

Cellular signalling pathways of matrix metalloproteinase gene expression by *Pseudomonas aeruginosa*-infected human bronchial epithelial cells

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KEY MESSAGES

1. *Pseudomonas aeruginosa* infection is associated with increased production of matrix metalloproteinase-9 (MMP-9) from the airway epithelium; inappropriate expression and activation of MMP-9 may be associated with tissue injury and airway remodelling.
2. *P. aeruginosa* exposure induces the phosphorylation of ERK, JNK, and p38 MAPKs in human airway epithelial cell line (BEAS-2B). Inhibition of ERK or JNK activity blocks IL-8 and MMP-9 mRNA and protein expression in BEAS-2B cells infected with *P. aeruginosa*.
3. Induction of IL-8 and MMP-9 in human bronchial epithelial cells by *P. aeruginosa* infection is mediated via the NF- κ B-mediated pathway, possibly through upstream ERK and JNK MAPK pathways.
4. *P. aeruginosa* infection initiates an intense

inflammatory response, such as the release of IL-8, which progressively destroys pulmonary tissue via MMP-9 activation and is associated with high mortality.

5. Inhibition of ERK or JNK may be an effective therapeutic strategy against the early stages of pulmonary *P. aeruginosa* infection, via attenuation of the inflammatory cascade.

Hong Kong Med J 2014;20(Suppl 4):S14-7

RFCID project number: 03040832

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Introduction

Pseudomonas aeruginosa is a Gram-negative bacillus and an opportunistic pathogen that causes pneumonia in immunocompromised humans and severe pulmonary damage in non-cystic fibrosis bronchiectasis.¹ Because of the severity of *P. aeruginosa* infection, it is important to understand the mechanisms of the resulting epithelial injury and repair processes during conditions of airway inflammation. *P. aeruginosa* is a pathogen in chronic obstructive pulmonary disease associated with an intense airway inflammation and poor prognosis.^{2,3} The initial step of bacterial infection, crucial for the development of permanent colonisation at later stages, is the adherence of the bacteria to epithelial cells. The infection is associated with an excessive inflammatory response, characterised by the accumulation of large amounts of the polymorphonuclear leukocyte chemokine IL-8 and its toxic products in the airways.⁴

The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade the main protein components of extracellular matrices and may lead to continued airway obstruction when

uncontrolled.⁵ They are produced by structural cells such as fibroblasts and epithelial cells, and by many inflammatory cells including macrophages, eosinophils, neutrophils, and mast cells. They are secreted as latent proenzymes and are converted to the active form by proteolytic cleavage of an amino-terminal domain. The MMPs are important in tissue remodelling in the airways through their ability to affect the integrity of the basal lamina and the degree of infiltration by inflammatory cells. The balance between activated MMPs and the endogenous tissue inhibitor of metalloproteinases (TIMPs) determine overall MMP proteolytic activity, and thus the turnover of airway extracellular matrix. Several MMPs including MMP-9 play an important role in the pathogenesis of many airway diseases such as asthma and chronic obstructive pulmonary disease.^{6,7} An up-regulation of MMP-8 and -9 has been reported in human bronchiectatic airways in vivo.⁸ Furthermore, MMP-2 and -9 expression is increased in mouse mycoplasma-infected airways in vivo.⁹

Studies have shown that mitogen-activated protein kinases (MAPKs) are involved in the

regulation of MMPs by various cell types.^{10,11} The MAPK family includes three major members: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinase (JNK)/stress-activated protein kinases, and p38.¹² Although the role of MAPKs in the regulation of MMPs has been studied in many cell types, little is known about how MAPKs regulate the production of MMPs after bacterial infection in airway epithelial cells. We hypothesised that *P aeruginosa* infection induces MMP-9 in human bronchial epithelial cells via NF- κ B activation. As NF- κ B is a candidate for therapeutic intervention in airway inflammation, we also investigated the role of NF- κ B in the induction of MMP-9 expression after *P aeruginosa* infection.

We investigated the expression and activity of MMP-9 in human bronchial epithelial cells using RT-PCR and gelatin zymography. The role of specific MAPK pathways in the activation of NF- κ B-dependent transcription was addressed using NF- κ B-dependent transcriptional reporters (ie BEAS-2B 6 κ Btk reporter cell line) and specific MAPK inhibitors.

