Chapter 3
Extracellular matrix proteins: a positive feedback loop in lung fibrosis?

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Marjolein E. Blaauboer
Fee R. Boeijen, Claire L. Emson, Scott M. Turner, Roeland Hanemaaijer, Theo H. Smit,
Reinout Stoop, Vincent Everts
Abstract

Lung fibrosis is characterized by excessive deposition of extracellular matrix. This affects not only tissue architecture and function, but it also influences fibroblast behavior and thus disease progression. Here we describe the expression of elastin, type V collagen and tenascin C during the development of bleomycin-induced lung fibrosis. We further report in vitro experiments clarifying both the effect of myofibroblast differentiation on this expression and the effect of extracellular elastin on myofibroblast differentiation.

Lung fibrosis was induced in female C57Bl/6 mice by bleomycin instillation. Animals were sacrificed at zero to five weeks after fibrosis induction. Collagen synthesized during the week prior to sacrifice was labelled with deuterium. After sacrifice, lung tissue was collected for determination of new collagen formation, microarray analysis, and histology. Human lung fibroblasts were grown on tissue culture plastic or BioFlex culture plates coated with type I collagen or elastin, and stimulated to undergo myofibroblast differentiation by 0 to 10 ng/ml transforming growth factor (TGF)β1. mRNA expression was analyzed by quantitative real-time PCR.

New collagen formation during bleomycin-induced fibrosis was highly correlated with gene expression of elastin, type V collagen and tenascin C. At the protein level, elastin, type V collagen and tenascin C were highly expressed in fibrotic areas as seen in histological sections of the lung. Type V collagen and tenascin C were transiently increased. Human lung fibroblasts stimulated with TGFβ1 strongly increased gene expression of elastin, type V collagen and tenascin C. The extracellular presence of elastin increased gene expression of the myofibroblastic markers α-smooth muscle actin and type I collagen.

The extracellular matrix composition changes dramatically during the development of lung fibrosis. The increased levels of elastin, type V collagen and tenascin C are probably the result of increased expression by fibroblastic cells; reversely, elastin influences myofibroblast differentiation. This suggests a reciprocal interaction between fibroblasts and the extracellular matrix composition that could enhance the development of lung fibrosis.
Introduction

Idiopathic pulmonary fibrosis (IPF) is a severely destructive lung disease, resulting in impaired architecture and function of lung tissue (Selman et al., 2004). The incidence of IPF is estimated to be 5 to 10 per 100,000 (Fernandez Perez et al., 2010) and appears to increase in recent years (Nalysnyk, Cid-Ruzafa, Rotella, & Esser, 2012). At the core of the fibrotic process are changes in both the structure and the composition of the extracellular matrix. The deposition of excessive amounts of type I collagen is classically seen as the main problem in fibrosing tissues (Meltzer & Noble, 2008).

Fibroblasts are responsible for maintenance of the extracellular matrix. During fibrosis development, they differentiate toward the myofibroblastic phenotype, characterized by an increased contractile capacity due to the expression of α-smooth muscle actin (αSMA) and by increased release of different types of extracellular matrix proteins (Hinz, 2007; Hinz et al., 2012; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). Extensive literature exists on how this process is regulated by growth factors, such as transforming growth factor (TGF)β1 (Todd, Luzina, & Atamas, 2012). However, myofibroblast differentiation is also affected by the extracellular matrix. Cell surface receptors like integrins allow cells to chemically and mechanically probe their environment and to respond to the composition of the extracellular matrix (Huang & Ogawa, 2012; Wells, 2013). The composition and architecture of the matrix determine which specific attachment sites are available to the cells and also influence the mechanical properties of the matrix and determine the mechanical loading experienced by the cells during, for example, breathing (Suki & Bates, 2008). Via this route, changes in the extracellular matrix during the development of fibrosis will influence cell behavior such as myofibroblast differentiation. This was confirmed by seeding lung fibroblasts into decellularized matrix from IPF patients and healthy controls, resulting in an increased expression of myofibroblast markers in IPF matrix (Booth et al., 2012), thus indicating that changes in the extracellular matrix composition determine disease progression.

