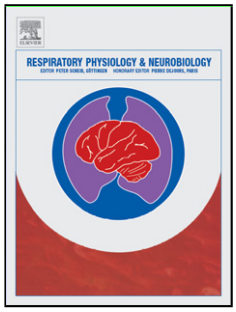

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Knockdown of versican 1 blocks cigarette-induced loss of insoluble elastin in human lung fibroblasts

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Highlights

We find extracellular matrix protein versican 1 is in creased by cigarette smoke extract solution in lung fibroblasts.

Versican 1 knockdown restore cigarette-related elastin loss through chondroitin sulfate decrease.

Exgenous chondroitin sulfate could block the beneficent effect brought by versican 1 knockdown.

Abstract

COPD lung is characterized by loss of alveolar elastic fibers and an increase in the chondroitin sulfate (CS) matrix proteoglycan versican 1 (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.

Key words: pulmonary fibroblast, COPD, elastin, versican

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; Hinek et al., 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 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 2,000/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 24h, 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 1µg 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'.

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 24h after siRNA transfection. Transfection was achieved by Lipofectamin™ 2000 (Invitrogen, CA, USA), siRNA concentration was 80nM.

2.7. CS stimulation

CS was used at concentrations ranging from 0-400μg/ml and applied to the culture medium alone or in combination with the siRNA (80nM) 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 (200μg/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 $p< 0.05$.

3. Results

3.1. Inhibition of fibroblast proliferation, viability, tropoelastin synthesis and insoluble elastin by CSE

Cells were treated with CSE at five concentrations (0%, 0.5%, 1%, 10%, 20% of stock CSE medium), and effect on proliferation was determined on days 3 and 14 (Fig. 1A). Proliferation was suppressed by CSE (Fig. 1A) in a concentration-dependent manner. Cell viability was also determined on days 3 and 14 (Fig. 1B), viability suppression was also concentration-dependent. Tropoelastin mRNA levels were significantly decreased in CSE-containing medium at 1% and 10% compared with control cultures (0%) at 3 and 14 days of culture (Fig. 1C). The decrease in mRNA was reflected in significant decreases in levels of soluble tropoelastin (in medium) and insoluble (in cell layer) elastin at day 14, with a non-significant trend for both at day 3 (Fig. 1D and E). By day 14, soluble elastin in 1% and 10% CSE was decreased by 58% and 64%, and insoluble elastin by 49% and 51% respectively.

3.2. V1 expression increased by CSE and blocked by siRNA

V1 mRNA levels were significantly increased following 3 days exposure to CSE at 1 and 10% (1.9 and 1.7 fold increase respectively compared with control). By day 14, however, this increase in mRNA was not significantly raised over control levels (Fig. 2A). Changes in V1 protein levels were generally consistent with changes in V1 mRNA. V1 levels in supernatants were 101.8, 127 and 121.8 ng/10^6 cells for control, 1% and 10% CSE cultures at day 3, and 21.6, 41.1 and 50.95 ng/10^6 cells at day 14. There was no significant difference between cultures exposed to 1% and 10% CSE (Fig. 2B). The V1 mRNA increase seen in 10% CSE was completely blocked by concomitant exposure to V1 siRNA on day 3. On day 14, siRNA reduced V1 mRNA of cells exposed to 10% CSE significantly below that of control levels (Fig. 2C), and concomitant culture of cells exposed to CSE and siRNA markedly reduced V1 levels in the culture medium (Fig. 2D). V1 decreased from 110.7 to 12.5 ng/10^6 cells on day 3, from 80.4 to 2.3 ng/10^6 cells on day 14. These results demonstrate the effectiveness of V1 knockdown. The scramble siRNA showed no knockdown efficacy (Fig. 2C and D).

3.3. Effect of V1 knockdown on soluble and insoluble elastin

Exposure of cells to 10% CSE for 3 days did not significantly reduce soluble or insoluble elastin levels (Fig. 3A and B), although did show a non-significant decrease similar to initial experiments (Fig. 1D and E). On day 14, however, and similar to initial experiments (Fig. 1D and E), both soluble and insoluble elastin levels were significantly decreased in 10% CSE. In the concomitant cultures on day 3, exposure to CSE plus V1 siRNA did not result in change to soluble or insoluble elastin levels (Fig. 3A and B), but at day 14 the siRNA blocked the decrease in insoluble elastin seen with CSE alone (Fig. 3B). There was no significant difference in cells transfected with scramble siRNA.

