IL-1β promotes A549 cell migration via MAPKs/AP-1- and NF-κB-dependent matrix metalloproteinase-9 expression

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A B S T R A C T

Matrix metalloproteinases (MMPs), in particular MMP-9, is induced by cytokines including IL-1β and contributes to airway injury and remodeling. However, the mechanisms underlying IL-1β-induced MMP-9 expression and cell migration in human A549 cells remain unclear. Here, we report that the IL-1β-induced MMP-9 gene expression was mediated through the activation of p42/p44 MAPK, p38 MAPK, and JNK1/2 in A549 cells, determined by zymographic, RT-PCR, and Western blotting. The involvement of MAPKs in the IL-1β-induced responses was further ensured by transfection with siRNA of MEK1, p42, p38, or JNK2. Moreover, the IL-1β-induced MMP-9 gene expression was also mediated through the translocation of NF-κB (p65) into the nucleus and the degradation of IκBα. In addition, the IL-1β-induced c-Jun phosphorylation was reduced by pretreatment with U0126 or SP600125. IL-1β stimulated the transcriptional activity of wild-type MMP-9 promoter in A549 cells, which was inhibited by U0126, SB203580, SP600125, and helenalin. In contrast, IL-1β had no effect on the cells transfected with a NF-κB-mutated MMP-9 promoter construct, suggesting that NF-κB is required for this response. Finally, the IL-1β-induced MMP-9 expression led to cell migration which was attenuated by pretreatment with U0126, SB203580, SP600125, helenalin, or MMP-2/9 inhibitor. These results suggested that in A549 cells, the activation of p42/p44 MAPK, p38 MAPK, JNK1/2, NF-κB, and AP-1 are essential for the IL-1β-induced MMP-9 gene expression and cell migration.

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1. Introduction

Airway remodeling and inflammation are key features of persistent asthma and characterized by the deposition of extracellular matrix (ECM) proteins in the airways [1]. Matrix metalloproteinases (MMPs) are a family of ECM-degrading enzymes [2] and play important roles in inflammation, tissue remodeling, angiogenesis, wound healing, tumor invasion, and metastatic progression [3]. In particular, MMP-9 is up-regulated by cytokines in a variety of IL-1β-induced MMP-9 gene expression was also mediated lung inflammatory diseases, including asthma, chronic obstructive pulmonary disease, and tuberculosis [4–6]. Raised levels of MMP-9 have been detected in bronchoalveolar lavage fluid, blood, and sputum from individuals with allergic asthma [7]. The up-regulation of MMP-9 gene has been shown to be associated with bronchial hyperresponsiveness [8]. The other gelatinase is MMP-2 that is constitutively expressed by several cell types and not induced by cytokines or growth factors [9,10]. Although MMP-2 and MMP-9 have similar substrate specificities [11], there is difference in the regulation of their expression. These studies have demonstrated that the expression of MMP-9 may be regulated during airway injury and remodeling. Airway epithelium plays an important role in airway injury through the production of a variety of lipid mediators, cytokines, chemokines, and ECM components. Airway epithelial cells are also an important source of MMP-9. The expression of MMPs has been shown to be regulated by several extracellular stimuli, including proinflammatory cytokines, such as IL-1β and TNF-α in various cell types [12–15]. IL-1β is also elevated in the lungs of asthmatic patients and plays a key role in airway inflammation [16]. Therefore, the expression of MMP-9 induced by IL-1β may be integrated to the signaling networks that augment airway remodeling through degradation of ECM. Moreover, the expression of MMP-9 appears to be highly regulated by mitogen-activated protein kinases (MAPKs), NF-κB, and AP-1 in various cell types [13,15,17]. Although cytokines, such as IL-1β are reported to activate all of MAPKs [18,19], including p42/p44 MAPK [20], and JNK1/2 [21], the mechanisms underlying IL-1β-induced MMP-9 gene expression in A549 cells were remain unclear. Therefore, in A549 cells, we investigated whether activation of these MAPKs are required for IL-1β-induced MMP-9 expression. In addition, it is of
interest that many of the genes regulated by these MAPK pathways are
dependent on NF-κB for transcription [22] and lead to MMP-9 gene
expression at the transcriptional level [13]. Although many cells from
the lung tissues can release MMP-9 [23], the intracellular signaling
mechanisms underlying MMP-9 expression induced by IL-1β in A549
cells are not completely characterized.

In this study, the experiments were undertaken to investigate the
roles of p42/p44 MAPK, p38 MAPK, JNK1/2, NF-κB, and AP-1 in IL-1β-
induced MMP-9 gene expression and cell migration in A549 cells. We
found that the activation of these MAPKs, NF-κB, and AP-1 are essential
for the maximal induction of MMP-9 gene expression by these cells.
Moreover, the activation of MMP-9 is essential for the A549 cell migra-
tion. These results provide new insights into the mechanisms of IL-1β-
action that the MAPKs, NF-κB, and AP-1 may be critical pathways
associated with the MMP-9 expression and then migration in A549 cells.