Possible candidates for specific proteins within the extracellular matrix regulating fibrotic cellular processes can be derived from our earlier study (Blaauboer et al., 2013). In that study we measured new collagen formation by analysis of deuterated water incorporation into hydroxyproline in mice with bleomycin-induced lung fibrosis. We combined this with microarray analysis and correlating these results allowed us to identify fibrosis-relevant changes in gene expression within this model. Interestingly, three extracellular matrix proteins were strongly correlated with new collagen formation: elastin, type V collagen and tenascin C. Patients with IPF and its histopathological equivalent usual interstitial pneumonia, also have increased levels of elastin (Cha et al., 2010), type V collagen (Parra et al., 2006) and tenascin C (Fitch, Howie, & Wallace, 2011; Kuhn & Mason,
Therefore, these proteins are attractive candidates in the search for regulatory roles of extracellular matrix proteins in fibrosis.

Direct effects of type V collagen on fibrosis-related pathways have been described in the literature. Exposure to this extracellular matrix protein results in inflammatory responses (Braun et al., 2010), that could affect the development of fibrosis, for example via fibrosis-relevant cytokine release by immune cells (Todd et al., 2012). This is in line with the fact that patients with IPF are more often hypersensitive to type V collagen (Bobadilla et al., 2008). Furthermore, immunization of rabbits with type V collagen was shown to result in scleroderma-like symptoms in the skin (Bezerra et al., 2006) and increased remodeling in the lung (Teodoro et al., 2004).

Also tenascin C could play a regulatory role in the development of fibrosis since it increases cell migration (Trebaul, Chan, & Midwood, 2007) and migration of cells is important for the recruitment of myofibroblasts. This was confirmed by observations that interstitial cells express tenascin C after myocardial infarction and myofibroblasts appear in tenascin C positive areas (Imanaka-Yoshida et al., 2001). Furthermore, tenascin C knockout mice have a decreased recruitment of myofibroblasts after myocardiac wounding (Tamaoki et al., 2005) or during the progression of hepatitis to liver fibrogenesis (El-Karef et al., 2007). Finally, fragments of tenascin C resulting from its breakdown, were shown to inhibit fibroblast migration (Trebaul et al., 2007).

These observations emphasize the reciprocal relationship between changes in matrix composition and cellular contributions to fibrosis development. In this study, we aimed to further unravel this reciprocal relationship between cells and matrix during fibrosis development. For this, we first analyzed the expression of elastin, type V collagen and tenascin C at different time points during the development of bleomycin-induced lung fibrosis. Then we addressed the role of lung fibroblasts and myofibroblasts in the changed expression of these extracellular matrix proteins. Since it is not known if elastin has similar fibrosis-inducing effects as type V collagen and tenascin C, we investigated the effect of elastin on lung fibroblasts and the differentiation to myofibroblasts.
Experimental Procedures

Animal procedures
All animal procedures were approved by TNO Animal Welfare Committee (#2738). Female C57Bl/6j mice (Charles River Laboratories, Germany) 10 to 12 weeks of age received intratracheal instillation of 30 μl bleomycin (Pharmachemie BV, Haarlem, The Netherlands; 1.25 U/ml in PBS). To label new collagen the mice received 35 μl deuterated water (D2O) / gram body weight (i.p.) at 7 days before sacrifice and normal drinking water was replaced with 8% deuterated water. Water was refreshed every second day.

Mice were sacrificed by CO2 asphyxiation at 1 (n=8), 2 (n=8), 3 (n=8), 4 (n=6) or 5 (n=7) weeks after bleomycin treatment. Untreated animals were used as control (t = 0 wk, n=7). After sacrifice, the left lung lobe was fixed with 10% formalin and processed for histology; the cranial lung lobe was stored at -80°C until determination of D2O incorporation in hydroxyproline while the caudal lobe was snap-frozen in liquid nitrogen for microarray analysis.

