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Matrix metalloprotease-9 induces transforming growth factor-β_1 production in airway epithelium via activation of epidermal growth factor receptors

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ABSTRACT

Aims: Matrix metalloprotease (MMP)-9 is present in abundance in various chronic airway disorders and is involved in lung remodeling. MMP may cleave membrane-bound precursor proteins and release epidermal growth factor-like ligands that subsequently bind to epidermal growth factor receptor (EGFR). We hypothesized that MMP-9 may stimulate the airway epithelium to produce fibrogenic mediators through activation of membrane-bound receptors.
Main methods: Human airway epithelial cells were grown on air–liquid interface culture inserts. MMP-9 was employed to stimulate the cells. Conditioned medium following MMP-9 stimulation was co-incubated with human lung fibroblasts.
Key findings: MMP-9 stimulated human airway epithelial cells to produce transforming growth factor (TGF)-β_1 at both the mRNA and protein level. Using a microarray, increased phosphorylation of EGFR tyrosine kinase (TK) was identified and further confirmed by immunoprecipitation and Western blot analysis. A significant increase in EGF and TGF-α release was observed after MMP-9 had been added for 30 min. Protease inhibitor, EGFR monoclonal antibody and EGFR-TK inhibitor blocked this action and subsequent TGF-β_1 production. Neutralizing antibodies against EGF and TGF-α substantially inhibited TGF-β_1 production following MMP-9 stimulation. MMP-9-induced TGF-β_1 production occurred through MAP kinase p44/42 phosphorylation. Selective p44/42 kinase inhibitor UO126 successfully inhibited TGF-β_1 production. Conditioned medium from epithelial cells treated with MMP-9 significantly induced Smad3 phosphorylation and subsequent fibroblast proliferation after 24 h culture.
Significance: These data indicate that MMP-9 induces TGF-β_1 production in the airway epithelium through the cleavage of EGF and EGF-like ligands and activating EGFR, suggesting potential targets of therapeutic intervention in airway fibrotic disorders.

Introduction

Matrix metalloproteinase (MMP)-9 has been shown to be involved in diverse pathologic conditions, particularly asthma, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease (COPD) (Atkinson and Senior, 2003). MMP-9, and increased MMP-9 activity has been detected in the airways of COPD and asthma patients (Araujo et al., 2008; Tang et al., 2006; Vernooy et al., 2004). Airflow limitation is a characteristic of COPD and is caused by a combination of small airway disorders and parenchymal destruction (Hogg, 2004). An imbalance between MMP-9 and a tissue inhibitor of metalloproteinase-1 is considered to be associated with the progression of asthmatic airway remodeling (Atkinson and Senior, 2003; Kelly and Jarjour, 2003). The mechanism of MMP-9 induction of airway fibrosis remains to be elucidated.

Transforming growth factor (TGF)-β_1, a critical mediator of lung fibrosis, is a strong extracellular matrix inducer and a chemoattractant for fibroblasts and neutrophils (Hannigan et al., 1998; Postlethwaite et al., 1987). Targeted overexpression of TGF-β_1 leads to progressive fibrosis (Sime et al., 1997). Increased expression of TGF-β_1 has been observed in the small airway epithelium of smokers and patients with COPD, and the expression of TGF-β_1 mRNA has been shown to be positively associated with the degree of small airway obstruction (Takizawa et al., 2001). Moreover, it is believed that TGF-β_1 may play an essential role in the airway remodeling that occurs in asthmatic patients (Bosse and Rola-Pleszczynski, 2007). In response to stimuli, the airway epithelium can produce TGF-β_1 (Perng et al., 2006, 2007, 2008) and contribute greatly to the pathogenesis of airway obstruction and remodeling.

The epidermal growth factor receptor (EGFR) is expressed in most tissue and mediates a variety of biological functions, such as growth,

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differentiation and survival (Zwick et al., 1999). These signals are initiated by receptor binding of EGF and EGF-like ligands, which are expressed as transmembrane precursor proteins and need to be cleaved by proteases to release the mature form (Chan et al., 2006). It has been reported that angiotensin II can mediate cardiomyocyte hypertrophic growth pathways via MMP-dependent heparin-binding EGF liberation and EGFR activation (Smith et al., 2004). In addition, inhibition of EGFR tyrosine kinase (TK) can diminish TGF-α-induced pulmonary fibrosis in the animal model (Hardie et al., 2008). Whether or not EGFR is involved in lung tissue remodeling is worthy of further investigation.

The relationship between MMP-9, EGFR and TGF-β1 in obstructive airway disorders is not yet understood. The airway epithelium is likely to be exposed to high levels of MMP-9, but the response of the epithelium following this exposure is not yet clear. We hypothesized that MMP-9 may stimulate the airway epithelium to produce fibrogenic mediators through activation of membrane-bound receptors.

Methods

Modified air–liquid interface culture for human airway epithelial cells (HAECs)

Preparation of the modified air–liquid interface culture for HAECs has been described in detail previously (Perng et al., 2003). Briefly, human bronchus, obtained from surgical lobectomy for lung cancer, was rinsed several times with Leibovitz's L-15 medium containing penicillin, streptomycin, and amphotericin B. The tissue was cut into pieces, which were planted onto six-well culture inserts and coated with type IV collagen. Two milliliters of culture medium were added to the basal chamber, and 100 μl to the insert. The culture medium in the basal chamber was changed every 48–72 h, while no medium was added to the insert. Cells were grown on the inserts and then dissociated and seeded in 24-well culture inserts in order to determine the extent of TGF-β_1 release following stimulation by various concentrations of MMP-9.