2. Methods

2.1. Materials

DMEM/F-12 medium, FBS, and TRIzol reagent were purchased from
Invitrogen (Carlsbad, CA, USA). Anti-MMP-9 monoclonal antibody was
purchased from NeoMarkers (Fremont, CA, USA). MMP-2/MMP-9
inhibitor II was from Calbiochem (San Diego, CA, USA). Anti-phospho-
p42/p44 MAPK, anti-phospho-p38 MAPK, anti-phospho-JNK1/2, anti-
phospho-k-Box, and anti-phospho-c-Jun antibodies were from Cell
Signaling (Beverly, MA, USA). Anti-k-Box and anti-NF-κB (p65) antibodies
were from Santa Cruz (Santa Cruz, CA, USA), U0126, SB203580,
SP600125, and helenalin were from Biomol (Plymouth Meeting, PA, USA).

2.2. Cell culture

A549 cells (human alveolar epithelial cell carcinoma) and human
pulmonary alveolar epithelial cells (HPAEPic) (primary cultured human
pulmonary alveolar epithelial cells) were purchased from the American
Type Culture Collection (Manassas, VA) and ScienCell[tm] (Cat. No. 3200),
respectively. The HPAEPics comprised of alveolar epithelial cells more
than 99% of the internal surface area of the lung. A549 cells were cultured
in DMEM/F-12 supplemented with 10% FBS and antibiotics at 37 °C in a
humidified 5% CO2 atmosphere. Human pulmonary alveolar epithelial
cells were cultured in the basal medium supplemented with growth
factors according to the instruction of the manufacturer. The cells were
plated onto 12-well culture plates and 10-cm culture dishes for the
measurement of protein expression and mRNA accumulation, respec-
tively. Culture medium was changed after 24 h, and then every 3 days.

2.3. MMP-9 gelatin zymogram

A549 cells were plated onto 12-well culture plates and made
quiescent at confluence by incubation in serum-free DMEM/F-12 for
24 h. Growth-arrested cells were incubated with IL-1β at 37 °C for the
indicated times. When inhibitors were used, they were added 1 h
prior to the application of IL-1β. After treatment, the culture medium
was collected and centrifuged at 10,000 x g for 5 min at 4 °C to remove
the cell debris. The MMP-9 expression was analyzed as previously
described [24]. Moreover, we have performed the XTT assay to
examine the cell survival after treatment of these inhibitors for 48 h.
These data showed the A549 cell survival rate was not changed by the
pretreatment with these inhibitors for 48 h (data not shown).

2.4. Western blot analysis

Growth-arrested A549 cells were incubated with IL-1β at 37 °C for the
indicated times. The cells were washed with ice-cold PBS, scraped,
collected, and centrifuged at 45,000 x g for 1 h at 4 °C to yield the
whole cell extract, as previously described [25]. Samples were
denatured, subjected to SDS-PAGE, and then blotted using an anti-
phospho-p42/p44 MAPK, anti-phospho-p38 MAPK, anti-phospho-
JNK1/2, or anti-GAPDH antibody. The immunoreactive bands detected
by ECL reagents were developed by Hyperfilm-ECL.

2.5. Transient transfection with siRNAs

SMARTpool RNA duplexes corresponding to human MEK1 (NM-
002755), p42 (NM_138895), p38 (NM_139013), JNK2 (NM_002752), and
scrambled #2 siRNA were from Dharmacon Research Inc (Lafayette, CO,
USA). Transient transfection of siRNAs was carried out using Metafectene
transfection reagent. siRNA (100 nM) was formulated with Metafectene
transfection reagent according to the manufacturer’s instruction.

2.6. RT-PCR analysis

Total RNA was isolated from A549 cells treated with IL-1β for the
indicated times in 10-cm culture dishes with Trizol according to
the protocol of the manufacturer as previously described [24]. MMP-9
and β-actin sequences were amplified for 35 and 30 cycles (468 and
636 bp for MMP-9 and β-actin, respectively). Amplification of β-actin,
a relatively invariant internal reference RNA, was performed in parallel,
and cDNA amounts were standardized to equivalent β-actin mRNA levels.

2.7. Western blot analysis of MMP-9

Conditioned media derived from untreated or IL-1β-treated A549 cells
were concentrated using trichloroacetic acid precipitation. After determi-
nation of protein contents, the samples were subjected to SDS-PAGE, and
then blotted with anti-MMP-9 antibody as previously described [25].