Microarray analysis
Gene expression data were retrieved from the microarray analysis dataset published in (Blaauboer et al., 2013) and accessible online through GEO Series accession number GSE37635 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37635).

Kinetic analysis
Deuterated water incorporation into hydroxyproline was used as a measure for new collagen formation and the analysis is described earlier in (Blaauboer et al., 2013).

Histology and immune-histochemical staining
Formalin-fixed tissues were embedded in paraffin, sectioned in 5 μm sections, and stained extracellular matrix proteins. To deparaffinize, the slides were immersed in xylene and rehydrated in decreasing ethanol concentrations.

To investigate expression of elastin, sections were incubated for 1 h in resorcin-fuchsin solution (Electron Microscopy Sciences, Hatfield, PA, USA), differentiated in 96% and 100% ethanol, followed by xylene.

The expression of type V collagen and tenasin C protein was assessed by immuno-histochemical staining. During the deparaffinization process, in between the 100% ethanol steps, endogenous peroxidase in the lung tissue was blocked (20 minutes at room temperature; 0.3% H2O2 in methanol). For type V collagen staining, the sections were incubated after deparaffinization in a citrate-buffer (10mM tri-sodium citrate dehydrate in H2O; pH = 6) at 100°C for 10 minutes for antigen retrieval. For both stainings, aspecific protein binding was blocked for 15 minutes with bovine serum albumin (BSA, 1% in PBS) at
room temperature. The sections were incubated overnight at 4°C with polyclonal rabbit anti-type V collagen antibodies with biotin label (Acris antibodies, Herford, Germany) or 2 h at room temperature with polyclonal rabbit anti-tenascin C (Millipore, Amsterdam, The Netherlands) diluted 1:200 in 1% BSA in PBS. As unspecific rabbit antibody Dako Universal Negative Control Rabbit (Dako, Heverlee, Belgium) was used diluted 1:100 in 1% BSA in PBS. Hereafter, bound immunoglobulin was detected by incubating for 45 minutes (tenascin C staining) or 60 minutes (type V collagen staining) at room temperature with the labeled polymer from the EnVision anti-rabbit Kit (Dako), followed by an incubation for 8 minutes in DAB substrate solution (Vector, Burlingame, CA, USA). Then nuclei were stained using Hematoxylin Mayer solution. Sections were dehydrated again using increasing ethanol concentrations, followed by xylene incubation. Finally the sections were covered with depex and a coverslip.

All sections were photographed with a CRi Nuance FX Multispectral Camera (Quorum Technologies, Ontario, Canada).

Cell culture
Primary normal human lung fibroblast (NHLF) cells were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA). Human fetal lung (HFL-1) fibroblasts were obtained from ATCC (ATCC, Wesel, Germany). NHLF cells were cultured in Dulbecco’s minimal essential medium (D-MEM; Invitrogen, Paisley, UK) and HFL-1 cells were cultured in F12K medium (ATCC), both supplemented with 10% fetal clone serum (FCS; HyClone, South Logan, UT, USA) and 1% antibiotic-antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (PSA), Sigma-Aldrich, St. Louis, MO, USA) in an incubator set at 37 °C, 95% humidity, and 5% CO₂. Once grown to confluency, cells were trypsinized using 0.5% trypsin (Sigma-Aldrich) and 0.1% EDTA (Merck, Darmstadt, Germany) in PBS (Invitrogen).

TGFβ₁-response experiments
Cells from passage 6 (NHLF cells) or passage 16 (HFL-1 cells) were seeded in D-MEM with 10% FCS and 1% PSA at a density of 50,000 cells/cm² in a 24 wells plate. After allowing the cells to attach for 24 h, medium was replaced by D-MEM with 1% FCS and 1% PSA and 24 h later myofibroblast differentiation was induced by replacing the medium with D-MEM supplemented with 1% FCS and 1% PSA containing 0 to 20 ng/ml recombinant human TGFβ₁ (PeproTech EC, London, UK). Fibroblasts were cultured for 24 h in the presence or absence of TGFβ₁ and mRNA samples were collected.

