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Novel combination of collagen dynamics analysis and transcriptional profiling reveals fibrosis-relevant genes and pathways

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ABSTRACT

Collagen deposition is a key process during idiopathic pulmonary fibrosis; however, little is known about the dynamics of collagen formation during disease development. Tissue samples of early stages of human disease are not readily available and it is difficult to identify changes in collagen content, since standard collagen analyses do not distinguish between 'old' and 'new' collagen. Therefore, the current study aimed to (i) investigate the dynamics of new collagen formation in mice using bleomycin-induced lung fibrosis in which newly synthesized collagen was labeled with deuterated water and (ii) use this information to identify genes and processes correlated to new collagen formation.

Lung fibrosis was induced in female C57Bl/6 mice by bleomycin instillation. Animals were sacrificed at 1 to 5 weeks after fibrosis induction. Collagen synthesized during the week before sacrifice was labeled with deuterium by providing mice with deuterated drinking water. After sacrifice, we collected lung tissue for microarray analysis, determination of new collagen formation, and histology. Furthermore, we measured *in vitro* the expression of selected genes after transforming growth factor (TGF) β_1-induced myofibroblast differentiation.

Deuterated water labeling showed a strong increase in new collagen formation already during the first week after fibrosis induction and a complete return to baseline at five weeks. Correlation of new collagen formation data with gene expression data allowed us to create a gene expression signature of fibrosis within the lung and revealed fibrosis-specific processes, among which proliferation. This was confirmed by measuring cell proliferation and collagen synthesis simultaneously using deuterated water incorporation in a separate experiment. Furthermore, new collagen formation strongly correlated with gene expression of e.g. elastin, Wnt-1 inducible signaling pathway protein 1, tenascin C, lysyl oxidase, and type V collagen. Gene expression of these genes was upregulated *in vitro* in fibroblasts stimulated with TGFβ_1.

Together, these data demonstrate, using a novel combination of technologies, that the core process of fibrosis, i.e. the formation of new collagen, correlates not only with a wide range of genes involved in general extracellular matrix production and modification but also with cell proliferation. The observation that the large majority of the genes which correlated with new collagen formation also were upregulated during TGFβ_1-induced myofibroblast differentiation provides further evidence for their involvement in fibrosis.

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1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating fibrosing lung disease and is characterized by excessive matrix deposition, destroying both tissue architecture and function. The incidence of IPF is estimated to be 5 to 10 in 100,000 (Fernandez Perez et al., 2010); most patients die within 2 to 5 years after diagnosis. This is related to the fact that IPF is often diagnosed quite late, due to the overcapacity of the lung tissue. Only once extensive damage to the lung tissue has taken place, symptoms such as shortness of breath occur leading to diagnosis.

Abbreviations: D_2O, deuterated water; EM1, natural abundance fraction; GC–MS, gas chromatography–mass spectrometry; GO, gene ontology; Hyp, hydroxyproline.

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Because of this late diagnosis, the etiology of IPF is largely unknown and little information is available about the early phases in the development of lung fibrosis. To gain more insight into these early phases of fibrosis development, research heavily relies on animal models (Chua et al., 2005).

The most extensively used model to study lung fibrosis uses intratracheal instillation of bleomycin into the lungs of mice as an inducer (Chua et al., 2005; Moeller et al., 2008). The proposed mechanism is that bleomycin-induced generation of reactive oxygen species results in extensive epithelial necropsy. This damage induces an inflammatory response with a peak around 1 week after bleomycin instillation, followed by a phase in which extensive fibrotic remodeling occurs (Chaudhary et al., 2006; Moeller et al., 2008). To elucidate which genes and processes are involved in the development of fibrosis, microarray analyses have been used (Kaminski et al., 2000; Hannivoort et al., 2012). These types of studies have shown that during the development of bleomycin-induced lung fibrosis, many changes in gene expression patterns occur. However, as with all animal models, it is difficult to distinguish which processes are important for fibrosis development in general and which represent model-specific changes. Therefore, the challenge is how to identify the fibrosis-specific pathways from the total changes in gene expression.

Collagen deposition is the hallmark of fibrosis. In the bleomycin model total collagen content of the lung, measured by the amount of hydroxyproline (Hyp), is a standard outcome parameter used to evaluate fibrosis (Chua et al., 2005). However, this parameter is sometimes less than optimal since healthy lung already contains substantial amounts of collagen, making it difficult to identify subtle but important changes in collagen deposition early in the process of fibrosis. Therefore, in the current study we focused on the formation of newly synthesized collagen during the 7 days prior to sacrifice by deuterated water labeling. The deuterated water is incorporated into all proteins produced during this period and after sacrifice the amount of labeled hydroxyproline is a parameter of newly deposited collagen (Gardner et al., 2007).