2.8. Isolation of cell fraction

Cells were harvested, sonicated for 5 s at output 4 with a sonicator
(Ultrasonics Inc, NY, USA), and centrifuged at 8000 rpm for 15 min at 4 °C.
The pellet was collected as the nuclear fraction. The supernatant
was centrifuged at 14,000 rpm for 60 min at 4 °C to yield the pellet
(membrane fraction) and the supernatant (cytosolic fraction).

2.9. Immunofluorescence staining

A549 cells were plated on six-well culture plates with coverslips. Cells
were treated with IL-1β- and washed twice with ice-cold PBS. Immuno-
fluorescence staining was performed as previously described [26].

2.10. Measurement of MMP-9 luciferase activity

For construction of the MMP-9-luc plasmid, human MMP-9 promoter,
a region spanning –720 to –11 bp was cloned into pGL3-basic vector
(Promega, Madison, WI, USA). In addition, the introduction of a double-
point mutation into the NF-κB-site to generate pGL3-MMP-9mNF-κB was
performed as previously described [24]. The mutant was generated using
the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).
MMP-9-luc activity was determined as previously described [24] using a
luciferase assay system (Promega, Madison, WI, USA). Firefly luciferase
activities were standardized for β-gal activity.

2.11. Migration assay

A549 cells were cultured to confluence in six-well plates and
starved with serum-free DMEM/F-12 medium for 24 h. The mono-
layer cells were scratched manually with a blue tip, and the detached
cells were removed with PBS. Serum-free DMEM/F-12 medium with
or without IL-1β was added to each dish as indicated after
pretreatment of MMP2/9 inhibitor, helenalin, and MAPKs inhibitors
for 1 h (containing 10 μM hydroxyurea in the whole course). Images
were observed and taken at 48 h with a digital camera and a
microscope (Olympus, Japan) as previously described [27].

2.12. Chromatin immunoprecipitation assay

To detect the association of nuclear proteins with human MMP-9
promoter, chromatin immunoprecipitation (ChIP) analysis was con-
ducted as previously described [28]. DNA immunoprecipitated by anti-c-
Jun antibody was purified. The DNA pellet was re-suspended in H2O and
subjected to PCR amplification with the forward primer 5’-
TGCTCTTTACTGCCTGCTCA-3’ and the reverse primer 5’-ACTC-
CAGGCTGTCCCTCCTCTCCTT-3’, which were specifically designed from
the MMP-9 promoter region. PCR products were analyzed on ethidium
bromide-stained agarose gels (1%).

2.13. Analysis of data

Data were expressed as the mean ± SEM and analyzed with a one-
way ANOVA to make comparisons with Bonferroni’s test at a P<0.05
level of significance.

3. Results

3.1. IL-1β induces MMP-9 expression

IL-1β has been shown to induce MMP-9 expression in various cell
types [14,29]. However, the mechanisms underlying IL-1β-induced
MMP-9 expression were not completely understood in A549 cells. To
determine the effect of IL-1β on MMP-9 expression, cells were treated
with IL-1β (15 ng/ml) for the indicated times. As shown in Fig. 1A, IL-1β-
induced MMP-9 expression in a time-dependent manner determined by
gelatin zymographic analysis. There was a significant increase in this
response within 16 h, and a maximal response was achieved within 48 h
during the period of these observations. The obvious up-regulation
occurred in the 92 kDa pro-form zymogen of MMP-9. A slight increase in
the 84 kDa active form of MMP-9 was also observed. There was no
significant change in proMMP-2 (72 kDa) level. To further confirm
whether IL-1β increased MMP-9 protein up-regulation, the condition
media were collected, precipitated with trichloroacetic acid, and then
analyzed by Western blot analysis. The results showed that IL-1β-
induced MMP-9 protein expression in a time-dependent manner
(Fig. 1B).

To examine whether IL-1β-induced MMP-9 mRNA expression, as
shown in Fig. 1C, IL-1β-induced MMP-9 mRNA expression in a time-
dependent manner in A549 cells. A maximal response was obtained
within 4–8 h and sustained over 12 h. We also found that IL-1β-
induced MMP-9 expression was significantly attenuated by pretreat-
ment with the transcription and translation inhibitors, actinomycin D
and cycloheximide, respectively (data not shown). These results
demonstrated that IL-1β-enhanced MMP-9 expression at transcription
and translation levels in A549 cells.

