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Relaxin attenuates silica-induced pulmonary fibrosis by regulating collagen type I and MMP-2

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ABSTRACT

Silicosis is one of the most prevalent occupational lung diseases, but the pathogenic mechanisms of silicosis are largely unknown and an effective treatment is not yet available. In this study, we investigated the potential effects of relaxin (RLX) on fibrosis by an *in vitro* model involving silica-induced and macrophage-mediated pulmonary fibroblasts. Following pre-treatment with DQ12 quartz, the culture supernatant of human monocytic THP-1 cells was added to human fetal lung fibroblast MRC-5 cells with or without RLX. DQ12 significantly induced an increase of *TGFB1* mRNA in THP-1 cells, coinciding with elevated TGF-β1 protein excretion in the supernatant, but RLX had no effect on DQ12-stimulated TGF-β1 secretion in THP-1 cells. Furthermore, RLX inhibited the proliferation of MRC-5 cells, and reduced the mRNA level and protein secretion of collagen type I, whereas it increased the mRNA level and protein activity of MMP-2 in MRC-5 cells treated with THP-1 cell culture supernatant. Our data suggest that RLX may inhibit TGF-β1-mediated fibrosis during the process of silicosis, providing evidence for the protective effect of RLX on silica-induced pulmonary fibrosis.

1. Introduction

Silicosis is a fibrotic pulmonary disease characterized by fibroblast proliferation and excessive collagen deposition, and is initiated by inhalation of crystalline silica dusts [1,2]. The pathogenic mechanisms of silicosis remain to be determined and an effective treatment is not available.

Accumulating evidence demonstrates that macrophages and fibroblasts play key roles in silica-induced fibrotic reactions [3]. Activation of macrophages following the ingestion of silica particles releases fibrogenic and inflammatory cytokines including transforming growth factor-1 (TGF-β1), which in turn stimulates pulmonary fibroblasts to produce collagen. This ultimately leads to the development of fibrosis [2–7]. Paradoxically, fibroblasts also secrete matrix-metalloproteinases (MMPs) to induce ECM degradation. Therefore, an imbalance between pro-fibrotic factors and anti-fibrotic factors might lead to excessive collagen deposition, tissue re-modeling and finally pulmonary fibrosis [8,9]. It has been proposed that a constant production of fibrotic factors by macrophages contributes to the progression of silica-induced lung fibrotic lesions by recruiting and stimulating the proliferation of fibroblasts [10]. It is thus intriguing to postulate that blocking the stimulatory effect of macrophages on fibroblasts after silica exposure, might provide an effective strategy to prevent silica-induced lung fibrosis.

Relaxin (RLX) was first identified in 1926 and subsequently regarded as a hormone that could re-model ECM in the female reproductive tract, in order to facilitate parturition [11]. Recent studies indicated that RLX plays an important role in connective tissue homeostasis [12], and exogenous RLX demonstrated anti-fibrotic effects in non-reproductive tissues including the heart [13], kidney [14], dermis [15] and lung [16]. *In vitro*, RLX decreased matrix accumulation by inhibiting the secretion and/or deposition of collagen while stimulating the expression of MMPs, and reduced the TGF-β-induced secretion of collagen and/or fibronectin in human lung fibroblasts [16]. *In vivo*, either RLX or RLX receptor gene-knockout mice demonstrated an increase in interstitial collagen in the lung [17,18]. Furthermore, RLX inhibited the bleomycin-induced fibrosis in a murine model [16], which shares some histopathological features of silica-induced pulmonary fibrosis [19]. These data suggest that RLX is a potential therapeutic drug for silica-induced pulmonary fibrosis.

In order to explore the protective effect of RLX on silica-induced pulmonary fibrosis, we established an *in vitro* model using macrophages (human monocytic THP-1 cells) and fibroblasts (human fetal lung fibroblast MRC-5 cells) to examine the effect of RLX on TGF-β1 synthesis in silica-induced THP-1 cells. We also assessed the effect on cell proliferation, collagen synthesis, and expression of MMP-2 in silica-induced and macrophage-mediated pulmonary fibroblasts.

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2. Materials and methods

2.1. Cell and reagents

Human monocytic cell line (THP-1) and human fetal fibroblast cell line (MRC-5) were obtained from the Institute of Cell Research, Chinese Academy of Science (Shanghai, China), and cultured in MEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. Dörentrup quartz, ground product no.12 (DQ12), with a mean diameter of 0.96 μm, was heated for 16 h at 220 °C to remove possible endotoxin contamination [20]. DQ12 was suspended in MEM (2 mg/mL) and sterilized. Phorbol myristate acetate (PMA), obtained from Sigma Chemical, was dissolved in DMSO (1 mg/mL) and stored in the dark at −20 °C. Relaxin (RLX, recombinant human gene-2 relaxin), purchased from R&D Systems (Minneapolis, MN, USA), was dissolved in phosphate-buffered saline (PBS) to make a solution of 1 μg/mL, and stored at −20 °C.

2.2. PMA-primed THP-1 cells

THP-1 cells (5.0×10^5/mL) were maintained in MEM complete medium at 5% CO_2 and 37 °C, and incubated with 10 ng/mL PMA for 48 h to facilitate differentiation into macrophages (differentiated THP-1 cells, which are hereafter referred to as diff THP-1) [21]. Following replacement with serum-free MEM, diff THP-1 were divided into 4 different treatment groups: MEM only (control), DQ12 (200 μg/10^6 cells) only, RLX (100 ng/ml) only and DQ12 plus RLX (100 ng/ml). The different cell types were incubated for 1, 3, 6 and 12 h [22]. After incubating with DQ12, the culture supernatants (CS) from diff THP-1 cells (THP-1 CS) were harvested, sterilized with 0.22-μm millipore filter, and stored in −80 °C for further use. Diff THP-1 were collected and prepared for total RNA extraction. The level of TGF-β1 in THP-1 CS was measured using human TGF-β1 ELISA kit (Westang, Shanghai, China) following the manufacturer's protocol.

