Expressional Switches Leading to Contractile Fibroblast Modulation

USAMAH S. KAYYALI*, ANNE MARIE SOUSA*, MATTHIAS GAESTEL#, AND TIEGANG LIU*
*Pulmonary and Critical Care Division, Department of Medicine/Tupper Research Institute, Tufts-New England Medical Center and Tufts University School of Medicine, Boston, MA 02111, USA
#Institute of Biochemistry, Medical School Hannover, 30625 Hannover, GERMANY

Abstract: - Fibroblasts are cells that mediate a wide variety of responses including wound repair, tissue remodeling and fibrosis. In response to certain types of injury or inflammation these cells undergo certain expressional changes that lead to phenotypical alteration. In that process fibroblasts upregulate the expression of a smooth muscle form of α actin (smα) that causes them to become more contractile, i.e., they turn into myofibroblasts. Understanding the mechanism by which this phenotypic switch occurs is of great importance to understanding a variety of diseases ranging from organ fibrosis to pulmonary hypertension and asthma. Several signaling pathways, including those which involve p38 MAP kinase, have been proposed to be involved in the response of fibroblasts to profibrotic signals. This paper focuses on the mechanism by which profibrotic agents such as TGFβ and hypoxia might produce a myofibroblastic phenotype. The responses to hypoxia or TGFβ will be characterized in cultured fibroblasts obtained from wild type mice and mice in which the expression of MK2 has been disrupted (MK2-/-). Our findings suggest that activation of the p38/MK2- pathway in fibroblasts mediates increased expression of smα, increased actin stress fiber formation and increased contractility of the fibroblasts.

Key-Words: - Myofibroblast, MK2, remodeling, pulmonary hypertension, p38, actin

1 Introduction

Pulmonary hypertension is a disease characterized by narrowing and increased resistance of the pulmonary arteries the etiology of which is not well understood. While the disease can be primary or secondary to other conditions, a common feature in different types of pulmonary hypertension is vascular remodeling [for review, see 1]. In particular, extension of smooth muscle cells to parts of pulmonary arteries that are not usually muscularized, and thickening of the walls of pulmonary arteries by proliferation of fibroblasts that produce extracellular matrix proteins are prominent features of the disease. Indeed fibroblasts have been reported to constitute the first type of cells to respond to pulmonary hypertension producing stimuli, e.g., chronic hypoxia in animal models [2] [1]. This paper addresses the role of fibroblasts in pulmonary hypertension by focusing on signaling mechanisms that mediate their differentiation into myofibroblasts in response to hypoxia or TGFβ.

Differentiation of fibroblasts into the smooth muscle like myofibroblast is a hallmark of wound repair and fibrosis. In addition to the role this process plays in conditions such as pulmonary fibrosis and lung tissue remodeling recent studies have highlighted it in the remodeling that occurs in certain vascular diseases [for review, see 3]. The relation of this differentiation to pulmonary hypertension has not been elucidated. Myofibroblasts occur in areas of active fibrosis and are responsible for production and deposition of extracellular matrix proteins [4]. Myofibroblasts are derived from fibroblasts through the action of growth factors, such as, TGFβ [5]. Recently, hypoxia which is also known to upregulate TGFβ, has been shown to directly trigger the differentiation of fibroblasts into myofibroblasts in vitro [6], suggesting potential synergy between hypoxia and TGFβ.

2 Problem Formulation

The problem this paper deals with is the role the kinase MK2 might play in pulmonary hypertension. The experiments carried out focus on demonstrating that MK2 mediates the differentiation of fibroblasts to myofibroblasts. The hypothesis
being tested, outlined in Fig. 1 below, is that MK2 disruption disrupts the pathway leading to myofibroblast differentiation. Remodeling and fibrosis occur in different organs in response to different types of injury, and been placed on signals that cause fibroblasts to differentiate into myofibroblasts such as those generated by TGFβ [5, 7-9]. TGFβ has been reported to be elevated in the lungs of idiopathic pulmonary fibrosis patients [10]. This growth factor belongs to