In addition to A549 cell lines, we also investigated whether IL-1β-
induced MMP-9 expression occurred in primary human normal pulmon-
ary alveolar epithelial cells (HPAEpiC). IL-1β (15 ng/ml) induced MMP-9
expression in a time-dependent manner determined by zymographic
assay (Fig. 1D). Moreover, the data revealed by RT-PCR showed that IL-1β-
also induced MMP-9 mRNA expression in HPAEpiCs (Fig. 1E). These results
indicated that IL-1β-induced responses in A549 cells were similar to those
of HPAEpiC. Thus, the following experiments were performed using A549
cells which can be applied as a model throughout this study.

3.2. IL-1β induces MMP-9 expression through MEK1/2-p42/p44 MAPK cascade

Some studies have indicated that IL-1β induces expression of many
genesis via the activation of diverse MAPKs [29,30]. We further
investigated whether p42/p44 MAPK was involved in IL-1β-induced
MMP-9 expression in A549 cells. The activation of p42/p44 MAPK is
mediated through an upstream molecule, MEK1/2. Therefore, a

Fig. 1. IL-1β induces MMP-9 expression in A549 cells. (A) A549 cells were treated with 15 ng/
ml IL-1β for the indicated times. The conditioned media were collected and analyzed by
gelatin zymography. (B) Cells were treated with 15 ng/ml IL-1β for various times. Conditioned
media were collected and concentrated by the addition of trichloroacetic acid. ProMMP-9
protein expression was analyzed by Western blot analysis. (C) Cells were incubated with
15 ng/ml IL-1β for the indicated times. The RNA samples were analyzed by RT-PCR for the
levels of MMP-9 mRNA. (D) HPAEpiCs were treated with 15 ng/ml IL-1β for 6 h. The RNA samples were analyzed by RT-PCR for the levels of MMP-9 mRNA. Data are expressed as mean±S.E.M. of at least three
independent experiments. *P<0.05; #P<0.01 as compared with the cells exposed to vehicle.
MEK1/2 inhibitor, U0126 [31] was used for this purpose. Pretreatment with U0126 significantly attenuated IL-1β-induced MMP-9 expression in a concentration-dependent manner (Fig. 2A).

To determine whether p42/p44 MAPK was involved in the MMP-9 expression, the activation of p42/p44 MAPK was assayed by Western blot analysis. As shown in Fig. 2B, IL-1β stimulated a time-dependent phosphorylation of p42/p44 MAPK in A549 cells. A maximal response was obtained within 10 min, and then slightly declined within 30 min. Pretreatment with U0126 blocked IL-1β-stimulated p42/p44 MAPK phosphorylation in a concentration-dependent manner (Fig. 2C).

**Fig. 2.** IL-1β induces MMP-9 expression via p42/p44 MAPK in A549 cells. (A) Cells were treated with 15 ng/ml IL-1β for 48 h in the absence or presence of U0126. The conditioned media were collected and analyzed by gelatin zymography. (B) Cells were incubated with 15 ng/ml IL-1β for the indicated times. The cell lysates were subjected to Western blot analysis using an anti-phospho-p42/p44 MAPK Ab. (C) Cells were pretreated with various concentrations of U0126 for 1 h, and then stimulated with IL-1β for 10 min. The cell lysates were subjected to Western blot analysis using an anti-phospho-p42/p44 MAPK Ab. (D) Cells were transfected with p42 siRNA, and then treated with IL-1β for the indicated times. The cell lysates were subjected to Western blot analysis using an anti-phospho-p42/p44 MAPK Ab. (E) Cells were transfected with MEK1 siRNA or p42 siRNA, and then incubated with IL-1β for 48 h. The conditioned media were collected and analyzed by gelatin zymography. The cell lysates were subjected to Western blot analysis using an anti-MEK1 or anti-p42 Ab. Data are expressed as mean ± S.E.M. of at least three independent experiments. *P<0.05; **P<0.01 as compared with the cells exposed to IL-1β alone.
To further ensure activation of p42/p44 MAPK required for IL-1β-induced MMP-9 expression, A549 cells were transfected with siRNAs of MEK1 and p42, and then treated with IL-1β for the indicated times. As shown in Fig. 2D, transfection with p42 siRNA significantly reduced p42/p44 MAPK phosphorylation stimulated by IL-1β. Moreover, transfection with siRNAs of MEK1 and p42 significantly knocked down the protein levels of MEK1 and p42 and attenuated IL-1β-induced MMP-9 expression (Fig. 2E). These data indicated that MEK1/2-p42/p44 MAPK cascade was involved in IL-1β-induced MMP-9 expression in A549 cells.

3.3. IL-1β induces MMP-9 expression via p38 MAPK

To determine whether p38 MAPK was also involved in the IL-1β-induced MMP-9 expression in A549 cells, a p38 MAPK inhibitor, SB203580 [32] was used. As shown in Fig. 3A, pretreatment with SB203580 significantly attenuated IL-1β-induced MMP-9 expression and activity. To ascertain that IL-1β promoted p38 MAPK phosphorylation, activation of this kinase was assayed. As shown in Fig. 3B, IL-1β stimulated a time-dependent phosphorylation of p38 MAPK with a maximal response within 10 min and sustained over 30 min in A549 cells.
cells. Pretreatment with SB203580 attenuated IL-1β-stimulated p38 MAPK phosphorylation in a concentration-dependent manner (Fig. 3C).

Furthermore, to ensure whether p38 MAPK was required for IL-1β-induced MMP-9 expression, A549 cells were transfected with p38 MAPK siRNA, and then treated with IL-1β for the indicated times. As shown in Fig. 3D, transfection with p38 siRNA significantly reduced p38 MAPK phosphorylation induced by IL-1β. Moreover, transfection with p38 siRNA almost completely knocked down the protein level of p38 MAPK and attenuated the IL-1β-induced MMP-9 expression (Fig. 3E). These results suggested that p38 MAPK activation was required for the IL-1β-induced MMP-9 expression in A549 cells.

3.4. JNK1/2 plays a crucial role in IL-1β-induced MMP-9 expression

To determine whether JNK1/2 was also involved in IL-1β-induced MMP-9 expression in A549 cells, a JNK inhibitor, SP600125 [33] was used. As shown in Fig. 4A, the pretreatment of A549 cells with SP600125 concentration-dependently blocked IL-1β-induced MMP-9 expression.
and activity. To further examine whether IL-1β-stimulated activation of JNK1/2, activation of JNK1/2 was assayed. As shown in Fig. 4B, IL-1β-stimulated phosphorylation of JNK1/2 in a time-dependent manner, and a maximal response within 30 min in A549 cells. This response was concentration-dependently inhibited by the pretreatment with SP600125 (Fig. 4C). To further determine whether the activation of JNK1/2 was essential for the IL-1β-induced expression of MMP-9, A549 cells were transfected with JNK2 siRNA, and then treated with IL-1β for the indicated times. As shown in Fig. 4D, transfection with JNK2 siRNA significantly blocked the IL-1β-stimulated JNK1/2 phosphorylation. Transfection with JNK2 siRNA knocked down the protein expression of JNK2 (Fig. 4E) and attenuated IL-1β-induced MMP-9 expression (Fig. 4E). These results demonstrated that the activation of JNK1/2 was involved in the IL-1β-induced MMP-9 expression in A549 cells.

3.5. Involvement of NF-κB in IL-1β-induced MMP-9 expression

Inflammatory responses following stimulation with cytokines, such as IL-1β, are highly dependent on the activation of the transcription factor NF-κB. Moreover, NF-κB is one of the major mediators of the intracellular functions of IL-1β. In addition, IL-1β has been shown to induce MMP-9 gene expression through NF-κB in various cell types [13,29]. Therefore, the involvement of NF-κB activation in MMP-9 expression following stimulation with IL-1β was investigated using a pharmacological approach.

![Fig. 5. NF-κB is essential for IL-1β-stimulated MMP-9 expression in A549 cells.](image)

(A) Cells were treated with 15 ng/ml IL-1β for 48 h in the absence or presence of helenalin. The conditioned media were collected and analyzed by gelatin zymography. (B) Cells were treated with 15 ng/ml IL-1β for the indicated times. The cytosolic and nuclear fractions were prepared and subjected to Western blot analysis using an anti-p65 or anti-κBα Ab. (C) Nuclear translocation of NF-κB (p65) determined by immunofluorescence staining. A549 cells were pretreated with 1 µM U0126, 30 µM SB203580, 10 µM SP600125, or 10 µM helenalin for 1 h, and then stimulated with 15 ng/ml IL-1β for 10 min. Cells were fixed, and then labeled with anti-p65 antibody and FITC-conjugated secondary antibody. Individual cells were imaged as described in Methods. Data are expressed as mean ± S.E.M. of at least three independent experiments. *P<0.05, **P<0.01 as compared with the cells exposed to IL-1β alone.
inhibitor of NF-κB, helenalin, a specific sesquiterpene lactone compound, is known to inhibit NF-κB [34]. As shown in Fig. 5A, pretreatment with helenalin caused a concentration-dependent attenuation of the IL-1β-induced MMP-9 expression and activity.

Cells activated by cytokines lead to the degradation of IκBα, accompanied by NF-κB translocation into the nucleus. To determine whether the IL-1β-induced IκBα degradation and NF-κB translocation, cells were stimulated with 15 ng/ml IL-1β for the indicated times. The cytosolic and nuclear fractions were used to determine the degradation of the IκBα and the translocation of NF-κB, respectively. The results showed that IL-1β rapidly stimulated IκBα degradation and NF-κB translocation (Fig. 5B), a maximal response was obtained within 3–30 min and gradually restored to the basal level.

MAPKs activation and NF-κB translocation were necessary for the MMP-9 expression induced by IL-1β in A549 cells, it would be important to differentiate whether the phosphorylation of these MAPKs was associated with the NF-κB activation. To examine this possibility, the translocation of NF-κB was assessed following IL-1β stimulation in the presence of the inhibitors of MEK1/2, p38 MAPK, and JNK1/2. IL-1β-stimulated translocation of NF-κB was significantly inhibited by pretreatment with helenalin, but not by U0126, SB203580, and SP600125 (Fig. 5C), suggesting that IL-1β-stimulated NF-κB translocation was independent of MAPKs activation. These results demonstrated that IL-1β-stimulated NF-κB translocation was essential for MMP-9 up-regulation and independent of p42/p44 MAPK, p38 MAPK, and JNK1/2 activation in A549 cells.

3.6. IL-1β stimulates MMP-9 promoter transcriptional activity via MAPKs and NF-κB

This regulation of the MMP-9 gene transcription through MAPKs and NF-κB pathways induced by IL-1β was further confirmed by gene luciferase activity assay. MMP-9 luciferase reporter gene was transfected into A549 cells, and then stimulated with IL-1β (15 ng/ml). Data in Fig. 6A showed that IL-1β significantly stimulated MMP-9-luciferase activity within 4 h, peaked at 16 h, and sustained for over 48 h. Moreover, IL-1β-induced MMP-9 promoter activity was inhibited by selective inhibitors, including U0126, SB203580, SP600125, and helenalin (Fig. 6B). To further confirm the functional role of NF-κB in IL-1β-mediated MMP-9 promoter activity, point-mutated MMP-9 promoter construct was used to test this induction by IL-1β. As shown in Fig. 6C, IL-1β-stimulated MMP-9 luciferase activity was totally lost in A549 cells transfected with mutated NF-κB promoter, indicating that the NF-κB binding site is required for the MMP-9 promoter activation induced by IL-1β. These results indicated the involvement of p42/p44 MAPK, p38 MAPK, JNK1/2, and NF-κB pathways in IL-1β-induced MMP-9 gene transcription.

3.7. MAPKs and NF-κB are required for IL-1β-induced MMP-9 mRNA expression

We also investigated whether these MAPKs and NF-κB signaling molecules were involved in the IL-1β-induced MMP-9 mRNA expression in A549 cells. As shown in Fig. 7, pretreatment of A549 cells with the

![Fig. 6. Involvement of MAPKs and NF-κB in IL-1β-induced MMP-9 promoter activity. (A) Cells were transiently transfected with MMP-9-luc reporter gene, and then challenged with IL-1β for the indicated times. The MMP-9 promoter activity was determined in the cell lysates. (B) Cells were transiently transfected with MMP-9-luc reporter gene, pretreated with U0126 (1 µM), SB203580 (30 µM), SP600125 (10 µM), or helenalin (10 µM) for 1 h, and then stimulated with 15 ng/ml IL-1β for 16 h. The MMP-9 promoter activity was determined in the cell lysates. (C) A549 cells were transfected with either pGL3-MMP-9-WT or pGL3-MMP-9-mNF-κB for 24 h, and then treated with IL-1β (15 ng/ml) for 16 h. The MMP-9 promoter activity was determined in the cell lysates. Data are expressed as mean ± S.E.M. of at least three independent experiments. *P<0.05; **P<0.01 as compared with the cells exposed to vehicle (A) or IL-1β alone (B,C).]
the activation of p42/p44 MAPK, p38 MAPK, JNK1/2, and NF-κB in A549 cells, IL-1β-induced MMP-9 mRNA accumulation. These results indicated that in immunoprecipitates of c-Jun-associated DNA from cells treated with IL-1β, samples were analyzed by RT-PCR, using the primers specific for MMP-9 and β-actin. Data are expressed as mean ± S.E.M. of at least three independent experiments.

Inhibitors of MEK1/2 (U0126), p38 MAPK (SB203580), JNK1/2 (SP600125), and NF-κB (helenalin) significantly blocked the IL-1β-induced MMP-9 mRNA accumulation. These results demonstrated that the induction of MMP-9 expression via MAPK- and NF-κB-dependent manners is essential for the IL-1β-induced A549 cell migration.

