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Knockdown of versican 1 blocks cigarette-induced loss of insoluble elastin in human lung fibroblasts

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ABSTRACT

COPD lung is characterized by loss of alveolar elastic fibers and an increase in the chondroitin sulfate (CS) matrix proteoglycan versican V1 (V1). V1 is a known inhibitor of elastic fiber deposition and this study investigates the effects of knockdown of V1, and add-back of CS, on CCL-210 lung fibroblasts treated with cigarette smoke extract (CSE) as a model for COPD. CSE inhibited fibroblast proliferation, viability, tropoelastin synthesis, and elastin deposition, and increased V1 synthesis and secretion. V1 siRNA decreased V1 and constituent CS, did not affect tropoelastin production, but blocked the CSE-induced loss in insoluble elastin. Exogenous CS reduced insoluble elastin, even in the presence of V1 siRNA. These findings confirm that V1 and CS impair the assembly of tropoelastin monomers into insoluble fibers, and further demonstrate that specific knockdown of V1 alleviates the impaired assembly of elastin seen in cultures of pulmonary fibroblasts exposed to CSE, indicating a regulatory role for this protein in the pathophysiology of COPD.

1. Introduction

COPD is characterized by persistent airway inflammation, irreversible airway obstruction and airway and parenchymal structural remodeling. Recurrent acute and chronic airway inflammation destroys lung tissue gradually, leading to air sac enlargement and eventually emphysema. Elastin is an essential structural protein in the lung extracellular matrix (ECM) of alveolar walls, ensuring elastic recoil and compliance (Maclay et al., 2012). Elastases (Metalloproteinases) from inflammatory cells, chiefly neutrophils and macrophages, degrade elastin and other connective tissue components, leading to development of balloon-like bullae or blisters that trap air, thereby giving rise to emphysema. Biochemical studies of dissected lung tissue show decreased expression of elastin in both centrilobular and panacinar emphysema (Snider, 2000). This widespread loss of elastin, which is reflected in reduced lung function, however, is not balanced by repair despite evidence that the lung fibroblasts retain their ability to synthesise tropoelastin (Zhang et al., 2012).

During elastic fiber formation, tropoelastin is secreted at the cell surface, complexed with an elastin binding protein that mediates delivery of tropoelstin onto an elastin microfibrillar scaffold where it is cross-linked to form insoluble elastin. Several studies have shown chondroitin sulfate (CS) rich matrix proteoglycans, such as versican, inhibit this process (Hinek et al., 1991, 2000; Huang et al., 2006; Black et al., 2008), and COPD lungs may also have increased content of CS proteoglycans in alveolar walls (Deslee et al., 2009).

Versican is a large ECM proteoglycan present in most tissues, secreted by numerous cells including lung fibroblasts, and interacting with various binding partners (Wight, 2002; Wu et al., 2005). The amino terminal globular end (G1) is characterized by a hyaluronan binding region. The carboxyl terminal globular domain (G3) consists of epidermal growth factor like domains, carbohydrate recognition domain and complement binding domain. Alpha and beta core protein domains between two globular terminals covalently link to CS glycosaminoglycan (GAG) chains. There are at least four splice variants of versican, named V0, V1, V2, V3, which differ

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in the size of their core proteins generated by alternative splicing of mRNA encoding the two GAG chain binding domains. V0 and V1 are distributed widely in adult tissues, in which V1 is the principal proteoglycan found in pulmonary ECM (Sampson et al., 1984). V2 seems to be expressed only in the central nervous system (Wu et al., 2005; Dours-Zimmermann et al., 2009). The V3 variant, which lacks GAG domains and thus CS chains, is generally expressed at very low levels in adult tissues (Cattaruzza et al., 2002). This complex structure of versican and its variants underlies multiple effects on proliferation, adhesion, locomotion and interactions with ECM components.

Several studies within the past decade have confirmed a significant role for V1 in regulating local cell interactions, and ECM metabolism (Wight, 2002; Wu et al., 2005). The carboxyl terminal G3 domain interacts with β1 integrin, activating focal adhesion kinase, thus promoting cell adhesion in for example glioma cells (Wu et al., 2002). Moreover, versican binds with CD44 through its GAG domain or G1 hyaluronan binding domain to promote cell proliferation, migration, adhesion and enhanced tumor spread (Yang et al., 1999; Ang et al., 1999). Versican also modulates other ECM components. Notably, the isoforms of versican containing CS, or CS alone (Hinek et al., 2000), inhibit elastin deposition at the cell surface, by inhibiting elastin binding protein-mediated assembly of tropoelastin (Hinek et al., 2000). Conversely, forced expression of V3, without CS chains, enhances elastin deposition (Merrilees et al., 2002) and concomitantly reduces pericellular versican (Schonherr et al., 1991). Similarly, knockdown of the isoforms containing CS by versican antisense also results in increased deposition of insoluble elastin (Huang et al., 2006). These findings indicate that control of versican could provide a potential therapeutic strategy for maintaining or enhancing elastin deposition in COPD lung.