Elastin and collagen coating
For each experiment, NHLF cells from passage 6 to 10 were seeded at a density of 3,000 or 10,000 cells/cm² on BioFlex type I collagen or elastin-coated six-well culture plates (Flexcell International Corp, McKeesport, PA, USA). After allowing the cells to attach for 24 h,
medium was replaced by D-MEM with 1% FCS and 1% PSA and 24 h later myofibroblast differentiation was induced by replacing the medium with D-MEM supplemented with 1% FCS and 1% PSA containing 0 or 10 ng/ml recombinant human TGFβ1. Fibroblasts were cultured for 48 h in the presence or absence of TGFβ1 and mRNA samples were collected.

Quantitative real-time PCR
mRNA was isolated from cell culture experiments using an RNeasy Mini Kit for RNA extraction (Qiagen, Hilden, Germany). The mRNA concentration was measured using a Synergy HT microplate reader (Biotek Instruments, Winooski, VT, USA). mRNA was reverse-transcribed to complementary DNA (cDNA) using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). mRNA expression of elastin (ELN), the α1 chain of type V collagen (COL5A1), tenascin C (TNC), αSMA (ACTA2), and the α1 chain of type I collagen (COL1A1) and the housekeeping gene GAPDH was analyzed by Real-Time PCR performed on a 7500 Fast Real-Time PCR system (Applied Biosystems). GAPDH mRNA expression was determined using TaqMan® Rodent GAPDH Control Reagents (Applied Biosystems). All other genes were analyzed using unique TaqMan® Assays-on-Demand™ Gene Expression kits (Table 1; Applied Biosystems) specific for human.

Table 1: Gene product, Gene, GenBank ID, and Assay-on-Demand™ used in this study.

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Statistics
Statistical analyses were performed using SPSS (version 20, IBM Corporation, Armonk, NY, USA). Results of the in vitro experiments were evaluated using two-way ANOVA or with Bonferroni-adjusted t-tests for multiple comparisons between groups.

Using Microsoft Office Excel (2007, Microsoft Corporation, Redmond, WA, USA) Pearson’s correlation coefficients were calculated over all data points of all time points to correlate new collagen formation to single gene data.
Results

Elastin, type V collagen, and tenascin C strongly correlate with new collagen deposition in bleomycin-induced lung fibrosis

In vivo new collagen formation, as measured by incorporation of deuterated water into hydroxyproline, correlated strongly with gene expression of elastin ($r = 0.93$, Figure 1A), the $\alpha_1$ chain of type V collagen ($r = 0.84$, Figure 1B) and tenascin C ($r = 0.88$, Figure 1C) during the development of bleomycin-induced lung fibrosis.

![Graphs showing correlations between gene expression and new collagen formation](image)

**Figure 1:** During bleomycin-induced lung fibrosis, gene expression of elastin, type V collagen, and tenascin C is highly correlated with new collagen formation; the core process of fibrosis. Correlation between new collagen formation as measured by deuterated water incorporation in hydroxyproline and A) elastin gene (ELN) expression, B) gene expression of the $\alpha_1$ chain of type V collagen (COL5A1), and C) tenascin C gene (TNC) expression. Each point represents data of one experimental animal, lines represent trend-lines from linear regression.
**Increased protein deposition of elastin, type V collagen and tenascin C during bleomycin-induced lung fibrosis**

Resorcin-fuchsin staining in lung sections of healthy control mice indicates the presence of elastin around blood vessels and at the tips of alveolar septae (Figure 2A). In fibrotic lung sections, elastin was increasingly found in fibrotic areas at all time points (Figure 2B-D).

Type V collagen was present in healthy lung tissue in blood vessel walls and in a thin layer around bronchioles (Figure 2E). In fibrotic lungs, during the first two weeks after fibrosis-induction by bleomycin instillation type V collagen was increased in fibrotic areas (Figure 2F and 2G). At 4 weeks, type V collagen immunostaining was decreased compared to the high levels in the first two weeks (Figure 2H).

