Protective Effects of Endothelin-2 Expressed in Epithelial Cells on Bleomycin-Induced Pulmonary Fibrosis in Mice

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学 位 論 文 の 内 容 要 旨

Protective Effects of Endothelin-2 Expressed in Epithelial Cells on Bleomycin-Induced Pulmonary Fibrosis in Mice

上皮細胞に発現するエンドセリン-2 はマウスにおけるブレオマイシンによって
誘導される肺繊維症に対し保護的作用を示す

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A. Background

Endothelins (ET) family consist of three 21 amino acid peptides named ET-1, ET-2, and ET-3. This peptide works as a ligand for G Protein Coupled Receptor (GPCR), Endothelin A (ET_A) and B (ET_B) receptors. Among the three peptides, ET-3 is the unique one as it has a low-affinity binding to ET_A. Meanwhile, ET-1 and ET-2 peptide structures differed in two amino acids and showed similar affinity binding to ET_A and ET_B. Moreover, both peptides elicit similar contractions in all human vessels tested. These evidences raise the presumption that ET-2 works similarly with ET-1.

Recent findings revealed a distinct role of ET-2 that notable role of ET-2 in ovulation and retinal angiogenesis. Furthermore, ET-2 global knock-out (KO) mice exhibited lung development impairment that is distinct from ET-1 KO mice, suggesting the unique role of ET-2 in lung development.

ET-1 promotes lung fibrosis, and treatment with an endothelin receptor antagonist could attenuate disease progression in the mice model of the bleomycin-induced lung fibrosis. However, endothelin receptor antagonist trials in interstitial lung disease and systemic sclerosis-related disease showed a detrimental outcome, even lead to death rate compared with placebo. This contradictive evidence leads to hypothesize that ET-2, which acts via the same receptor as ET-1, may play a different role from ET-1 in lung fibrosis. Thus, the study to elucidate the role of ET-2 in pulmonary fibrosis was needed to provide a novel insight into other Endothelin ligand's effects in pulmonary fibrosis pathophysiology.

B. Methods

For *in vivo* studies, 11-12 weeks old ET-$2^{flox/flox}$; SHH-$Cre^{+/-}$ and ET-$2^{flox/flox}$ littermate mice were administered either 5 mg/kg bleomycin in 60 µl normal saline vehicle or only the vehicle intratracheally. After designated days, lung mechanic parameters were measured and sacrificed. Then, the lungs were harvested for histology, quantitative RNA analysis, or measuring ET-1/ET-2 levels.

For *in vitro* studies, A549 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum. Cells were transfected with siRNA-mediated silencing for *ET-1, ET-2,* or negative siRNA as control. In some experiments, the cells were treated with 500 µM H_2O_2 for 6 h or 300 µM H_2O_2 for a specified duration. In addition, mouse lung fibroblasts were isolated and cultured in Dubelco's Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells were cultured until passage 3 for experiments and then treated with the indicated agents and harvested for protein quantitative RNA analysis or immunoblotting. In some experiments, cells were migrated using either 100 nM recombinant ET-1 or ET-2 peptide, or treated with 100 nM recombinant ET-1 or ET-2 peptide, while 10ng/ml Transforming Growth Factor-β1 (TGF-β1) was used to induce fibroblast activation.

C. Results

1. ET-2 expressed in mouse lung epithelial cells

Determination of ET-2 expression in the lung was studied using ET-2-iCre mice. In the lung of ET-2-iCre; ROSA26LacZ mice, X-Gal-stained ET-2 expressions were detected in the bronchial epithelium, and dispersed punctate expressions were observed in pneumocytes. This result was later confirmed in ET-2-iCre; Ai9 mice by colocalization expression of RFP-stained ET-2 and pro-SFTPC-stained alveolar epithelial type II cells in pneumocytes. To investigate whether ET-2 in epithelial cells plays a role in lung development, ET-$2^{flox/flox}$; SHH-$Cre^{+/-}$ mouse strain was generated in which ET-2 expression was deleted specifically in epithelial cells. ET-2 mRNA expression was markedly reduced in the lungs and small intestines of ET-$2^{flox/flox}$; SHH-$Cre^{+/-}$ mice. These mice did not show any growth retardation compared to control ET-$2^{flox/flox}$ littermate mice, judged by body weight and food consumption. Lung morphology and mechanics were also found similar in both mice strains. These results indicate that despite being primarily expressed from epithelial cells, ET-2 from epithelial cells is not essential for growth and normal lung development.