3.4. Effect of V1 is via CS

CS in the culture medium was measured to determine whether the effect of versican V1 on insoluble elastin was mediated through its CS chains. CS in V1 siRNA treated cultures decreased from 1.7 ng/10^6 cells to 0.4 ng/10^6 cells by day 3, and from 0.7 ng/10^6 cells to 0.1 ng/10^6 cells by day 14 (Fig. 4A). The addition of exogenous CS to cell culture medium significantly decreased insoluble elastin in a concentration-dependent manner over 14 days (Fig. 4B), while the addition of 200 µg/ml of CS in the presence of 10% CSE and V1 siRNA countered the protection conferred by the siRNA, resulting in a significant decrease in insoluble elastin (Fig. 4C).

4. Discussion

In the present study we show that CSE applied to cultured human lung fibroblasts, an *in vitro* model for COPD, inhibits proliferation, viability and secretion of soluble and insoluble elastin, and increases versican variant V1. We further show that the decrease in insoluble elastin induced by CSE can be blocked by a siRNA directed at V1, which in turn can be countered by exogenous CS, the component of V1 and other CS-rich matrix proteoglycans known to inhibit the deposition of insoluble elastin (Hinek et al., 2000; Huang et al., 2006; Black et al., 2008).

4.1. Cigarette causes poor proliferation ability, viability and elastin lose in fibroblasts

CSE has been shown to repress fibroblast proliferation through re-initiation of mRNA translation of CCAAT/enhancer-binding proteins, which coincides with increased interleukin-8 and an increase in degraded elastin (Miglino et al., 2012). In a further study, on primary COPD lung fibroblasts exposed to CSE at 0.5% and 5% over 72 hours, elastin was not affected. In this current study, with CSE levels of 1% and 10%, changes in both soluble and insoluble elastin after 3 days of exposure were also small and not significant, but were significant at 14 days, which is consistent with other studies on elastin production by cultured cells in which insoluble elastin takes a week or more to be deposited in significant amounts in the ECM (Black et al., 2008; Merrilees et al., 2002). Cigarette smoke induced development of emphysema via the recruitment of elastase-producing leukocytes or inactivation of lung elastase-inhibitors (Janoff, 1985). In acute stage, neutrophils release elastase which is mainly in charge of the acute insoluble elastin degradation, in late stage, matrix metalloproteinase- (MMP-) 12 and MMP-9 secreted by other cells (macrophages et al) are involved (Dhami et al., 2000; Churg et al., 2002; Shapiro et al., 2003; Valenca et al., 2004; Churg et al., 2007). It is reported that cigarette smoke also induces IFN-gamma-inducible protein-10 release of CD8+ T cells, which induces production of macrophage elastase (MMP-12) that degrades elastin (Maeno et al., 2007). Interestingly, CSE is expected to have dose-dependent effect on elastin synthesis and deposit. However, in our experiment, this effect was not observed, which was consistent with other findings (Chen et al., 2005; Putnam et al., 2002). Cigarette smoke was a complex compound, different doses of CSE might inspire different intracellular signal pathways (Messner et al., 2012). Explanation of this phenomenon is still unclear.

4.2. Cigarette stimulates an increase in V1 expression which could be suppressed by siRNA

The mechanism by which CSE stimulates versican production is not clear. It is possible that components of CSE act through several enhancers located at the 5'-flanking sequence of versican, such as AP2, CCAAT binding transcription factor and SP1 (Naso et al., 1994). In colon carcinoma tissue, the versican gene is hypomethylated. This may be another mechanism involved in versican overexpression in the CSE model (Adany and Iozzo, 1990). Cigarette smoke

also triggers NF-κB-dependent inflammatory responses, (Goncalves et al., 2011; Rom et al., 2013) which are associated with increases in versican (Wight et al., 2014). Moreover, versican is also regulated by several microRNAs (Morton et al., 2008; Wang et al., 2010; Rutnam et al., 2013). Versican expression, however, has been reported to be inhibited by cadmium chloride, a component of CSE (Chambers et al., 2013). Our findings show a significant up-regulation of versican by CSE at 3 days, and further up-regulation at 14 days, but with overall lower levels at this later time period. The reason for the decrease in versican at day 14 is not clear, but CS production is known to decrease with increasing cell density, and this may be a confounding factor in how CSE affects versican levels over time (Merrilees, 1987).