In healthy lung tissue minimal tenasin C staining was present in only a few alveolar structures (Figure 2I). Extracellular tenasin C was observed at high levels in fibrotic areas 1 and 2 weeks after bleomycin instillation (Figure 2J and 2K). At 4 weeks, extracellular tenasin C levels were reduced (Figure 2L). From 2 weeks onward, tenasin C was also visible intracellularly, which was especially obvious at the later time points (Figure 2K and 2L).

**Elastin, type V collagen, and tenascin C mRNA expression is increased by TGFβ1 stimulation in human lung fibroblasts**

Culturing normal human lung fibroblasts (NHLFs) and human fetal lung fibroblasts (HFL1s) in the presence of TGFβ1 to mimic fibrotic conditions resulted in differentiation into the myofibroblastic phenotype, as indicated by a dose-dependent increase in mRNA expression of αSMA (ACTA2) and the α1 chain of type I collagen (COL1A1) (Figure 3A-D). Under these fibrotic conditions, mRNA expression of elastin (ELN), the α1 chain of type V collagen (COL5A1), and tenasin C (TNC) was dose-dependently increased as well (Figure 3E-J). Especially elastin mRNA expression was strongly upregulated in NHLFs (up to 24-fold at 10 ng/ml TGFβ1) and HFL1s (up to 62-fold at 10 ng/ml TGFβ1).

**Elastin coating increases TGFβ1-induced mRNA expression of α-smooth muscle actin, elastin, and tenascin C in human lung fibroblasts**

NHLF cells cultured on elastin-coated surfaces had an increased mRNA expression of αSMA (ACTA2) and type I collagen (COL1A1) compared to NHLF cells cultured on collagen coated surfaces in the presence of 10 ng/ml TGFβ1 (Figure 4A and 4B), indicating increased myofibroblast differentiation in the presence of extracellular elastin. Extracellular elastin did also increase mRNA expression of elastin (ELN) both in the absence and presence of 10 ng/ml TGFβ1 (Figure 4C).
Extracellular matrix proteins: a positive feedback loop in lung fibrosis?

Figure 2: The extracellular matrix proteins are visible at the protein level on histological staining in fibrotic areas of lungs with bleomycin-induced lung fibrosis.

In healthy tissue, elastin and type V collagen are present around blood vessels (closed arrow heads). Elastin is also visible at the tips of alveolar septae (open arrow heads) and in the walls of the bronchioles. Tenascin C is not present in healthy tissue, except for very small areas (open arrow). In fibrotic tissue, elastin is increasingly present extracellularly during the time course of bleomycin-induced lung fibrosis, localized at higher levels at the same locations compared to in healthy tissue. Fibrotic areas (closed arrows) are extensively stained for elastin. Type V collagen and tenascin C are present extracellularly in fibrotic areas at increased levels 1 and 2 weeks after bleomycin induction and reduce thereafter. From 2 weeks on, tenascin C was also found intracellularly (line arrows). A-D) Elastin staining, blue-purple, E-H) immunohistochemical staining of type V collagen, dark brown, and hematoxylin staining of nuclei, blue-purple, I-L) immunohistochemical staining of tenascin C, brown, and hematoxylin staining of nuclei, blue-purple, in control mice lungs (A, E, I), and 1 week (B, F, J), 2 weeks (C, G, K), and 4 weeks (D, H, L) after induction of bleomycin-induced lung fibrosis. Scale bar: 100 µm.
Figure 3: mRNA expression of elastin, type V collagen, and tenasin C is highly upregulated by TGFβ₁ stimulation in lung fibroblasts.

mRNA expression of A-B) αSMA (ACTA2), C-D) the α₁ chain of type I collagen (COL1A1), E-F) elastin (ELN), G-H) the α₁ chain of type V collagen (COL5A1), and I-J) tenasin C (TNC) in NHLF cells (A, C, E, G, and I) and HFL-1 cells (B, D, F, H, and J) after 24 h of stimulation with 0 to 20 ng/ml TGFβ₁. Gene expression is shown relative to mRNA expression of the household gene GAPDH. Data are normalized to 0 ng/ml TGFβ₁ and shown as mean ± standard deviation (n = 4 or 5). Significant differences with unstimulated control are indicated. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 4: mRNA expression of elastin, type V collagen, and tenascin C is increased on elastin coated surfaces.