**Hypoxia, Cytokines (TGFβ)**

![Diagram of Hypoxia, Cytokines (TGFβ)](image)

**Figure 1. Hypothesis: Disruption of MK2 signaling inhibits myofibroblast differentiation**

Current thinking on the mechanism by which they might arise includes a model of abnormal wound healing. For example, in response to injury or some unknown stimulus, inflammation occurs leading to the recruitment of fibroblasts. It is also possible that persistent hypoxia or persistent pulmonary arterial vasoconstriction which are both characteristics of pulmonary hypertension trigger fibroblast recruitment and activation without a full inflammatory reaction. These fibroblasts proliferate and differentiate into myofibroblasts, and when unresolved lead to vascular remodeling or fibrosis. Myofibroblasts occur in areas of active fibrosis and are responsible for production and deposition of extracellular matrix proteins [4]. The factors that determine the path to resolution versus persistence of fibroblasts and deposition of extracellular matrix are not fully understood, however considerable focus has

a large family of proteins, which include members such as bone morphogenetic proteins, and that are important in a variety of diseases, such as, cancer and pulmonary hypertension. TGFβ has been shown to stimulate a pathway that proceeds from cell surface receptors to the nucleus through Smad proteins [for review, see 11]. Yet TGFβ can signal independently of Smad proteins by activating Ras and Erk [12, 13], Rho GTPase and JNK [14], as well as p38 MAP kinase [15]. Indeed TGFβ-induced myofibroblast differentiation has been suggested to be mediated by p38, and inhibition of p38 reduced pulmonary [16, 17] and renal [18] fibrosis in animal models.

p38 is a stress-activated kinase that is activated in response to stimuli such as ultraviolet radiation and hyperosmolarity. Once p38 is activated, it phosphorylates a variety of substrates,
including the kinase MK2. Upon its phosphorylation by p38, MK2 becomes activated, and in turn, phosphorylates additional substrates, such as the small heat shock protein HSP27. When phosphorylated, HSP27 no longer blocks the polymerization of actin, thus resulting in the stabilization of actin fibers [19]. Recent studies both in vivo and in vitro [18, 20] have suggested that p38 plays an important role in the pathogenesis of fibrosis, even though some researchers reported no effect of inhibiting p38 on differentiation of lung fibroblasts into myofibroblasts [21]. We launched the experiments described in this report to examine if signaling downstream of the p38 pathway plays a role in fibroblast differentiation into myofibroblasts.

Our laboratory has already published that the p38-MK2-HSP27 pathway mediates cytoskeletal changes in endothelial cells exposed to hypoxia, which lead to increased actin stress fiber formation [22, 23]. Since similar actin reorganization is believed to occur when fibroblasts switch to myofibroblasts, we decided to examine whether embryonic fibroblasts from MK2-/- knockout mice differed in their filamentous actin distribution when compared to embryonic fibroblasts from wild type mice.

### 3 Problem Solution

Immortalized mouse embryonic fibroblasts (MEF) from wild type and MK2-/- knockout mice were prepared as described earlier [24]. These cells were treated with TGFβ (1 ng/mL) or hypoxia (3% oxygen) as we described earlier [23]. The immunocytochemical analysis and actin staining and immunolabeling have also been described earlier [23].

![Figure 2](image-url)

**Figure 2**: MEF from wild type mice contain more stress fibers than MEF from MK2-/- mice. Hypoxia caused filamentous actin to increase in wild type MEF, while it is redistributed to cortical regions in MK2-/- MEF.
3.1. Components downstream of p38 mediate actin cytoskeleton reorganization: We have described cytoskeletal changes in rat endothelial cells (EC) in response to hypoxia, and potential mechanisms involved in this process [23]. Hypoxia-induced actin redistribution is mediated by components downstream of p38, which we have shown to be activated in EC by hypoxia [23]. Our results indicate that MK2 (a substrate of p38) becomes activated by hypoxia, leading to the phosphorylation of one of its substrates, HSP27 [23]. Since HSP27 phosphorylation is known to alter actin distribution in response to other stimuli, we postulated that it also causes the actin stress fiber formation observed in hypoxia. This notion is supported by our observations that similar actin redistribution occurs in cells overexpressing constitutively active (ca)MK2 or phospho-mimicking (pm)HSP27 mutants [23]. We found that overexpressing dominant negative (dn)MK2 blocks the effects of hypoxia on the actin cytoskeleton [23].