Human lung fibroblast culture

Lung parenchyma, obtained from surgical lobectomy for lung cancer, was rinsed several times with Leibovitz's L-15 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The tissue was cut into 1- to 2-mm^2 pieces, and 3–4 pieces of tissue were planted onto six-well culture plates. The culture medium contained antibiotics/antimycotics, glutamine (2 mM), and 10% FBS in DMEM. Cells were detached from the plates by trypsinization and seeded onto 24-well culture plates for the proliferation studies.

Measurement of EGF, TGF-α and TGF-β_1 after MMP-9 stimulation

Cells (5×10^4 cells/100 μl) were seeded onto 24-well culture inserts and grown in culture medium (500 μl per basal chamber). At confluence, MMP-9 was added to the apical compartment at varying concentrations (0–100 ng/ml). Supernatants were collected at regular intervals and stored at −80 °C. Levels of EGF, TGF-α and TGF-β_1 were determined at various time points by enzyme-linked immunosorbent assay according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). To measure the total (latent and active) TGF-β_1 concentration, freshly collected supernatants were acidified by 1 N HCl and neutralized by NaOH to activate the latent TGF-β_1 to the immunoreactive form. Active TGF-β_1 was directly examined without acidification/neutralization. To suppress the effect of TGF-β_1 release induced by MMP-9, cells were pretreated with various concentrations of EGFR monoclonal antibody (cetuximab, 10–1000 μg/ml, Merck KgaA, Darmstadt, Germany), EGFR tyrosine kinase inhibitor (gefitinib, 0.01–10 μM, AstraZeneca, Macclesfield, UK), protease inhibitor (Roche Molecular Biochemicals, Mannheim, Germany), neutralizing antibody (R&D Systems) against EGF and TGF-α (Pai et al., 2002) and UO126 (1,4-diamino-2,3-dicyano-1,4-bis (2-amino phenylthio) butadiene), a selective p44/42 inhibitor from Promega (Madison, WI, USA). Supernatants were collected and stored at −80 °C until assayed for mediators.

Phosphorylation of receptor tyrosine kinases (RTKs) array

Expression of phosphorylated RTKs was detected using a RayBio Phosphorylation Antibody Array I Kit (RayBiotech, Inc., Norcross, GA, USA). Airway epithelial cells (2×10^7 cells/ml), untreated/treated with MMP-9, were lysed in 1× RIPA lysis buffer containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Cell lysates were centrifuged at 14,000 *g* for 10 min at 4 °C, and the supernatants collected and stored at −80 °C. Five-hundred μg of pooled total protein was incubated with RTK array membranes spotted with various anti-phospho-RTK antibodies. The procedures were performed according to the manufacturer's instructions. After incubation with HRP-conjugated streptavidin, the signals were visualized by chemiluminescence and quantified by densitometry.

Semiquantative reverse transcription-polymerase chain reaction (RT-PCR) for TGF-β_1, EGF and TGF-α mRNA expression

Following the removal of supernatants for mediator detection, total cellular RNA was isolated from cell monolayers using a High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany), and the RNA (1 μg) was then reverse-transcribed into cDNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). An aliquot of cDNA was then subjected to 28 cycles of PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a standard procedure: denaturing at 94 °C for 2 min, annealing at 55 °C for 30 s, and elongating at 72 °C for 1.5 min. Further aliquots of cDNA were subsequently subjected to 35 cycles of PCR for TGF-β_1 and 30 cycles for EGF and TGF-α using a standard procedure: denaturing at 94 °C for 2 min, annealing at 55 °C for 40 s, and elongating at 72 °C for 5 min. The specific primers (Gibco BRL Life Technologies, Gaithersburg, MD, USA) for TGF-β_1, EGF, TGF-α and GAPDH are shown in Table 1. The respective amplified products were electrophoresed in a 2% agarose gel containing ethidium bromide (0.5 μg/ml) and viewed under a UV illuminator. Images were photographed, stored, and analyzed by a photodocumentation system using Photo-Capt software (ETS Vilber-Lourmat Inc., Marne LuVallee Cedex, France). Each band was normalized by calculating the ratio of the target cDNA signal to the GAPDH control, and mRNA expression was presented as a percentage of the GAPDH signal.

Real-time quantitative polymerase chain reaction (QPCR)

The cDNAs (1 μl) were amplified and detected using an Applied Biosystems Prism 7000 Sequence Detection System (Foster City, CA). The cycle threshold value used to assess the quantity of target gene expression was determined by how much the fluorescence exceeded a preset limit. The amount of TGF-β_1 RNA message in each sample was calculated on the basis of the relative standard curve generated with the RNA from epithelial cells. The data have been normalized to the expression of GAPDH.

Table 1
Primers used for RT-PCR analysis.

Primer	Sequence	Product
TGF-β_1	F: 5′-GGGACTATCCACCTGCAAGA-3′ R: 5′-CCTCCT TGGCGTAGTAGTCG-3′	240 bp
EGF	F: 5′-AACTGCTTGGTGTTCGTGTCG-3′ R: 5′-TCGGTGTTGATGCACTTGGAG-3′	393 bp
TGF-α	F: 5′-CGCTGTGGGTATTGTGTTGG-3′ R: 5′-GGTCCGCTGATTTCTCTCT-3′	680 bp
GAPDH	F: 5′-ATCAAGAAGGTGGTGAAGCAGG-3′ R: 5′-GCAACTGTGAGGAGGGGAGATT-3′	385 bp