mRNA expression of A) α-smooth muscle actin (ACTA2), B) the α1 chain of type I collagen (COL1A1) and C) elastin (ELN) in NHLF cells growing on either type I collagen or elastin coating after 48 h of stimulation with 0 or 10 ng/ml TGFβ1. Data are shown as mean ± standard deviation (n = 6 for collagen coating, n = 12 for elastin coating). Significant differences between type I collagen and elastin coating are indicated. * p < 0.05, ** p < 0.01, *** p < 0.001.
Discussion

In this study, we aimed to shed light on the reciprocal relationship between the cells and the changing extracellular matrix following induction of lung fibrosis with bleomycin. We found increased gene expression of elastin, type V collagen and tenascin C in mice with active fibrosis and confirmed this finding at the protein level in histological sections. Furthermore, fibroblastic cells increased expression of these extracellular matrix proteins under fibrotic conditions in vitro, suggesting a contribution of myofibroblasts in the increased levels found in vivo. Finally, we uncovered a regulatory role for elastin in myofibroblast differentiation, emphasizing the possibility that a positive feedback loop plays a role in the progressive nature of lung fibrosis in patients.

In an earlier study, we quantified the core process of fibrosis – new collagen formation – in the bleomycin-induced lung fibrosis model by measuring deuterated water incorporation in hydroxyproline. We used this measure of fibrotic activity in the lung to select fibrosis-relevant genes from microarray results by correlating new collagen formation values with gene expression data from the same mice (Blaauboer et al., 2013). Interestingly, gene expression of three extracellular matrix proteins was strongly correlated with new collagen formation: elastin, the α₁ chain of type V collagen and tenascin C. An upregulation of mRNA expression of elastin and tenascin C is in line with data presented by others: bleomycin-induced lung fibrosis increased levels of elastin mRNA in mice (Lucey et al., 1996) and tenascin C mRNA levels in rats (Zhao et al., 1998). To our knowledge, we are the first to report increased type V collagen mRNA levels during bleomycin-induced lung fibrosis. This indicates that increased staining of type V collagen protein could at least in part be the result of new collagen production, besides the possibility of increased epitope availability of type V collagen protein already present in the tissue, due to increased remodeling of the tissue. Furthermore, the clear correlation of elastin, type V collagen and tenascin C mRNA expression to new collagen formation indicates that the highest levels of mRNA expression of these matrix proteins are present specifically in mice with very active fibrosis development, further emphasizing the relevance of this finding.

By histology we further confirmed the presence of elastin, type V collagen and tenascin C at the protein level. In healthy lung tissue, elastin was present in blood vessels walls, bronchiolar walls and alveolar septae, as described before (Mariani et al., 1997). During the development of fibrosis elastin levels increased. The patterns of expression in healthy and fibrotic lung tissue correspond to earlier descriptions of end-stage lung fibrosis induced by bleomycin (Collins et al., 1981; Dolhnikoff et al., 1999; Laurent et al., 1981; Starcher et al., 1978), by overexpression of TGFβ₁ (Sime et al., 1997; Tarantal et al., 2010) and by butylated hydroxytoluene with 70% oxygen (Hoff et al., 1999). In the last model, the abnormal elastic
fiber morphology persisted in the mice lungs for at least 6 months. This is in line with the seemingly permanent changes in deposited elastin in the current study, where levels of elastin were increasing until five weeks after fibrosis-induction. Earlier, it was suggested that elastin proteins level in liver fibrosis were regulated at the level of degradation by MMP12 (Pellicoro et al., 2012). Here, we also find a clear elevation in mRNA levels of elastin, suggesting that in bleomycin-induced lung fibrosis elastin is regulated at least in part at the transcriptional level or at the posttranscriptional level. This posttranscriptional level is an interesting candidate, since it has been reported before that TGFβ1 regulates the stability of elastin mRNA (Kähäri et al., 1992).

