-1-stimulated condition. Dexamethasone attenuated the expression of MCP-1 mRNA and protein in both conditions (Fig. 4). Thyrocytes obtained from two Graves' tissues responded well to such proinflammatory cytokines and characteristic of the responses was very similar in each of these thyrocytes.

4. Discussion

The expression of MCP-1 mRNA and protein in human thyrocytes was strongly up-regulated by either IL-1 or TNF-α and IFN-γ potentiated IL-1- or TNF-α-induced MCP-1 expression. TSH was rather inhibitory on the cytokine(s)-induced MCP-1 expression. In contrast, the expression of TG, a thyroid-specific protein, mRNA and protein was remarkably stimulated by TSH and this TSH-dependent up-regulation of TG expression was completely blocked by a co-administration of IL-1 as reported previously [27]. Furthermore, over 95% of the cultured cells incubated with TSH alone were positively stained with anti-TG antibody (data not shown). These findings indicate that human thyrocytes themselves can produce

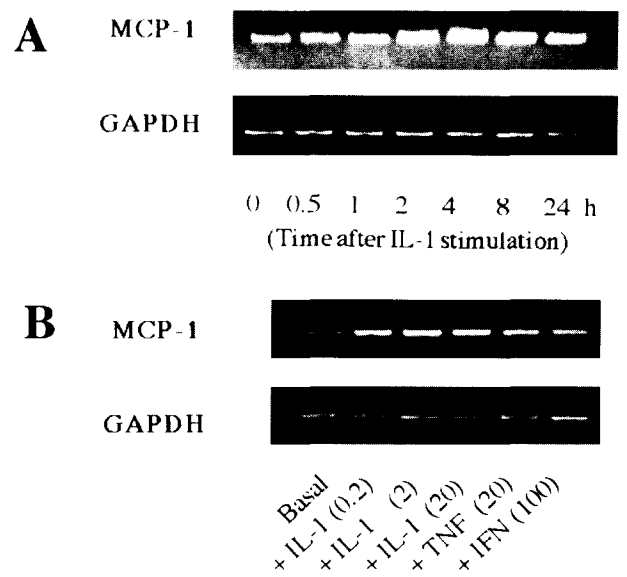

Fig. 2. Changes in mRNA levels for MCP-1 in human thyrocytes. Time course changes in the abundance of MCP-1 mRNA in IL-1-stimulated thyrocytes (A). Expression of MCP-1 mRNA in thyrocytes 4 h after stimulation of IL-1, TNF-α or IFN-γ (B). Thyrocytes were cultured in 60-mm dish in 2 ml of Ham F-12 medium containing 10% FBS. RNA was extracted at the indicated times after IL-1 (20 ng/ml) stimulation or 4 h after stimulation with varying doses of IL-1 (0.2–20 ng/ml), TNF-α (20 ng/ml) or IFN-γ (100 U/ml). The abundance of mRNAs for MCP-1 and GAPDH as reference was evaluated by RT-PCR.

MCP-1, suggesting a possible role of thyrocytes to participate in the recruitment of monocytes and T-lymphocytes from the blood stream into thyroid stroma through MCP-1 production. Activated monocytes/macrophages secrete IL-1 and TNF, and activated lymphocytes produce IFN-γ. These potent proinflammatory cytokines are expressed in the tissues of autoimmune thyroid disease [17,18], indicating that not only recruitment but also activation of mononuclear cells really takes place in vivo. This would constitute an amplification loop of the immune and inflammatory responses through a paracrine way. This is also proposed to be the case in destructive thyroiditis, although direct evidence has been scarcely obtained because such thyroiditis should not be surgically treated.

Inhibition by actinomycin D of MCP-1 mRNA expression in both IL-1-stimulated and unstimulated cells, can be likely explained by transcriptional control of MCP-1 gene expression. Since the mRNA for MCP-1 contains AUUUA sequences in their 3′ untranslated region which are typical for immediate early genes and cause instability of mRNA [28], the cycloheximide-dependent MCP-1 mRNA expression in unstimulated thyrocytes can be interpreted as stabilization of the message due to the reduced synthesis of RNAases as has been already shown in smooth muscle cells, endothelial cells and mesangial cells [29–31]. In contrast, a slight inhibition by cycloheximide of the IL-1-induced expression of MCP-1 mRNA suggests that action of IL-1 might be partly dependent on new protein synthesis in thyrocytes. Regulation of the gene expression by cycloheximide seems to be cell-specific, because in peripheral blood monocytes cycloheximide caused inhibition [29,30]. Dexamethasone, a potent immunosuppressive agent, inhibited both the gene expression and protein secretion by thyrocytes as has been shown in other cell types [32]. The inhibition of gene expression by glucocorticoids is generally caused through an interaction with glucocorticoid-responsive elements in the promoter regions of genes, a modulation of AP-1 DNA binding activity [33] or/and through production of IκB, an inhibitory molecule for nuclear factor kappa B (NF-κB) [34,35]. Accordingly, these mechanism(s) may probably operate in thyrocytes.