3.8. Nuclear localization of AP-1 bound to MMP-9 promoter region

To differentiate the relationship between AP-1 and MMP-9 protein expression, the abilities of AP-1 to regulate MMP-9 promoter activities were investigated. Firstly, IL-1β-induced phosphorylation of c-Jun was inhibited by pretreatment with U0126 and SP600125 (Fig. 8A), but not SB203580 (data not shown). Furthermore, to detect whether nuclear localized c-Jun bound to MMP-9 promoter region in response to IL-1β, CHIP assay was carried out with an anti-c-Jun antibody. The results showed that nuclear localization of AP-1 bound to MMP-9 promoter region in a time-dependent manner (Fig. 8B). Pretreatment with U0126 or SP600125 attenuated IL-1β-induced c-Jun binding to MMP-9 promoter region (Fig. 8C). These results suggested that IL-1β induces AP-1 nuclear translocation and binding to MMP-9 promoter region through p42/p44 MAPK and JNK1/2.

3.9. IL-1β induces cell migration in a MMP-9-dependent manner

To examine the functional response of the MMP-9 induced by IL-1β, A549 cell migration was evaluated. As shown in Fig. 9A, IL-1β-induced cell migration was significantly inhibited by pretreatment with an MMP-2/9 inhibitor, suggesting that MMP-9 participated in the induction of cell migration by IL-1β in A549 cells. An analysis of the conditioned media showed that MMP-9 activity was attenuated by pretreatment with this inhibitor (Fig. 9B). To rule out the interference with cell proliferation, cells were treated with a proliferation inhibitor hydroxyurea (10 µM) during exposure to IL-1β for 48 h. Moreover, we also found that IL-1β-induced A549 cell migration was significantly blocked by pretreatment with U0126, SB203580, SP600125, and helenalin (Fig. 9A), indicating that MAPKs and NF-κB indeed participate in IL-1β-induced cell migration. These results demonstrated that the induction of MMP-9 expression via MAPK- and NF-κB-dependent manners is essential for the IL-1β-induced A549 cell migration.

4. Discussion

MMPs have been demonstrated to be the major components of the enzyme cascade responsible for the degradation of the ECM and basement membrane proteins. Proteolysis of these proteins participates in the processes of cell migration, proliferation, differentiation, and tissue remodeling related to airway injury [3]. Lung epithelial cells are one of the important sources of MMPs, such as MMP-2 and MMP-9 appear to be differentially expressed during airway remodeling [35]. Despite an obviously important role of cytokines in airway injury and diseases [36], the processes by which IL-1β are implicated in lung epithelial functions are not completely understood. In this study, we investigated the intracellular signaling pathways by which IL-1β induced MMP-9 expression associated with the cell migration in a lung epithelial cell line A549. Firstly, we showed that IL-1β induced MMP-9 up-regulation, but there was no effect on the MMP-2 expression in A549 cells. The IL-1β-induced MMP-9 expression was significantly attenuated by pretreatment with the inhibitors of MEK1/2 (U0126), p38 MAPK (SB203580), JNK1/2 (SP600125), and NF-κB (helenalin), or transfection with their respective siRNAs. Moreover, the activation of NF-κB induced by IL-1β was independent of these MAPK pathways. On the other hand, our results suggested that IL-1β induces AP-1 nuclear translocation and binding to MMP-9 promoter region through p42/p44 MAPK and JNK1/2. Thus, we demonstrated that MAPKs, NF-κB, and AP-1 participate in IL-1β-induced MMP-9 up-regulation leading to the cell migration in A549 cells.

p38 MAPK and JNK1/2 have been shown to be activated by various stimuli, such as heat shock, osmotic shock, UV light, and DNA damaging agents, while activation of p42/p44 MAPK plays an important role in mediating cell proliferation in response to growth factors and mitogens [37,38]. IL-1β has been shown to activate all of these MAPK pathways in many cell types [18,19,39]. MMP-9 gene
expression has been shown to be regulated by diverse MAPKs, such as p42/p44 MAPK, p38 MAPK, or JNK1/2 in different cell types [29,40,41]. Since IL-1β plays an important role in different cellular responses, the phosphorylation of these MAPKs may not directly imply an involvement of these MAPKs in the IL-1β-induced MMP-9 expression. Therefore, the roles of MAPKs involved in the IL-1β-induced MMP-9 expression were investigated by using the selective pharmacological inhibitors of these MAPKs and the transfection with siRNAs. Our results showed that pretreatment of these MAPKs inhibitors, such as U0126, SB203580, and SP600125 significantly blocked the IL-1β-induced MAPKs phosphorylation and MMP-9 expression. Furthermore, transfection with siRNAs of these MAPKs also significantly reduced these MAPKs phosphorylation and MMP-9 expression induced by IL-1β, suggesting that the activation of these MAPKs cascades, including p42/p44 MAPK, p38 MAPK, and JNK1/2 are required for the IL-1β-induced MMP-9 up-regulation in A549 cells.