2.3. Proliferation of MRC-5 cells by conditioned medium

Proliferation of MRC-5 cells was determined using WST-8 dye (Beyotime Inst Biotech, China) according to the manufacturer's instructions. Briefly, MRC-5 cells were cultured in 96-well plates (4×10^3 cells/well) for 24 h, and then the culture mediums were switched to MEM supplemented with 0.2% lactalbumin hydrolysate for 24 h. Next, cells were divided into 4 different treatment groups including MEM only (control), THP-1 CS only, RLX only and THP-1 CS plus RLX. The RLX concentration was 1, 10 and 100 ng/mL, respectively. After 48 h, MRC-5 cells were photographed with an Olympus microscope at ×400 magnification. 10 μl WST-8 dye was then added to each well, cells were incubated at 37 °C for 2 h and the absorbance was determined at 450 nm using a microplate reader (Synergy 2, Bio-Tek Instrument, Inc., Winooski, VT, USA).

2.4. Measurement of MMP-2 activity and collagen type I concentration in culture supernatant from MRC-5 cells

MMP-2 activity was analyzed by zymography as previously described [23]. Firstly, MRC-5 cells were cultured in 6-well plates (1.2×10^5 cells/well) for 24 h, and then the cells were incubated in MEM supplemented with 0.2% lactalbumin hydrolysate for another 24 h. Lastly, the cells were divided into 4 different treatment groups including MEM only (control), THP-1 CS only, RLX only and THP-1 CS plus RLX. After 48 h, supernatants of MRC-5 cells were harvested for determination of collagen type I concentration and MMP-2 activity. Samples were subjected to electrophoresis on a 4% acrylamide stacking gel/7% acrylamide separating gel containing 1 mg/mL gelatin in the presence of sodium dodecyl sulfate (SDS) under non-reducing conditions. After electrophoresis, gels were washed twice in 2.5% Triton X-100 and then incubated at 37 °C overnight in substrate buffer (50 mM Tris, 10 mM $CaCl_2$, 200 mM NaCl, 0.05% polyethylene glycol monododecyl ether, pH 8.0). The gels were stained with Coomassie Blue R250, and de-stained in a solution of 40% methanol and 10% acetic acid. MMP-2 activity appeared as bright bands against a blue background. Density of the band was graphed to estimate the degree of MMP-2 induction by RLX and its biphasic dose-dependency. Results were expressed as arbitrary units of relative intensity. MRC-5 cells were collected and prepared for total RNA extraction. The collagen type I concentration in culture supernatant from MRC-5 cells was measured using the Human Collagen type I ELISA kit (Westang, Shanghai, China).

2.5. Isolation of RNA and quantitative real-time polymerase chain reaction

Total cellular RNA was isolated and evaluated as previously described [24]. Briefly, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized from 2 μg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, MD, USA) in a total volume of 20 μL. The primers of the genes (Table 1) used in the experiments were synthesized by TaKaRa Biotechnology (Dalian, China). QRT-PCR analysis was carried out on the ABI Prism 7900 Sequence Detection System (Applied Biosystem, CA, USA) using SYBRR Green qPCR SuperMix-UDG kit (Invitrogen, Beijing, China) according to the manufacturer's instructions. Each reaction was run in triplicate with appropriate negative controls. The conditions for PCR were: initial denaturation step at 50 °C for 2 min followed by 40 cycles of 15 s at 95 °C for denaturation, 30 s at 60 °C for annealing and 15 s at 72 °C for extension. The melting curve analysis was performed for identification of the gene. All samples were normalized to the β-actin values and data were analyzed by the $2^{-\Delta\Delta T}$ method [25].

2.6. Statistical analysis

All the experiments were independently performed at least three times, and related measurements were expressed as mean ± standard deviation. All data were processed with SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's *t* test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. DQ12 increases the expression of TGF-β1 in the differentiated THP-1 cells

THP-1 cells were floating and round in appearance before PMA treatment. After incubation with PMA (10 ng/mL) for 48 h, more than 90% of THP-1 cells became adherent to the culture dish (data not shown). To analyze the effect of DQ12 quartz particles and RLX on the expression of TGF-β1 in the differentiated THP-1 cells (diff THP-1), *TGFB1* mRNA in diff THP-1and protein levels of TGF-β1 in the supernatant was determined by QRT-PCR and ELISA, respectively. *TGFB1* mRNA in the diff THP-1 significantly increased after treatment with DQ12 for 12 h ($P < 0.05$)

Table 1
Forward (F) and reverse (R) primer sequences used for QRT-PCR.

Gene		Primer sequence	Product size (bp)
TGFB1	F	AGCGACTCGCCAGAGTGGTTA	130
	R	GCAGTGTGTTATCCCTGCTGTCA	
COL1A1	F	TCTAGACATGTTCAGCTTTGTGGAC	134
	R	TCTGTACGCAGGTGATTGGTG	
MMP2	F	CTCATCGCAGATGCCTGGAA	167
	R	CAGCCTAGCCAGTCGGATTTG	
ACTB	F	TGGCACCCAGCACAATGAA	186
	R	CTAAGTCATAGTCCGCCTAGAAGCA	