p38-MK2-HSP27 pathway mediates the formation of stress fibers in response to stimuli such as hypoxia. We suspected that cells from MK2\(^{-/-}\) mice would contain less stress fibers than wild type because the p38-MK2-HSP27 pathway is disrupted in these cells. MEF were grown on collagen-coated cover slips and then fixed and stained for filamentous actin using rhodamine-phalloidin as we described earlier. As shown in Figure 2, MEF from wild type mice contain a significant amount of stress fibers. MEF from MK2\(^{-/-}\) mice contain significantly less stress fibers and filamentous actin appeared to be concentrated at cortical regions. This difference became more significant when fibroblasts were exposed to hypoxia (1 hr), which we have shown to induce actin stress fiber formation through the p38-MK2-HSP27 pathway.

3.2. Mouse embryonic fibroblasts from MK2\(^{-/-}\) knockout mice contain less stress fibers than wild type MEF: One hallmark of differentiation of fibroblasts into myofibroblasts is the increased expression of \(\alpha\)-actin (sm\(\alpha\)). When

Thus we have demonstrated that in endothelial cells, hypoxia stimulates the p38-MK2-HSP27 pathway leading to significant alteration in the actin cytoskeleton.

3.3 MEF from MK2\(^{-/-}\) mice express very little sm\(\alpha\) compared to wild type: One hallmark of differentiation of fibroblasts into myofibroblasts is the increased expression of \(\alpha\)-actin (sm\(\alpha\)). When cell lysates were immunoblotted for sm\(\alpha\), the protein was barely detectable in MK2\(^{-/-}\) MEF, compared to wild type MEF which expressed significant levels of sm\(\alpha\) (Fig. 3). These results indicate that MK2\(^{-/-}\) MEF are less differentiated than WT MEF.

![Figure 3](image-url)
3.4 Unlike wild type MEF, MK2−/− MEF do not differentiate into myofibroblasts in response to TGFβ or hypoxia. When MEF were exposed to hypoxia or TGFβ, the level of smooth muscle α-actin (smα) was significantly increased in wild type MEF, an event that indicates differentiation into myofibroblasts (Fig. 3). Such differentiation in response to TGFβ or hypoxia is relevant to the pathogenesis of pulmonary hypertension. When MK2−/− MEF were exposed to either TGFβ or hypoxia there was no increase in smα (Fig. 3). These results suggest that MK2 is critical for differentiation of fibroblasts into myofibroblasts and highlight its relevance as a target for intervention in pulmonary hypertension.

4 Conclusion

Differentiation of fibroblasts into myofibroblasts has been a subject of interest because of the role this step is believed to play in wound healing, remodeling, and fibrosis. Several signaling pathways have been implicated in this process. Our results indicate that MK2 plays an important role in myofibroblast differentiation as defined by the expression of smα. In particular we have demonstrated that embryonic fibroblasts derived from MK2-deficient mice lack stress fibers and smα. In addition, unlike their wild type counterparts the MK2-null cells do not respond to TGFβ or hypoxia by increasing smα production.

As to the physiological significance of our findings, MK2 might turn out to be a key switch in the differentiation of fibroblasts into myofibroblasts, and hence in normal and pathological processes that involve myofibroblast differentiation such as wound repair, and pulmonary hypertension. In conclusion, our findings indicate that MK2 plays an important role in the development of the myofibroblast phenotype, and implicate the kinase as a target for the treatment of disease processes such as pulmonary hypertension.

References:


