Possible Involvements of Nuclear Factor-κB and Activator Protein-1 in the Tumor Necrosis Factor-α–Induced Upregulation of Matrix Metalloproteinase-12 in Human Alveolar Epithelial A549 Cell Line

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Abstract. Matrix metalloproteinase-12 (MMP-12) has been suggested to play an important role in airway inflammatory diseases. Tumor necrosis factor-α (TNF-α) is known to cause an upregulation of MMP-12 via an activation of activator protein-1 (AP-1) in monocytes. In the present study, we investigated the effect of TNF-α on the expressions of MMP-12 in airway epithelial cells, one of the sources of MMP-12 in the airway, and its underlying mechanism. MMP-12 mRNA and protein expressions induced by TNF-α in the absence or presence of BMS-345541 (a selective IκB kinase inhibitor) or SP600125 [a selective c-Jun N-terminal kinase (JNK) inhibitor] were measured by quantitative real-time PCR and Western blotting, respectively. Furthermore, siRNAs for p65 and JNK2 were used to confirm the involvements of nuclear factor-κB (NF-κB) and AP-1 in the MMP-12 mRNA expression induced by TNF-α in A549 cells. Both MMP-12 mRNA and protein were upregulated by the treatment with TNF-α in time- and concentration-dependent manners. Both BMS-345541 and SP600125 inhibited the upregulation of MMP-12 induced by TNF-α. Furthermore, both the depletion of p65 and JNK2 by siRNAs significantly attenuated the upregulation of MMP-12 induced by TNF-α. These findings suggest that both NF-κB and JNK/AP-1 pathways are important for the MMP-12 upregulation induced by TNF-α in A549 cells.

Keywords: matrix metalloproteinase-12 (MMP-12), tumor necrosis factor-α (TNF-α), nuclear factor-κB (NF-κB), activator protein-1 (AP-1)

Introduction

Airway remodeling is a key feature of the pathogenesis of chronic asthma and chronic obstructive pulmonary disease (COPD) (1). The irreversible structural changes such as subepithelial collagen deposition, goblet cell hyperplasia, and epithelial desquamation were observed in these diseases (2), indicating that epithelium is an important place for airway remodeling. Furthermore, epithelium is the primary target for the environmental agents such as antigen and house dust, and it also takes part in proinflammatory mediator recruitment.

The abnormal degradation of extracellular matrix (ECM) was observed in the airways of asthma and COPD patients, which indicates that ECM plays a critical role in the airway remodeling in chronic airway inflammatory diseases (3). Matrix metalloproteinases (MMPs) are a family of enzymes that degrade ECM and non-matrix proteins (4). All MMPs contain pro-domains and catalytic domains, which bind to the Zn²⁺ through the cysteine and histidines, respectively. Following cleavage of the pro-domain, the catalytic domain is free to act on substrates (5). MMP-12, also called macrophage elastase, mainly degrades ECMs such as elastin and collagen in the airway when it is activated by taking off its propeptide and hemopexin domains (6). Besides macrophages, it is also reported that MMP-12 is secreted from bronchial epithelial cells and airway smooth muscle cells (7, 8).

Increased levels of proinflammatory cytokines in the airways are characteristic of asthma and COPD (9). In human monocytes, the expression of MMP-12 has been reported to be regulated by tumor necrosis factor-α...
(TNF-α), which is known as one of the proinflammatory cytokines (10), but the detailed mechanism underlying MMP-12 expression induced by TNF-α in airway epithelial cell is still unclear.

In the present studying, the expressions of MMP-12 induced by TNF-α were analyzed in A549 cells. Furthermore, the involvements of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) in the TNF-α–induced MMP-12 expressions were also investigated.

Materials and Methods

Chemicals

All biochemicals were of analytical grade and were purchased from commercial suppliers: recombinant human TNF-α (Peprotech, Paris, France) and SP600125 and BMS-345541 (Sigma-Aldrich, St. Louis, MO, USA). Recombinant human TNF-α was dissolved in sterile phosphate-buffered saline (PBS). SP600125 and BMS-345541 were dissolved in 0.1% DMSO in sterile PBS.

Cell culture

A549 cells (a human alveolar epithelial cell line) were cultured in RPMI-1640 (Sigma-Aldrich), supplemented with 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 μg/mL fungizone at 37°C in a humidified 5% CO2 atmosphere. The cells were plated in 6-well culture plates (1 mL/well) (Becton Dickinson Labware, Franklin Lakes, NJ, USA) for the measurement of protein or mRNA expression. Culture medium was changed every 3 days. After reaching 90% – 95% confluence in 5% FBS RPMI-1640, cells were incubated in serum-free medium for 24 h. Then cells were treated with TNF-α in the absence or presence of inhibitors used for the indicated time periods.

Real-time polymerase chain reaction (PCR)

Total RNAs were extracted from cultured A549 cells with a one-step guanidinium – phenol – chloroform extraction procedure using TRI Reagent™ (Sigma-Aldrich). The mRNA levels of MMP-12 were examined by real-time PCR. Briefly, cDNAs were prepared from the total RNA (1.0 μg) by using a QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The cDNAs were amplified by quantitative real-time PCR (Bio-Rad, Hercules, CA, USA) using SYBR Green PCR Master Mix Reagent (Bio-Rad). The primer sets used were as follows: 5′-CACCTTCTTGGTGTCGAAAGTGA-3′ (sense) and 5′-GGAGGCTGCGATCAGCTC-3′ (antisense) for MMP-12, 5′-GGAGCCTAAGGGGTATCATCTCT-3′ (sense) and 5′-AGGGATGATGTGTCTGGAGAGCC-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were designed from published sequences (Accession No. NM_002426 and NM_145071, respectively). Each primer was used at a concentration of 0.1 μM for real-time PCR at each reaction. Cycling conditions are as follows: step 1, 3 min at 95°C; step 2, 15 s at 95°C; step 3, 15 s at 55°C; step 4, 1 min at 72°C; step 5, 7 min at 72°C; steps 2 to 4 were repeated for 45 cycles. Data from the reaction were collected and analyzed by the complementary computer software (Bio-Rad). Relative quantitations of gene expression were normalized to GAPDH in each sample using the 2−ΔΔCT method (11).

Western blotting

Total cell proteins were extracted on ice with 1 × SDS sample buffer. Samples were denatured at 100°C for 4 min. The mixtures were subjected to 10% SDS-PAGE. Proteins were then transferred for 2 h onto polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham, Buckinghamshire, UK) in transfer buffer (20% methanol containing 25 mM Tris and 192 mM glycine). After repeatedly washing with Tris buffer (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TTBS), the membranes were incubated with blocking buffer (5% skim milk in TTBS) for 3 h at room temperature. Then membranes were incubated with primary rabbit anti-MMP-12 antibody (1:1,000 dilution, C-terminal; Sigma-Aldrich), in antibody buffer (2% bovine serum albumin) in TTBS for 12 h at room temperature. After washing with TTBS for 10 min, 6 times, the membranes were then incubated with horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG (1:5,000 dilution, Amersham) for 1.5 h at room temperature, and were washed with TTBS for 10 min 6 times. The blots were detected with an enhanced chemiluminescent method (ECL System, Amersham) and quantitated by a cooled CCD camera system (Atto Densitograph; Atto Co., Tokyo). To normalize the MMP-12 contents by an internal control protein, GAPDH, immunoblotting was also performed on the same gel by using monoclonal mouse anti-GAPDH (1:5,000 dilution; Chemicon, Temecula, CA, USA) and goat anti-mouse IgG (1:5,000 dilution, Amersham).