4.3 Versican knockdown restores CSE-related loss of insoluble elastin

A reciprocal relationship between versican with constituent CS chains and elastin has been reported previously (Wight and Merrilees, 2004), including in lung parenchyma of patients with mild to moderate COPD (Merrilees et al., 2008). The increase in versican in the parenchyma of COPD lung, and its ability to block formation of elastic fibers may explain why restoration of a functional elastic fiber network does not occur in lungs affected by COPD (Merrilees et al., 2008) or in other emphysematous diseases (Merrilees et al., 2004). It has also been reported that versican production is higher in lung fibroblast cells isolated from COPD patients (GOLD stage IV), consistent with the lack of elastic fiber repair and a negative influence on the elastic recoil, and suggesting that the relationship exists in patients with severe COPD (Hallgren et al., 2010). A caveat to this proposed relationship, however, is that lower levels of versican have been reported in distal parenchyma of COPD patients compared with nonsmokers and non-obstructed smokers even though elastic fiber fractional areas are also low (Annoni et al., 2012). The current experimental findings directly, however, support a central role for CS matrix proteoglycans in preventing elastic fiber repair and point to manipulation of matrix levels of CS as a possible therapeutic strategy for resolving, or slowing progression of COPD.

4.4 Exogenous CS prevents insoluble elastin accumulation

Our findings show that CSE has significant and reciprocal effects on versican and elastin. These

changes under conditions of cell, in which there is an absence of the inflammatory changes seen *in vivo*, point to a key role for versican in inhibiting elastin repair in lungs challenged with cigarette smoke. Once versican is knocked down, however, the CSE-induced decrease in insoluble elastin is restored. Interestingly, the expression of soluble tropoelastin remains depressed. Synthesis and assembly, however, need not be linked in the formation of elastic fibers; the final stage of assembly depends on the ECM environment. Both in lung tissue and in vessel wall there is evidence that the lack of elastic fibers in damaged or repair tissue is not a consequence of reduced synthesis of tropoelastin. Tropoelastin may be expressed at high levels but the monomers are not assembled onto the microfibrillar framework. CS has a controlling influence on this latter process.

The process of elastic fiber formation involves elastin binding protein that chaperones tropoelastin to the cell surface and mediates transfer to the microfibrils. This process is inhibited in the presence of CS (Hinek et al., 2000). Elastin binding protein, an inactive variant of galactosidase, has a binding pocket for CS which, when occupied, releases tropoelastin prematurely and before transfer to the microfibrils (Hinek et al., 2000; Black et al., 2008). A consequence of this action is that potential repair of elastic fibers does not take place in the presence of elevated levels of pericellular versican or CS, even though cells in repair situations still have the capacity to synthesise tropoelastin. In some diseases enhanced synthesis of tropoelastin, may occur, as observed by in situ hypridization for alveolar wall fibroblasts in COPD (Deslee et al., 2009; Lucey et al., 1998) and for vascular smooth muscle in neointima formed following angioplasty (Nikkari et al., 1994). In both situations, however, insoluble elastin is not deposited in the ECM. As we demonstrate in this current study, manipulation of versican and or CS levels is an effective strategy for modulating elastin deposition by lung fibroblasts.

Study *in vivo* shows increased versican and corresponding disrupted elastin deposit in COPD patients (Merrilees et al., 2008). In rats suffered from balloon injury, the neointimae contains low levels versican, increased elastin deposits (Huang et al., 2006). Next, we will study V1 knockdown effect *in vivo*.

Therefore, we conclude that targeting versican with siRNA to reduce versican core protein, and consequently CS levels, maintains insoluble elastin production in the face of the CSE challenge.

Exogenous CS overrides the beneficial effect of siRNA. This result points to a possible therapeutic strategy whereby targeting versican may allow for restoration of elastic fibers and improved repair of COPD lung.