Considering the information aforementioned, and cigarette smoking is the leading independent risk factor for COPD, we hypothesize that cigarette smoking might lead to loss of COPD elastin through versican variation, and this effect is connected with CS changes. Main isoform of versican family expressed in lung is V1, and clinical specimen of COPD patients' lungs show progressively increased immuno-staining for V1 and correspondingly decreasing values of FEV1. There are urgent needs to investigate what would happen after V1 knockdown. In this study, we report that insoluble elastin deposit is devastated by exposure to cigarette smoke extract (CSE), and V1 expression is stimulated by CSE. V1 knockdown in lung fibroblasts overcomes elastin decrease caused by CSE. This beneficial effect is lost by exposure of cells to CS.

2. Materials and methods

2.1. Cell culture and reagents

Human lung fibroblast cells (CCL-210) were obtained from American Type Culture Collection, (Rockville, MD, USA). Fibroblasts were cultured in DMEM supplemented with 10% FCS, penicillin and streptomycin (100 ng/ml) at 37 °C and 5% CO_2. Cells were treated with CSE (1 and 10%), and with versican siRNA (Invitrogen, CA, USA) and/or CS (Sigma, St Louis, MO, USA). Kentucky reference cigarettes (2R1, University of Kentucky, KY, USA) were used to produce the CSE. Smoke from 3 2R1 cigarettes was thoroughly bubbled through 10 ml culture medium (Muller, 1995), and medium containing CSE was freshly made before each experiment.

2.2. Cellular proliferation and viability

Cell proliferation and viability were measured using the Alarma Blue (AB) colorimetric assay (Invitrogen, CA, USA) (Al-Nasiry et al., 2007). Briefly, fibroblasts were seeded at 2000/well into 96 well plates and treated with CSE medium diluted to provide a range of concentrations from 0 to 20%. AB was added at a final concentration of 10% and over 24 h, the %AB reduction measured. Proliferation was calculated as ratio target reading/baseline reading. Viability was calculated as the percentages of living cells in treated cultures to those in untreated cultures.

2.3. Quantification for elastin

Cells were seeded at 50,000 cells/well in 6-well plates and cultured for 3 and 14 days. Supernatants were collected for measurement of soluble elastin. Cell layers were collected for measurement of insoluble elastin. For both soluble and insoluble elastin, elastin levels were determined using the Fastin Elastin Assay Kit from Biocolor (Carrickfergus, County Antrim, UK).

2.4. Real-time PCR

Briefly, total RNA was extracted using Trizol Reagent (Invitrogen, CA, USA) and 1 μg of total RNA was transcribed. The relative mRNA expression of target genes in each sample were quantified and normalized to the GAPDH mRNA levels by the $2^{-\Delta\Delta Ct}$ method (Fink et al., 1998). Fold difference was calculated as ratio target gene in experimental/control-1. The primer pairs used were as follows: elastin (forward primer) 5′-TCT GAG GTT CCC ATA GGT TAG GG-3′, (reverse primer) 5′-CTA AGC CTG CAG CAG CTC CT-3′; V1 (forward primer) 5′-CCC AGT GTG GAG GTG GTC TAC-3′, (reverse primer) 5′-CGC TCA AAT CAC TCA TTC GAC GTT-3′; GAPDH (forward primer) 5′-GAGTCCACTGGCGTCTTCA-3′, (reverse primer) 5′-GGGGTGCTAAGCAGTTGGT-3′.

2.5. ELISA

For ELISA analyses, a V1 kit from MYBiosource (Vancouver, B.C., CA) was purchased. The level of V1 in culture supernatants was determined according to the manufacturer's instructions.

2.6. siRNA transfection

The sequence 5′-GAG GCT GGA ACT GTT ATT A-3′ had the best efficacy of 3 sequences tested and was chosen for the studies. Real-time PCR and ELISA were used to evaluate the knockdown effect 24 h after siRNA transfection. Transfection was achieved by Lipofectamin™ 2000 (Invitrogen, CA, USA), siRNA concentration was 80 nM.

2.7. CS stimulation

CS was used at concentrations ranging from 0 to 400 μg/ml and applied to the culture medium alone or in combination with the siRNA (80 nM) and CSE (10%). Continuous concentration gradient CS was added in culture medium for 14 days, cells were collected and insoluble elastin was tested. The concentration (200 μg/ml) that had best efficacy was chosen to stimulate cells in combination with siRNA and CSE for 14 days, insoluble elastin was detected again.

2.8. Statistics

All statistical analyses were performed using GraphPadPrism v5.0. All data were expressed as mean ± SD, and one-way analysis of variance followed by Bonferroni post-test performed to determine whether differences between groups were statistically significant. Differences were considered significant at the level of $p < 0.05$.