Transfection of siRNAs

Negative control siRNA and siRNAs corresponding to p65 (sense: GCCCGUAUCCUUUUAACGUCAtt, antisense: UGACGUAAGGGUAUGGCGctg) and c-Jun N-terminal kinase (JNK) 2 (sense: GCAUUCAGGUAGUAUA UUt, antisense: AAUUAUACCCGUGAAGGc) were purchased from Applied Biosystems (Foster City, MA, USA). After reaching 25% – 30% confluence, cells were transiently transfected with siRNAs using Lipo-fectamine 2000 transfection reagent (Invitrogen, CA,
USA). Briefly, 100 pmol of siRNAs was formulated with Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions. The transfection complex was diluted into 500 μL of OPTI-MEM reduced serum medium (Invitrogen) and added directly to the cells. The medium was replaced with serum-free medium at 24 h before the treatment with TNF-α. At 72 or 96 h after the transfection, samples were prepared for real time PCR and Western blot analyses, respectively.

Statistical analysis

Data were expressed as the mean ± S.E.M. Statistical significance of difference was determined by Bonferroni/Dunn test. A value of P less than 0.05 was considered as significant.

Results

Enhancement of the expressions of MMP-12 by TNF-α in A549 cells

To determine the effect of TNF-α on the expressions of MMP-12 in A549 cells, the mRNA and protein expressions of MMP-12 were analyzed after the treatment with various concentrations of TNF-α. As shown in Fig. 1A, the mRNA level of MMP-12 was increased by the treatment with TNF-α in concentration- and incubation time-dependent manners. When the cells were treated with 30 ng/mL of TNF-α, a significant increase was observed within 12 h with a maximal response at 48 h during the period of observation. Western blot analyses revealed the bands corresponding to the proenzyme (54 kD) and intermediate form (45 kD) of MMP-12 (Fig. 1B). The protein expression of MMP-12 was also increased by TNF-α treatment in concentration- and incubation time-dependent manners. A significant increase in MMP-12 protein expression was observed at 48 h after the TNF-α treatment when 30 ng/mL concentration was used.

Effects of BMS-345541 and SP600125 on the TNF-α–induced upregulation of MMP-12

As shown in Fig. 2A, the increased MMP-12 protein expressions induced by TNF-α were inhibited by a selective IκB kinase inhibitor (BMS-345541) and a selective JNK inhibitor (SP600125) in concentration-dependent manners. Furthermore, the TNF-α–induced mRNA expressions of MMP-12 were also suppressed by BMS-345541 and SP600125 (Fig. 2B). These findings suggest the possible involvements of NF-κB and AP-1 in the TNF-α–induced upregulation of MMP-12.

Effects of siRNAs for p65 and JNK2 on the TNF-α–induced upregulation of MMP-12

To further ensure the involvements of NF-κB and AP-1 in the TNF-α–induced expressions of MMP-12, the effects of siRNAs for p65 and JNK2 were also investigated. As shown in Fig. 3, A and B, the enhanced MMP-12 mRNA and protein expressions induced by TNF-α were significantly inhibited by the depletion of p65 or JNK2.
It has been suggested that the proteolysis of MMPs are involved in the process of cell migration, proliferation, and tissue remodeling associated with the pathogenesis of airway inflammation (12). MMP-12, which is also called macrophage elastase, is thought to play important roles in airway inflammatory diseases such as COPD and asthma (13). Besides macrophages, it has also been reported that airway smooth muscle and bronchial epithelial cells are important sources of MMP-12, which is thought to be deeply associated with the remodeling of these airway structural cells in chronic airway inflammatory diseases (7, 8). In the present study, both the mRNA and protein expressions of MMP-12 were detected in the human alveolar epithelial cell line A549. Indeed, airway