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Figure legends

Figure 1 Effect of CSE on fibroblast proliferation, viability and elastin expression and deposition. A Proliferation indices, determined by Alarma Blue assay, for control and CSE exposed cultures at 3 (left) and 14 (right) days. B The effect of CSE (0-20%) on cell viability is delineated. Cell viability decreased with raised concentrations. C Fold change in elastin mRNA in fibroblast cultures exposed to CSE, calculated as experimental 2-ddCt/control 2-ddCt－1. D and E Effect of CSE on soluble and insoluble elastin production by fibroblasts cultured for 3 and 14 days. *, $p<0.05$; **, $p<0.01$.

Figure 2 Effect of CSE and V1 siRNA on expression and secretion of versican V1 by cultured lung fibroblasts. A Fold difference from control cultures for V1 mRNA expression at 3 and 14 days. B V1 levels in medium at 3 and 14 days. C Effect of V1 siRNA (80nM) on V1 expression at 3 and 14 days, expressed as fold difference from control cultures. D Effect of V1 siRNA on V1 levels in medium at 3 and 14 days. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Figure 3 Effect of V1 siRNA (80nM) on soluble and insoluble elastin. A and B Effect of V1 siRNA on soluble (A) and insoluble (B) elastin levels produced by lung fibroblasts cultured for 3 and 14 days and exposed to 10% CSE. *, $p<0.05$.

Figure 4 V1 siRNA restored insoluble elastin repair was countered by chondroitin sulfate (CS). A Effect of V1 siRNA on CS levels in medium of fibroblasts cultured for 3 and 14 days and exposed to 10%CSE. B Effect of exogenous CS on production of insoluble elastin by lung fibroblasts cultured for 14 days. C Effect of exogenous CS on V1 siRNA-treated control (0% CSE) and CSE (10%) exposed fibroblasts cultured for 14 days. *, $p<0.05$; **; $p<0.01$.

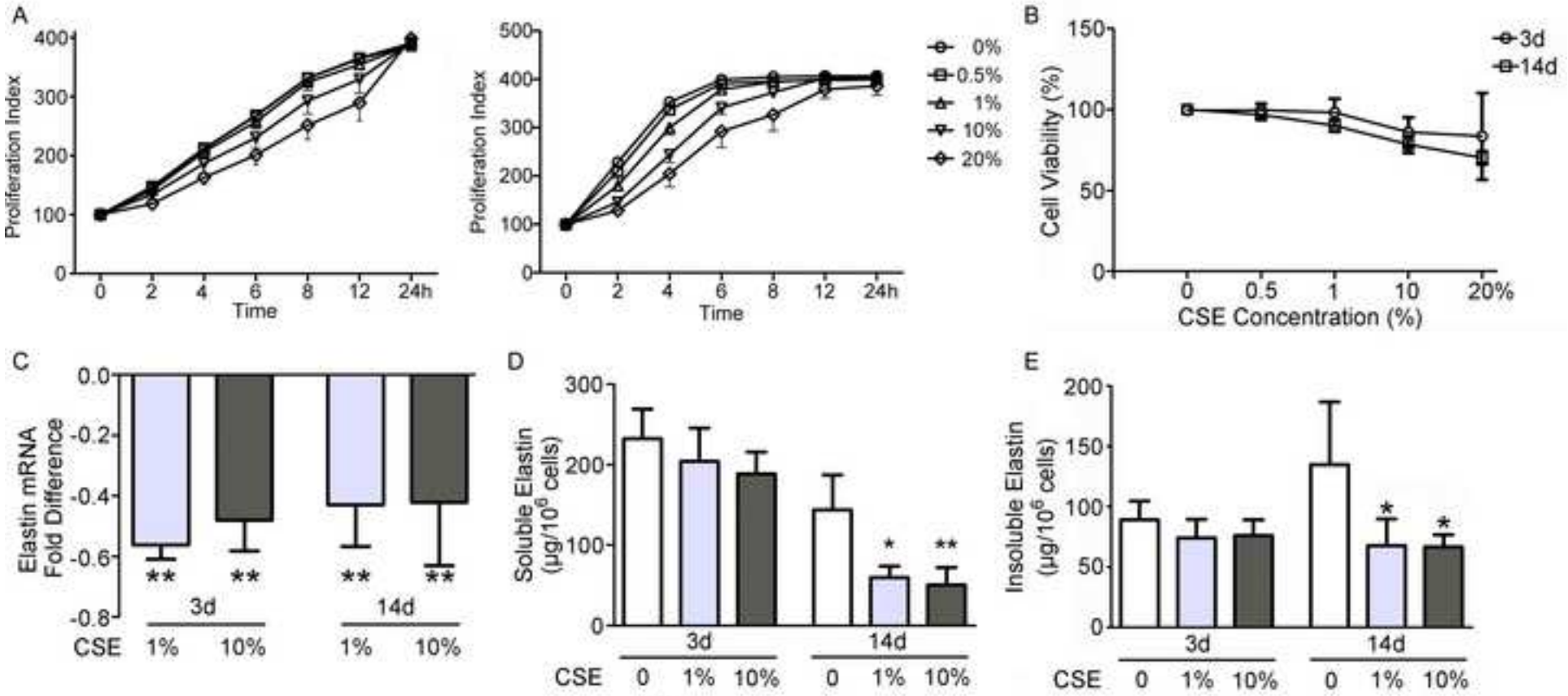
A
Proliferation Index
Time
0%
0.5%
1%
10%
20%
B
Cell Viability (%)
CSE Concentration (%)
3d
14d
C
Elastin mRNA Fold Difference
3d
14d
CSE
1%
10%
D
Soluble Elastin (μg/10^6 cells)
E
Insoluble Elastin (μg/10^6 cells)
CSE
0
1%
10%

Figure 1

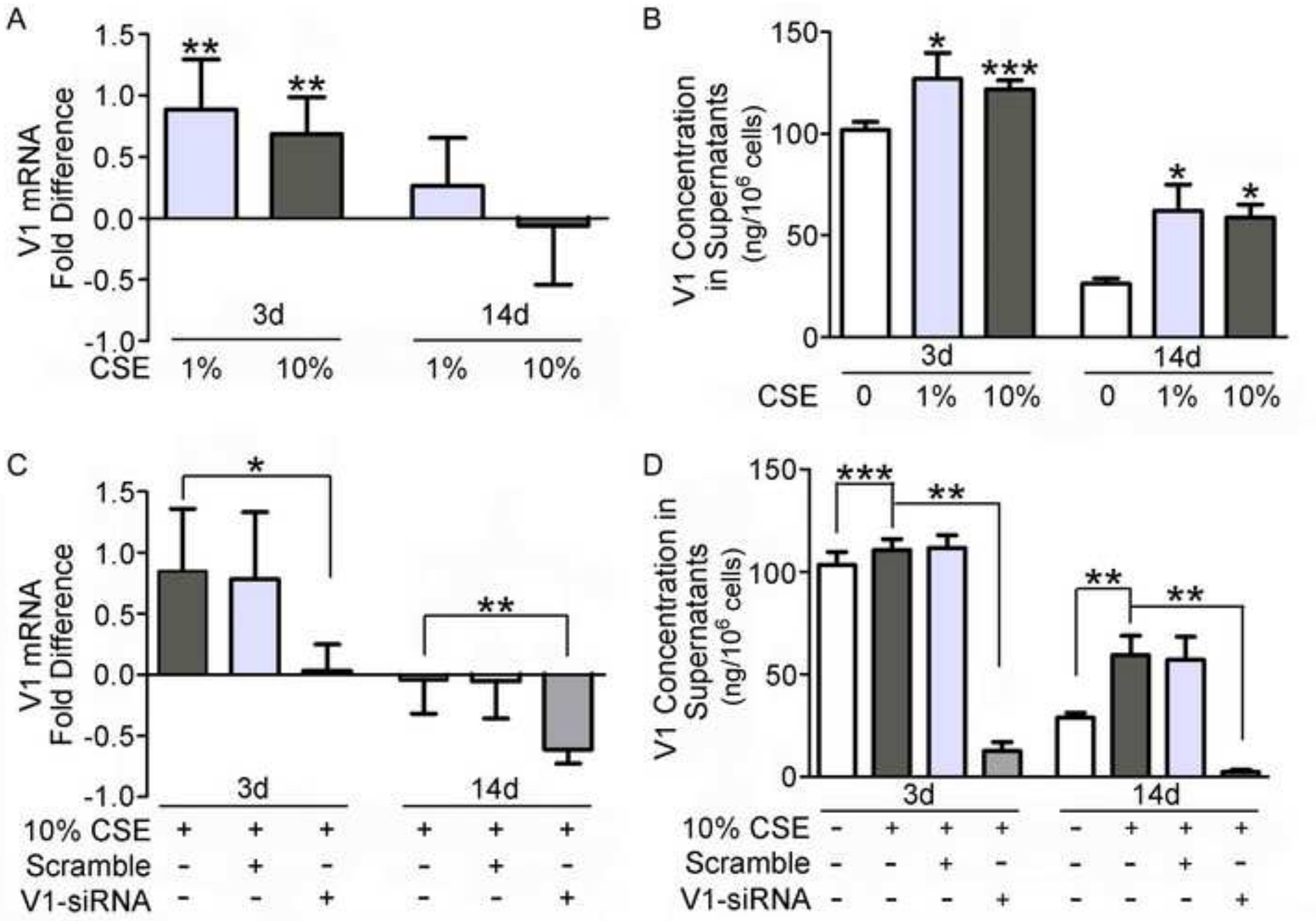
A
V1 mRNA Fold Difference
1.5
1.0
0.5
0.0
-0.5
-1.0
**
**
3d
14d
CSE 1% 10% 1% 10%
B
V1 Concentration in Supernatants (ng/10^6 cells)
150
100
50
0
*
***
*
*
3d
14d
CSE 0 1% 10% 0 1% 10%
C
V1 mRNA Fold Difference
*
**
3d
14d
10% CSE + + + + + +
Scramble - + - - + -
V1-siRNA - - + - - +
D
V1 Concentration in Supernatants (ng/10^6 cells)
***
**
**
**
3d
14d
10% CSE - + + + - + + +
Scramble - - + - - - + -
V1-siRNA - - - + - - - +