2. Exacerbation of lung fibrosis in ET-$2^{flox/flox}$; SHH-$Cre^{+/-}$ mice after bleomycin instillation

The role of ET-2 in pulmonary fibrosis was studied using bleomycin-induced pulmonary fibrosis mice model to ET-$2^{flox/flox}$; SHH-$Cre^{+/-}$ mice and control littermate. After 14 and 21 days of 5mg/kg bleomycin intratracheal instillation, ET-$2^{flox/flox}$; SHH-$Cre^{+/-}$ mice showed detrimental pulmonary fibrosis judged by enhanced collagen deposition, increase tissue damping, and suppressed static compliance in the lung compared to the control littermate. In addition, myofibroblast marker was increased in lung tissue, and mRNA expression level of fibroblast activation marker, *ACTA2*, and collagen marker, *COL1A1*, in the lungs. It is noteworthy that ET-1/ET-2 peptide expression levels were similar in both strains. Taken together, these results indicate that deletion of ET-2 in epithelial cells aggravates bleomycin-induced pulmonary fibrosis in mice.

3. Silencing of *ET-2* enhances apoptosis in A549 epithelial cells

To investigate the mechanism of exacerbated pulmonary fibrosis in mice with ET-2 deletion and to contrast ET-1 and ET-2 role in epithelial cells, A549 as an alveolar epithelial cells model and siRNA-mediated silencing were used. Cells with *ET-2* knockdown showed increased H_2O_2-induced cell apoptosis as indicated by the increasing ratio of cleaved-caspase-3/caspase-3. In addition, the cleaved-caspase-8/caspase-8 ratio as intrinsic and cleaved-caspase-9/caspase-9 ratio as extrinsic apoptosis markers also increased in *ET-2* knockdown cells. Furthermore, excessive oxidative stress and increased AMPKα activation were observed in *ET-2* knockdown cells. Moreover, in H_2O_2-treated cells, *ET-2*, but not *ET-1* mRNA expression was increased transiently. Collectively, these results suggest that ET-2 prevents oxidative stress accumulation and inhibits apoptosis in alveolar epithelial cells.

4. Exogenous ET-2 inhibits fibroblast migration and hampers TGF-β-induced fibroblast activation

ET-1 acting in promoting fibroblast to myofibroblast activation was established in several previous study. Interestingly, ET-$2^{flox/flox}$; SHH-$Cre^{+/-}$ mice showed increased myofibroblast raised the hypothesis whether ET-2 exerts a different role with ET-1 in lung fibroblast. Chemotaxis migration assay with recombinant ET-1 or ET-2 peptide to mouse lung fibroblast was done. As expected, ET-1 increases fibroblast migration, while, interestingly, ET-2 inhibits fibroblast migration. Furthermore, it showed that exogenous ET-1 upregulated *ACTA2* and *COL1A1* mRNA expression levels following TGF-β stimulation; in contrast, exogenous ET-2 showed lower *ACTA2* and *COL1A1* expression induced by TGF-β. Mechanistically, SMAD2/3 activation was found increased in TGF-β-treated mouse lung fibroblast with exogenous ET-1, but decreased with exogenous ET-2 treatment. As such, these results collectively suggest that ET-1 promotes fibroblast migration and TGF-β-induced activation but ET-2 hampers fibroblast migration and activation following TGF-β induction.

D. Discussion

In this study, ET-2 was expressed in lung epithelium, contrasting to the previous publications that showed ET-1, ET_A and ET_B receptor expressed in the lung mesenchyme. Furthermore, after bleomycin treatment, ET-2 expression was decreased, while ET-1, ET_A, and ET_B receptors were increased. This result might reflect the cell population shift on the lungs, as epithelial cells were damaged and decreased while mesenchymal cells were expanded.

Mice with ET-2 deletion in epithelial cells showed an exacerbated pulmonary fibrosis, suggesting the distinct role of ET-1 and ET-2 in the disease progression. As epithelial damage and apoptosis are known to initiate pulmonary fibrosis pathophysiology, the in vitro experiment in this study showed that ET-2 but not ET-1, inhibits epithelial cells apoptosis. Furthermore, these results suggest that ET-2 might transiently upregulate after injury to prevent oxidative stress accumulation, and it will further inhibit apoptosis in alveolar epithelial cells.

Fibroblast transformation to myofibroblast is known as an important feature in pulmonary fibrosis. Previous studies showed that ET-1 promotes fibroblast migration and activation. However, this study showed the distinct effects of ET-2, as it suppresses fibroblast activation potentially by inhibiting TGF-β / SMAD2/3 pathway. Furthermore, it is known that certain ligands and GPCR exert specific signaling pathways. Therefore, the different effects of ET-1 and ET-2 in lung fibroblast might be because of the different signal transduction triggered by these ligands. Hence, future studies investigating whether Endothelin ligands and receptors activate biased signaling will be important to elucidate the unique nature of Endothelin peptides and receptors.