Type V collagen and tenascin C were present at low levels in healthy lung tissue. This corresponds to reports in the literature, that there is normally little type V collagen (Parra et al., 2006) and almost no tenascin C (Kaarteenaho et al., 2010) present in healthy lung tissue. The small spots of tenascin C we observed in healthy lung sections might be areas with minor damage to the tissue, occurring during normal use of the lungs, since expression of tenascin C has been related to tissue damage (Chiquet-Ehrismann and Chiquet, 2003).

Interestingly, intense immunostaining of both type V collagen and tenascin C was present only in the first few weeks after fibrosis induction and levels decreased from 3 weeks on. In the case of type V collagen, it is unclear if this decrease in immunostaining is the result of a decrease in type V collagen protein levels in the tissue, or the result of masking of the type V collagen epitope by type I collagen, since type V collagen is often present in tissues as a component buried within type I collagen fibrils (Birk, 2001; Fichard et al., 1995). The transient upregulation of tenascin C reported here is supported by earlier findings in rat lungs during bleomycin-induced lung fibrosis (Zhao et al., 1998): immunohistochemical staining of tenascin C in this rat model was strongest in intensity at 8 days, and reduced at 12 days after induction.

In patients with pulmonary fibrosis expression of type V collagen and tenascin C seems to be more permanent than in the rodent bleomycin model. These patients are considered to be at the end stage of fibrosis development. Even though, in patients with usual interstitial pneumonia, the histopathological equivalent of IPF, expression of type V collagen within fibroblastic foci is increased and the most important predictor of survival, with higher levels of type V collagen being related to a lower chance of survival (Parra et al., 2006). Also, tenascin C is present throughout the matrix of the fibroblastic focus in IPF patients (Kuhn and Mason, 1995) and in patients with usual interstitial pneumonia (Fitch et al., 2011), who also have higher levels of tenascin C in their bronchoalveolar lavage fluid (Kaarteenaho-Wiik et al., 1998).

It is attractive to speculate about the meaning of this difference in time-dependence of expression of type V collagen and tenascin C during IPF and the development of bleomycin-induced lung fibrosis. Both type V collagen and tenascin C could have a regulatory role during the development of pulmonary fibrosis: type V collagen increases
inflammation and tenasin C increases myofibroblast recruitment. Therefore, the transient appearance of these extracellular matrix proteins in the rodent model versus the prolonged presence in human lung fibrosis patients could explain why lung fibrosis in the rodent bleomycin model is resolving (Moore et al., 2013), while patients have not been shown to improve.

Using whole lung RNA for the microarray analysis that includes RNA of the many cell types present within the lung allowed us to explore fibrosis relevant cellular processes in all these cell types in this in vivo model. However, as a consequence these data do not reveal the cell type(s) responsible for the increased expression. To investigate the possible role of fibroblastic cells in the increased expression of elastin, type V collagen and tenasin C during the development of lung fibrosis, we cultured human lung fibroblasts under fibrotic conditions, i.e. in the presence of TGFβ1, to induce myofibroblast differentiation. Under these fibrotic conditions, we found a strong increase in gene expression of elastin, type V collagen and tenasin C. This increased expression of these extracellular matrix proteins could be phenotypical for myofibroblasts, suggesting a fundamental role in different types of fibrosis. Such is confirmed by reports in the literature describing increases in gene expression in response to TGFβ1 in different cell types: for elastin in neonatal rat lung fibroblasts (McGowan and McNamer, 1990), for the α2 chain of type V collagen in human lung fibroblasts (Blaauboer et al., 2011) and for tenasin C in chick embryo fibroblasts (Pearson et al., 1988) and the alveolar epithelial cell line A549 (Fitch et al., 2011). Moreover, transdifferentiation of hepatic stellate cells into myofibroblasts by culturing on plastic also increased expression of elastin (Kanta et al., 2002). The fact that in the in vitro model human fibroblasts are used, indicates that our results from the in vivo mouse model are relevant for human pulmonary fibrosis.