Discussion

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epithelial cells seem to be an important target of the production and proteolysis of MMP-12 in the pathogenesis of airway inflammatory diseases (14, 15). Currently, we investigated the effect of TNF-α on the expressions of MMP-12 in A549 cells. TNF-α, one of the proinflammatory cytokines, is well-known as an activator of two classical inflammatory transcription factors, NF-κB and AP-1 (16). Both the mRNA and protein expressions of MMP-12 are upregulated by TNF-α in time- and concentration-dependent manners (Fig. 1). The TNF-α–induced MMP-12 protein expressions in cell lysates were significantly increased although not so apparently, which may be because the MMP-12 protein is also secreted into the medium (7, 8). In our preliminary experiment, the TNF-α–induced MMP-12 content in medium was increased but not so apparently as measured by zymography. The human MMP-12 is composed of the 54-kD proenzyme, 45-kD intermediate form, and 22-kD activated form (6). It’s also reported that the activity of MMP-12 changed in parallel with the protein expressions (7, 8, and 17). Only the 54-kD proenzyme and 45-kD intermediate form of MMP-12 were detected by Western blotting because the antibody currently used recognizes the C-terminal of MMP-12 that does not exist in the active form.

In monocytes, the upregulation of MMP-12 by tissue plasminogen activator (tPA) was confirmed to be due to the activated transcriptional regulation of AP-1 on its promoter (10). Activated JNKs bind to the NH2-terminal region of c-Jun and phosphorylate Ser63 and Ser73 located within its transactivation domain, which leads to the homodimerization of c-Jun or heterodimerization of c-Jun and other AP-1 proteins (18). Due to its higher affinity, JNK2 phosphorylates c-Jun more efficiently than JNK1 (19). SP600125 was demonstrated to potently and selectively suppress the activation of JNK 1/2 (20). It has been also reported that TNF-α induced the activation of JNK in A549 cells (21). In the present study, SP600125 inhibited the increased mRNA and protein expressions of MMP-12 induced by TNF-α (Fig. 2). Furthermore, the involvement of JNK2 in the MMP-12 upregulation induced by TNF-α was also confirmed by the depletion of JNK2 (Fig. 3). These findings suggest that the upregulation of MMP-12 induced by TNF-α is regulated by the JNK / AP-1 pathway in A549 cells.

It has been known that inflammatory responses following exposures to cytokines such as TNF-α are highly dependent on the activation of NF-κB and lead to gene expression (22). The sequester of NF-κB is regulated by IκB-α in the cytoplasm and IκB-α phosphorylation by IκB kinase, which leads to proteasomal degradation of itself and the translocation of the heterodimers of p65 and p50, which belong to the NF-κB family, resulting in the transactivation of NF-κB (23). The activation and translocation of NF-κB by TNF-α into nucleus is essential for the expression of several inflammation-related genes such as interleukin 8 (IL-8) in A549 cells (24). In the present study, the involvement of NF-κB in the TNF-α–induced upregulation of MMP-12 was investigated. The increased mRNA and protein expressions of MMP-12 by TNF-α was inhibited by the treatment with BMS-345541, which is reported to be a selective inhibitor of IκB kinase and block the NF-κB–dependent transcription (25). Moreover, the upregulated MMP-12 mRNA expression induced by TNF-α was also attenuated by the depletion of p65 (Fig. 3). These findings indicate that in addition to the involvement of JNK / AP-1 pathway described above, the activation of NF-κB is also required for the upregulation of MMP-12 induced by TNF-α in A549 cells.

Currently, both the inhibition of NF-κB and JNK by the respective chemical inhibitors and siRNAs completely inhibited the upregulation of MMP-12 induced by TNF-α (Figs. 2 and 3). Although its mechanism is unclear now, crosstalk between the NF-κB and JNK pathways may be involved and further studies are required to make this clear.

In conclusion, activations of the JNK / AP-1 and NF-κB pathways are both required for the upregulation of MMP-12 induced by TNF-α in A549 cells. The mechanism by which TNF-α induced the upregulation of MMP-12 might be an important link in the pathogenesis of airway inflammatory disease.

References

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