Figure 2

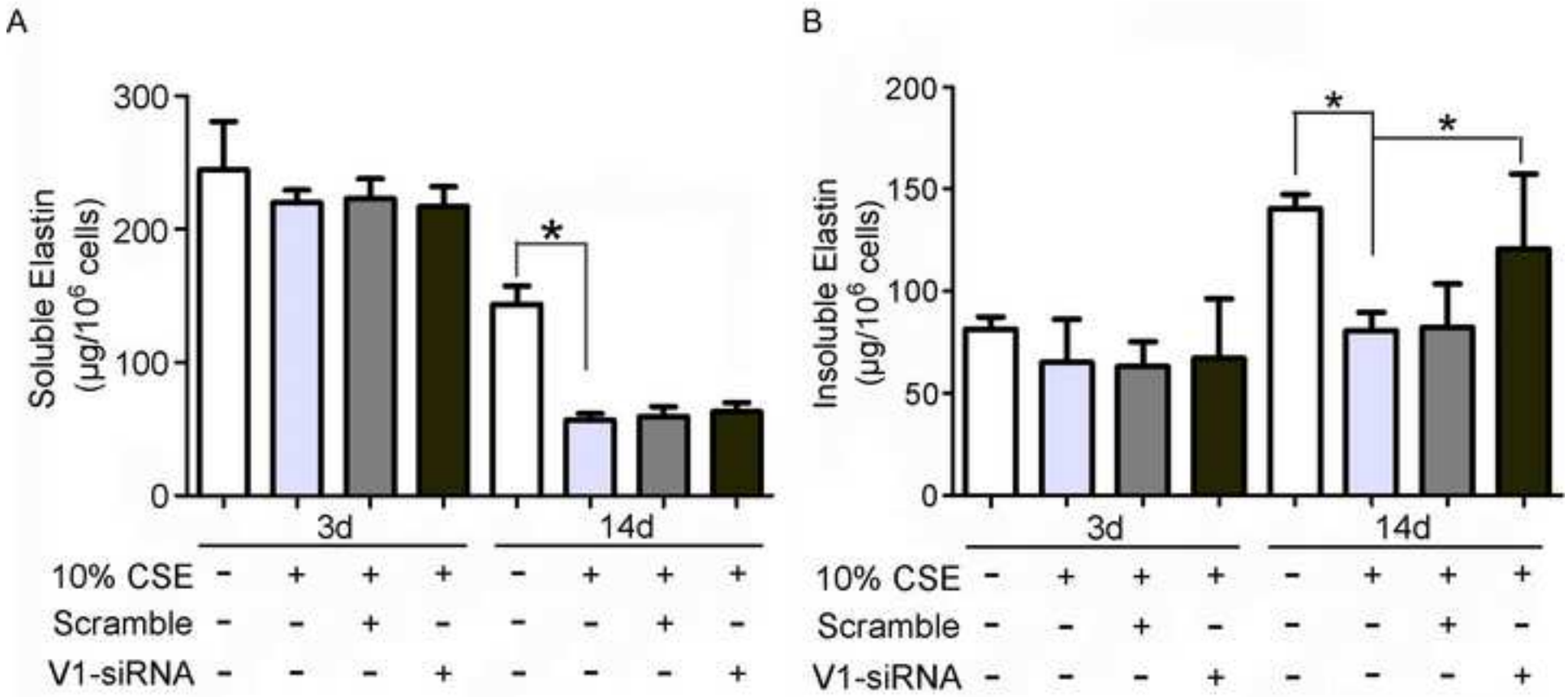
A
Soluble Elastin (µg/10^6 cells)
300
200
100
0
*
3d
14d
10% CSE - + + + - + + +
Scramble - - + - - - + -
V1-siRNA - - - + - - - +
B
Insoluble Elastin (µg/10^6 cells)
200
150
100
50
0
*
*
3d
14d
10% CSE - + + + - + + +
Scramble - - + - - - + -
V1-siRNA - - - + - - - +