Many processes have been described to be upregulated during experimental lung fibrosis, such as activation of macrophages and neutrophils (Moeller et al., 2008), inflammatory cytokine production (Moeller et al., 2008), transforming growth factor (TGF) β-signaling (Degryse et al., 2011), and epithelial-to-mesenchymal transition (Tanjore et al., 2009). The contribution of these processes to the fibrotic process occurring in the lung is still unclear. Therefore, we aimed in this study to identify which genes and processes specifically relate to the collagen deposition during bleomycin-induced lung fibrosis by combining new collagen formation with gene expression data at different time points after bleomycin treatment. Our results

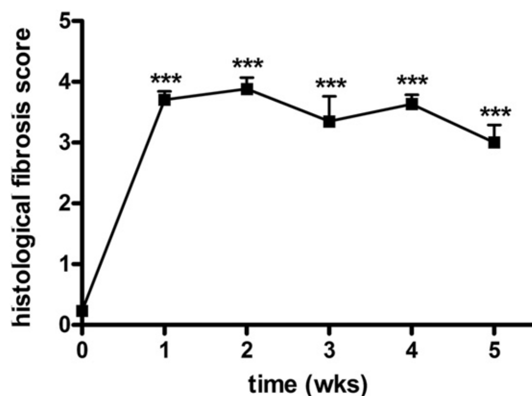

Fig. 1. Fibrosis induction in mice treated with bleomycin. Histological fibrosis score was determined from Masson's trichrome stained paraffin sections collected at 1 ($n = 8$), 2 ($n = 8$), 3 ($n = 8$), 4 ($n = 6$), and 5 ($n = 7$) weeks after intratracheal bleomycin instillation or from untreated animals at time point 0 ($n = 7$) as control. Results are given as mean ± SEM. *** $p < 0.001$.

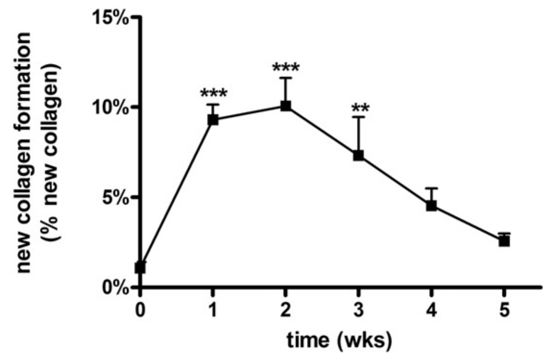

Fig. 2. Kinetics of new collagen formation during bleomycin-induced lung fibrosis in mice. Percentage of Hyp containing deuterated water as a measure of collagen deposited in the last 7 days before sacrifice at 1 ($n = 8$), 2 ($n = 8$), 3 ($n = 8$), 4 ($n = 6$), and 5 ($n = 7$) weeks after intratracheal bleomycin instillation or from untreated animals at time point 0 ($n = 7$) as control. Results are given as mean ± SEM. ** $p < 0.01$, *** $p < 0.001$.

indicate a strong correlation of new collagen formation with processes such as cell proliferation and extracellular matrix production and with specific genes. We provided further evidence for involvement of these genes in lung fibrosis by showing increased expression after $TGF\beta_1$-induced myofibroblast differentiation *in vitro*. This provides new leads towards the understanding of the etiology of lung fibrosis.

2. Results

2.1. Fibrosis induction

Light microscopic analysis and the use of the modified Ashcroft fibrosis score (Fig. 1) showed that fibrosis was strongly induced between week 0 and week 1, and remained high for at least 5 weeks after fibrosis induction by bleomycin.

2.2. New collagen formation

New collagen, determined as incorporation of deuterated water in Hyp, was significantly increased (9.3%, $p < 0.001$) one week after bleomycin administration in comparison to the level in control mice (1.1%) (Fig. 2). The maximal formation of collagen was found two weeks after the bleomycin administration, when 10.1% ($p < 0.001$) of the collagen contained labeled Hyp. After this period the collagen deposition decreased steadily, leading eventually to 2.4% new collagen five weeks after bleomycin exposure, which is similar to control non-bleomycin treated mice ($t = 0$).

These data indicate that new collagen is mainly formed within the first weeks after bleomycin exposure.

2.3. Correlation between new collagen formation and gene expression

To study which processes are involved in collagen deposition in bleomycin-induced lung fibrosis, we correlated gene expression data from microarray analysis with the level of newly synthesized collagen in the individual animals. The genes that are most highly positively or negatively correlated with the deposition of collagen are shown in Table 1.

Extracellular matrix-related proteins were well represented in the group of genes highly positively correlated to collagen deposition. In this group there are extracellular matrix proteins such as elastin ($r = 0.88$), tenascin C ($r = 0.85$), and collagen type V ($r = 0.81$), but also proteins related to the collagen deposition machinery, such as thrombospondin 2 ($r = 0.83$), playing a role in fibril formation and lysyl-oxidase ($r = 0.84$), important for the crosslinking of

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