The present study demonstrates that in cultured human thyrocytes, MCP-1 production and gene expression are remarkably up-regulated by proinflammatory cytokines like IL-1 and TNF-α in the presence or absence of IFN-γ. The production of MCP-1 besides IL-8 by human thyrocytes may play a role in the recruitment of monocytes and T-lymphocytes at immune inflammatory sites in thyroid gland from the blood, thus providing a possible mechanism by which thyrocytes themselves may participate in the processes of thyroid autoimmune and inflammatory disease.

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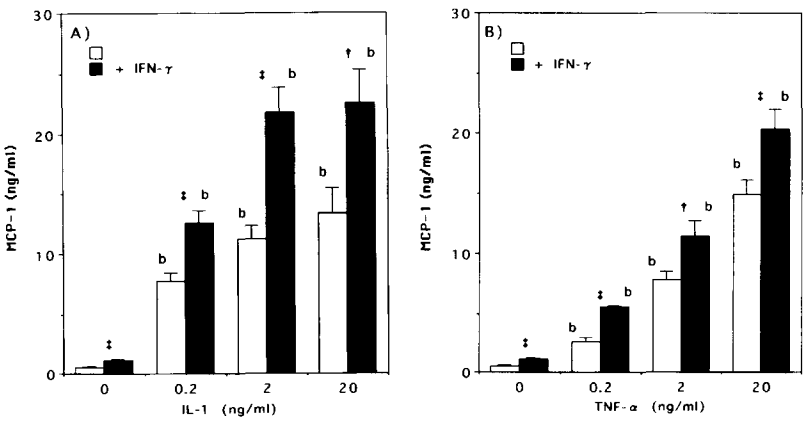

Fig. 3. Effects of IL-1 or TNF-α on the secretion of MCP-1 by thyrocytes in the presence or absence of IFN-γ. Thyrocytes in 48-well plate were stimulated with graded doses of IL-1 (A) or TNF-α (B) in the presence or absence of IFN-γ (100 U/ml) in Ham F-12 medium containing 10% FBS for 48 h. The data represented are a mean ± S.D. of three wells. b: $P < 0.01$ vs the respective value in control, †$P < 0.05$ and ‡$P < 0.01$ vs the respective value in the absence of IFN-γ.

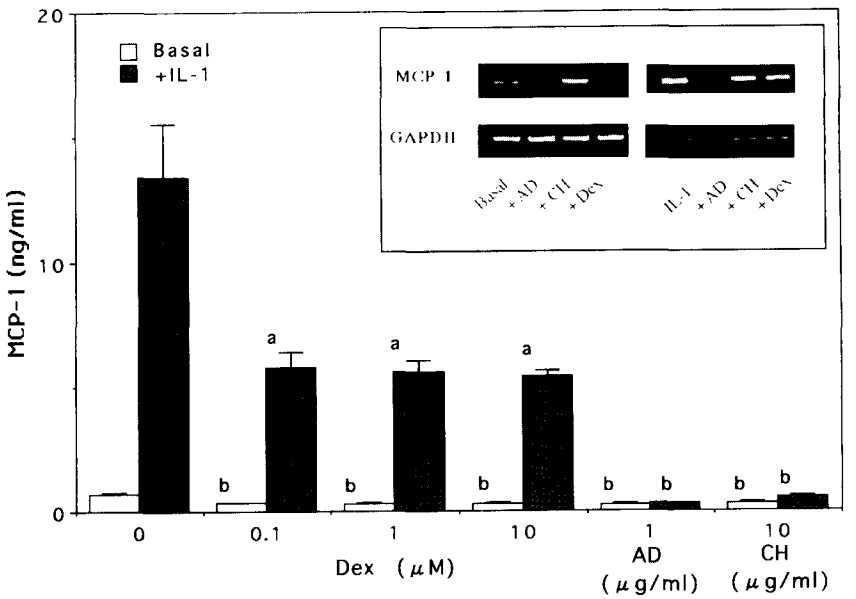

Fig. 4. Effects of dexamethasone, actinomycin D and cycloheximide on the expression of MCP-1 protein and mRNA in unstimulated or IL-1-stimulated thyrocytes. Thyrocytes in 48-well plate were incubated in the medium with or without dexamethasone (Dex; 0.1–10 μM), actinomycin D (AD; 1 μg/ml) or cycloheximide (CH; 10 μg/ml) in the presence or absence of 20 ng/ml IL-1 for 48 h. The data represented are a mean ± S.D. of three wells. a: $P < 0.05$ and b: $P < 0.01$ vs the respective control value. Expression of MCP-1 mRNA was analyzed in thyrocytes cultured in 60-mm dish for 4 h in the medium with or without AD (1 μg/ml), CH (10 μg/ml) or Dex (10 μM) in the presence or absence of 20 ng/ml IL-1.

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