Involvement of p42/p44 MAPK in these responses, is consistent with the previous reports indicating that p42/p44 MAPK plays a pivotal role in the MMP-9 expression induced by IL-1β in astrocytes [29] and by MTb in pulmonary epithelial cells [5]. Next, our results demonstrated that IL-1β-induced MMP-9 expression was mediated through the activation of p38 MAPK pathway, consistent with a study showing that p38 MAPK is crucial for the IL-1β-induced MMP-9 expression in rat mesangial cells [13]. In addition, we also found that involvement of JNK1/2 in IL-1β-induced MMP-9 expression revealed by the pretreatment with SP600125 or transfection with JNK2 siRNA, consistent with the report indicating that JNK1/2 has been shown to involve in MMP-9 expression in several cell types [42]. These results demonstrated that the activation of p42/p44 MAPK, p38 MAPK, or JNK1/2 is involved in the IL-1β-induced MMP-9 expression in A549 cells. Recently, several lines of evidence have implicated that specific MAPKs control the activation of AP-1 family (c-Fos and c-Jun) protooncogenes or other transcription factors [43,44], a converging point that regulates the expression of genes involved in cell proliferation, differentiation, transformation, inflammation, pulmonary defense, and cell migration [45]. Moreover, we also found that pretreatment with U0126 and SP600125 inhibited IL-1β-induced c-Jun bound to VCAM-1 promoter region. These results suggested that the IL-1β-induced AP-1 nuclear translocation and binding to MMP-9 promoter region through p42/p44 MAPK and JNK1/2.

Various responses following exposure to cytokines are highly dependent on NF-κB activation and lead to expression of several mediator genes [19,30]. The sequestration of NF-κB by IκBα in the cytoplasm and IκBα phosphorylation leading to proteasomal degradation of IκBα, resulting in the activation and the translocation of NF-κB into nucleus is essential for the expression of several genes, such as MMP-9 in brain astrocytes [29] and in human TSMCs [46]. In this study, the involvement of NF-κB in the IL-1β-induced MMP-9 expression was revealed by an NF-κB inhibitor, helenanin. These data showed that IL-1β...
induced MMP-9 expression via IκB degradation, NF-κB translocation and the activation in A549 cells, consistent with recent reports showing that IL-1β-induced MMP-9 expression is mediated through a NF-κB-dependent pathway in astrocytes [29] and in human TSMCs [46]. Interestingly, the activation of p42/p44 MAPK, p38 MAPK, and JNK1/2 as well as the NF-κB were required for the IL-1β-induced MMP-9 expression in A549 cells. It has been shown that MEKK1 induces activation of IKKα and IKKβ leading to the NF-κB activation [19,22]. Thus, the MAPKs activation might be required for the NF-κB activation stimulated by IL-1β. The results of the NF-κB immunofluorescence staining demonstrated that IL-1β-stimulated NF-κB translocation is independent of p42/p44 MAPK, p38 MAPK, and JNK1/2, indicating that in A549 cells, a simultaneous activation of MAPKs and NF-κB is involved in the IL-1β-induced MMP-9 expression, consistent with our previous studies showing that IL-1β-induced the MMP-9 expression is mediated through these three MAPKs and NF-κB pathways in astrocytes [29] and in human TSMCs [46]. Furthermore, the MMP-9 promoter reporter assay demonstrated that the activation of MAPKs and NF-κB signaling molecules by IL-1β are important for the initiation of the MMP-9 promoter. In addition, NF-κB binding sites have been identified in the MMP-9 promoter region [47]. Therefore, we constructed an MMP-9 promoter mutant containing a NF-κB binding site point-mutation to further confirm that the NF-κB binding site within the MMP-9 promoter region is crucial for this response. The data showed that the IL-1β enhanced wild-type MMP-9 promoter activity which was almost totally lost in A549 cells transfected with NF-κB-mutated MMP-9 promoter, indicating that NF-κB plays an important role for the IL-1β-induced MMP-9 promoter transcriptional activity. In conclusion, IL-1β directly induced MMP-9 expression via the activation of these three MAPKs (p42/p44 MAPK, p38 MAPK, and JNK1/2), NF-κB, and AP-1 pathways, which resulted in the promotion of the cell migration in the A549 cells. Based on the observations from literatures and our findings, Fig. 10 depicts a model for the molecular mechanisms implicated in the IL-1β-induced proMMP-9 expression and cell migration in A549 cells. These findings concerning the IL-1β-induced proMMP-9 expression and cell migration in A549 imply that the IL-1β might play a critical role in the development of lung injury and remodeling. Pharmacological approaches suggest that targeting MMP-9 and their upstream signaling components should yield useful therapeutic targets for lung injury, tumor, and remodeling progress.

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