Methods

Cell culture and *Pseudomonas aeruginosa* infection

BEAS-2B cells were grown in Keratinocyte-SFM containing 5 ng/ml epidermal growth factor and 50 μ g/ml bovine pituitary extract, and then incubated at 37°C in a humidified incubator with 95% air and 5% CO₂. Passages 41-49 were used. At 90% confluence, the culture was changed to growth factor-free medium for 24 h prior to treatment.

Different *P aeruginosa* strains (one clinical isolate from a local patient, PAO1 as a laboratory strain [ATCC15692] and ATCC27853 as a reference strain) were used at 10⁷ to 10⁵ colony-forming units for various time periods.

Semi-quantitative RT-PCR

Total cDNA was prepared by first-strand cDNA synthesis from 1 μ g of total RNA isolated from cells according to standard protocols. For PCR quantification, appropriate primers for IL-8, MMPs (MMP-2, MMP-8, and MMP-9) and TIMPs (TIMP-1 and TIMP-2) were designed according to the published sequences. Following amplification, PCR products were run on an ethidium bromide-stained agarose gel. Bands were quantified by densitometric analysis using glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) as a housekeeping gene. Data were expressed as the ratio of gene of interest/GAPDH.

ELISA

IL-8 was measured in the supernatant using

commercially available ELISA kits (BD OptEIASet).

Gelatin zymography

Levels of gelatinolytic MMPs, secreted into the media, were separated by electrophoresis through a 10% polyacrylamide gel containing 0.1% gelatine. Positions of gelatinolytic activity were unstained on a darkly stained background. The clear bands on the zymograms were scanned and the signals quantified by densitometric scanning to determine the intensity of MMP-9 activity as arbitrary units.

Western blot analysis

After infection with *P aeruginosa*, protein was extracted from cells. Samples (20 μ g) were run on 10% on SDS-polyacrylamide gels with prestained molecular weight markers (Bio-Rad) and transferred to Hybond-ECL membranes using standard techniques. Membranes were blocked with 5% nonfat milk and probed with primary antibodies to phosphorylated or total ERK1/2, JNK1/2 and p38 (Cell Signaling Technology) at a dilution of 1:1000 and incubated overnight at 4°C. HRP-conjugated goat anti-rabbit or anti-mouse antibody was used as a secondary antibody. In all cases, proteins were visualised using enhanced chemiluminescence reagent (Amersham) according to the manufacturer's instructions.

Luciferase activity assay

The BEAS-2B 6 κ Btk reporter cell line was generated as described previously.¹³ Luciferase activity was determined using a commercially available luciferase reporter gene assay system (Promega) under luminescence (FLUOstar Plate Reader).

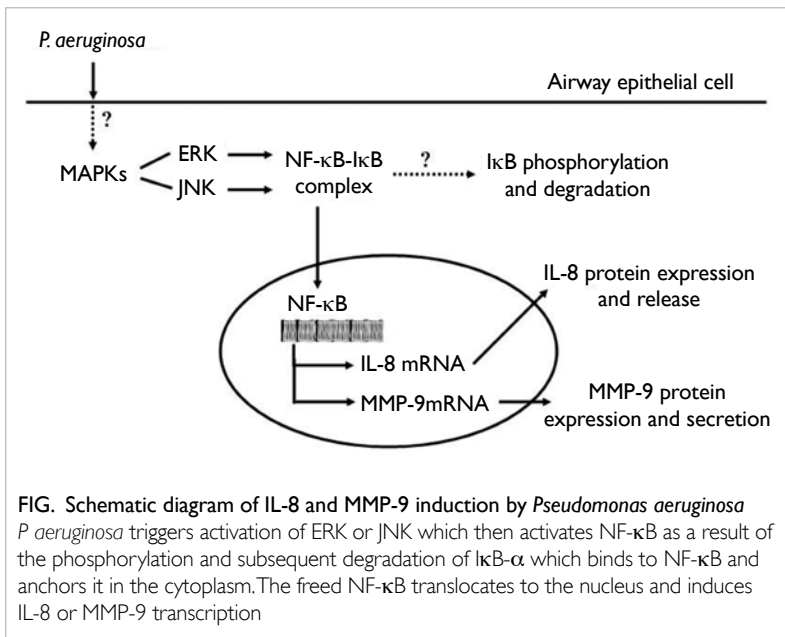
Statistical Analysis

Values were expressed as means \pm SEM. Data analysis was performed using Prism software (GraphPad Software). All experiments were carried out with materials collected from at least four to six separate cell cultures in duplicate or triplicate. Comparison of more than two groups was carried out with one-way analysis of variance, followed by Bonferroni's multiple comparison post hoc tests. A P value of <0.05 was considered statistically significant.