Fibrotic changes in the composition of the extracellular matrix can have a regulatory role during fibrosis development, thereby completing a positive feedback loop explaining the progressive nature of fibrotic diseases. Here, we report that in addition to a reported role for type V collagen and tenasin C (Braun et al., 2010; Trebaul et al., 2007), also elastin has a pro-fibrotic effect, since culturing human lung fibroblasts on elastin-coated culture plates resulted in increased expression of the myofibroblast marker α-smooth muscle actin and type I collagen, indicating an increased myofibroblast differentiation in the presence of extracellular elastin. Furthermore, this effect is self-amplifying, since elastin coating also increased mRNA expression of elastin itself. Thus, the effect of an increased presence of elastin in the fibrotic lung on myofibroblast differentiation is one more example of how changes in the extracellular matrix contribute to the progressive development of lung fibrosis.
Extracellular matrix proteins: a positive feedback loop in lung fibrosis?

We have shown before that cyclic mechanical stretch, mimicking the in vivo breathing movement, reduces TGFβ1-induced myofibroblast differentiation (Blaauboer et al., 2011). In that study, mRNA levels of the α2 chain of type V collagen and tenascin C were also decreased by cyclic mechanical stretch. Here we found that gene expression of elastin and the α1 chain of type V collagen were also reduced after cyclic mechanical stretch (Supplemental data). The relevance of the reduced gene expression after mechanical loading is related to the fact that, during the development of lung fibrosis, the lung tissues increases in stiffness (Booth et al., 2012; Ebihara et al., 2000; Liu et al., 2010). This could result in lower cyclic mechanical stretch for the fibroblasts in the lung tissue. The lower cyclic mechanical stretch can increase myofibroblast differentiation both directly, via an increase of α-smooth muscle actin and type I collagen expression, and indirectly, via an increase in elastin expression, which, in turn, also increases myofibroblast differentiation.

In summary, elastin, type V collagen and tenascin C are increasingly expressed in mice suffering from active fibrosis development, as characterized by high levels of new collagen formation. In vitro studies indicate a role for fibroblastic cells in this increased expression. Furthermore, the presence of extracellular elastin further increases myofibroblast differentiation, contributing to disease progression. Reduced levels of mechanical stimulation of lung fibroblasts due to stiffening of fibrotic tissue will further increase levels of elastin, type V collagen and tenascin C, and their effects on fibroblasts. From these data we conclude that the development of lung fibrosis could depend on a feedback loop due to the reciprocal interaction between fibroblasts and the extracellular matrix composition, in particular elastin, type V collagen and tenascin C.
Acknowledgements

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Supplemental data

**Supplemental Figure 1:** TGFβ1-induced mRNA expression of elastin and type V collagen is reduced by cyclic mechanical stretch.

mRNA expression of A) elastin (ELN) and B) the α1 chain of type V collagen (COL5A1) in NHLF cells after cyclic mechanical stretch. NHLF cells from passage 6 were seeded at a density of 3,000 or 10,000 cells/cm² on BioFlex type I collagen coated six-well culture plates (BioFlex, Flexcell International Corp, McKeesport, PA, USA). After allowing the cells to attach for 24 h, medium was replaced by D-MEM with 1% FCS and 1% PSA and 24 h later cells were subjected to 48 h of cyclic mechanical strain in a Flexercell FX4000 apparatus (Flexcell International Corp) placed in an incubator set at 37°C, 95% humidity and 5% CO₂. Cyclic mechanical loading was applied in a sinusoidal pattern with a frequency of 0.2 Hz and a maximum elongation of 10%. Control cells not receiving cyclic mechanical loading were placed in the same incubator next to the loading station of the Flexercell apparatus. During mechanical loading, 50% of the samples were stimulated with 10 ng/ml TGFβ1. After mechanical loading mRNA samples were collected and analyzed as described in the Experimental Procedures. Gene expression is shown relative to mRNA expression of the household gene GAPDH. Data are shown as mean ± standard deviation (n = 6). Significant differences with static control are indicated. * p < 0.05, ** p < 0.01, *** p < 0.001.
References