Figure 3

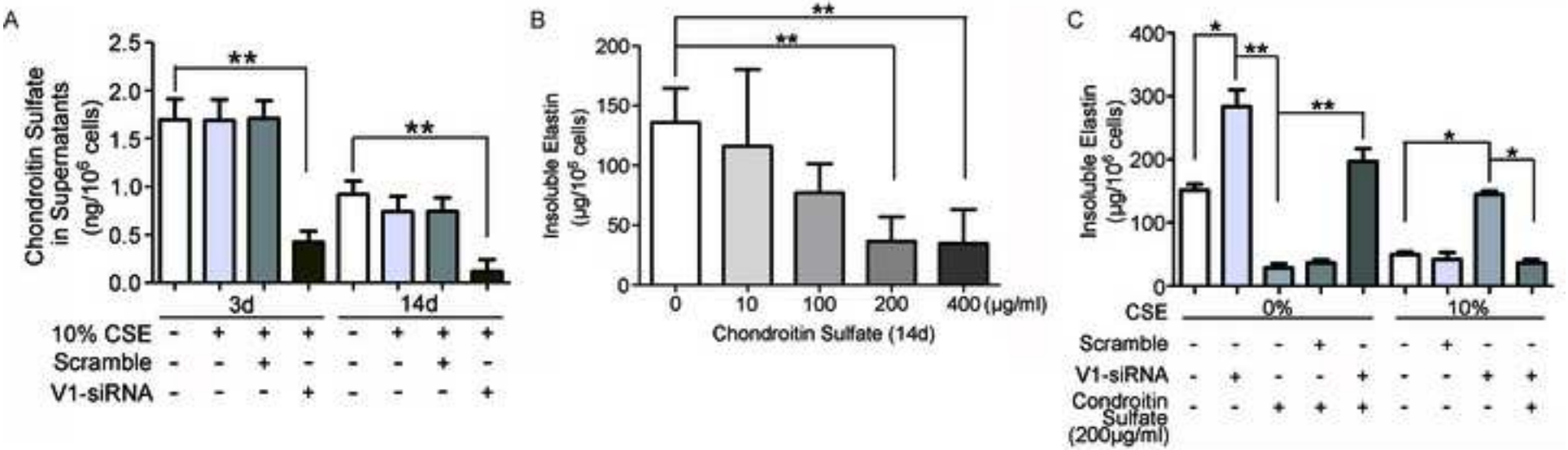

Figure 4