Results

P aeruginosa-induced IL-8 was released in a dose- and time-dependent manner. As the three different strains of *P aeruginosa* released comparable amount of IL-8, PAO1 was chosen for further study. Induction of IL-8 mRNA expression appeared as early as 1 h after exposure to *P aeruginosa* (preceding the release of IL-8) and continued to increase for up to 4 h.

Cells were found to express MMP-2 and MMP-9, which were secreted in their proforms



(proMMP; MMP-2 proform, 72 kD; MMP-9 proform, 92 kD). *P. aeruginosa* caused a dose-dependent increase in MMP-9 production, but the MMP-2 was not affected after 6h. Induction of MMP-9 mRNA expression was detectable at 1 h and reached plateau at 2 h after infection, indicating transcriptional regulation.

Although phosphorylation of the MAPKs ERK, JNK, and p38 at Thr²⁰²/Tyr²⁰⁴, Thr¹⁸³/Tyr¹⁸⁵, and Thr180/Tyr182 was detectable at 1 h and continued to increase up to 2 h after *P. aeruginosa* exposure. The MEK and JNK activity inhibitors, PD98059 and SP600125 respectively, blunted IL-8 release and MMP-9 activity in BEAS-2B cells treated with *P. aeruginosa* but not p38 MAPK inhibitor SB203580. The *P. aeruginosa*-induced IL-8 mRNA also diminished in the presence of PD98059 and SP600125 but not SB203580, suggesting that *P. aeruginosa*-induced ERK and JNK activity partially contributed to *P. aeruginosa*-induced IL-8 release and MMP-9 activity in BEAS-2B cells.

Treatment with pyrrolidine dithiocarbamate (a NF-κB inhibitor) before *P. aeruginosa* infection attenuated *P. aeruginosa*-induced IL-8 release and MMP-9 activity. *P. aeruginosa* (either PAO1 or a clinical isolate) alone caused a dose-dependent increase in NF-κB-dependent transcription, which was diminished at ≥10 μM for PD98059 and SP600125.

Discussion

The enzymes of MMP family are important in tissue development and regeneration. Inappropriate MMP regulation and activation during chronic

inflammation may lead to disturbances in the turnover and remodelling of pulmonary extracellular matrix, which could contribute to the structural remodelling that occurs after bacterial infection. In the present study, *P. aeruginosa* infection induced the expressions of IL-8 and MMP-9, the pivotal inflammatory mediators through a mechanism that involved ERK1, JNK, and NF-κB activation in BEAS-2B cells.

Lung epithelial cells can be activated directly by some microbes, such as *P. aeruginosa*.¹⁴ A variety of cellular signalling pathways can be altered by acute lower respiratory tract infection including MAPK signalling and NF-κB activation, among others.¹⁵ The MAPKs are important transducers of extracellular stimuli to the nucleus. The mechanisms of MAPK-mediated gene expression include the modulation of transcription factor activity.¹⁶ NF-κB is also an important transcription factor activating the IL-8 promoter.¹⁷ In support of NF-κB involvement, *P. aeruginosa* infection induced the activity of a reporter construct governed by a 6x tandem repeat of the NF-κB response element in BEAS-2B cells, suggesting that NF-κB might be involved in *P. aeruginosa*-induced IL-8 and MMP-9 promoter activity.

To our knowledge, there is no study addressing the significance of ERK and JNK signalling after *P. aeruginosa* infection. In accordance with our findings, ERK has been linked to production of MMP-9, and to IL-8 release in response to lipopolysaccharide or *Helicobacter pylori* through in vitro studies of primary monocytes and macrophage cell lines.^{18,19} The present study indicated that inhibition of ERK or JNK suppressed *P. aeruginosa*-induced increases in the luciferase activity of an NF-κB-dependent reporter, supporting the possibility of the role of ERK or JNK as an upstream activator of NF-κB. An ERK- or JNK-NF-κB-linked cascade was important in the case of human airway epithelial cells (Fig). We therefore suggest that inhibition of ERK or JNK may be an effective therapeutic strategy against the early stages of pulmonary *P. aeruginosa* infection, via attenuation of the inflammatory cascade.

Acknowledgement

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#03040832).

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