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Modulation of Smad signaling by non-TGFβ components in myofibroblast generation during wound healing in corneal stroma☆

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ABSTRACT

Corneal scarring/fibrosis disturbs normal transparency and curvature of the tissue and thus impairs vision. The lesion is characterized by appearance of myofibroblasts, the key player of the fibrogenic reaction, and excess accumulation of extracellular matrix. Inflammatory/fibrogenic growth factors or cytokines expressed in inflammatory cells that infiltrate into injured tissues play a pivotal role in fibrotic tissue formation. In this article the pathogenesis of fibrosis/scarring in the corneal stroma is reviewed focusing on the roles of myofibroblast, the key player in corneal stromal wound healing and fibrosis, and cytoplasmic signals activated by the fibrogenic cytokine, transforming growth factor β (TGFβ). Although it is established that TGFβ/Smad signal is essential to the process of keratocyte-myofibroblast transformation in a healing corneal stroma post-injury. This article emphasizes the involvement of non-TGFβ molecular mechanisms in modulating Smad signal. We focus on the roles of matricellular proteins, i.e., osteopontin and tenascin C, and as cellular components, the roles of transient receptor potential (TRP) cation channel receptors are discussed. Our intent is to draw attention to the possibility of signal transduction cascade modulation (e.g., Smad signal and mitogen-activated protein kinases, by gene transfer and other related technology) as being beneficial in a clinical setting to reduce or even prevent corneal stromal tissue fibrosis/scarring and inflammation.

1. Overview of tissue fibrosis/scarring

Physiological integrity of a tissue is maintained by a regulated interplay between cells and a well-organized extracellular matrix (ECM). Following primary post-injury wound healing, cells actively remodel tissues for the restoration of normal structure and function. However, dysregulated inflammation induced by an injury results in excessive and continuous inflammatory/fibrogenic growth factor upregulation, which often leads to formation of a fibrotic lesion causing a failure of tissue remodeling and dysfunction due to overabundant accumulation of extracellular matrix and tissue contraction. A fibrotic lesion is characterized by persistent inflammation and presence of myofibroblasts (Jester et al., 2010, 2012; Wilson, 2012), generated by local mesecnhymal cells in many organs, *i. e.,* corneal stroma, or via epithelial–mesenchymal transition (EMT) in certain epithelial tissues, both of which must decline for the healing process to be complete and restore normal tissue functions.

2. Myofibroblast, the main player of corneal stromal wound healing

The cornea, an avascular tissue of the eyeball shell, located at the anterior part of the eye must remain transparent and preserve its regular curvature for it to properly refract light. An organized ECM structure composed of collagen fibers and proteoglycans among the fibers is essential to the maintenance of its transparency and the regular curvature (Chen and Birk, 2013). The resident components involved in tissue repair are stratified epithelium and a collagenous matrix containing mesenchymal cells lying beneath it, but the tissue lacks vasculature unlike skin. Transparency and normal curvature of the cornea are disturbed by stromal fibrosis/scarring, leading to the impairment of the patients' vision (Saika, 2004). Growth factors/cytokines are involved in the pathogenesis of corneal scarring diseases, *i. e.,* post-alkali burn scarring or Stevens-

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Johnson's syndrome. In the majority of these diseases the components of the disease process include inflammation, fibroblast activation and ECM accumulation. Myofibroblasts, the key cell type playing a central role in the process of tissue fibrosis/scarring, were originally considered to be derived from keratocytes that are activated by various cytokines upon tissue injury. The most critical feature of myofibroblast is expression of α-smooth muscle actin (αSMA) (Fig. 1). Reports suggest that circulating bone marrow derived cell types (so-called fibrocytes) could differentiate and become myofibroblasts in a healing tissue, but this is still a controversial issue in corneal stroma (Brenner et al., 2012; Habiel et al., 2014). Although this article focuses on mainly the pathophysiology of the keratocyte-derived myofibroblast, pericytes of neovascularization in an inflammed corneal stroma are also capable of transformation to myofibroblast. The alteration of curvature of the cornea during wound healing is attributable to the contractile force of a scarred tissue due to αSMA-containing cytoskeletal machinery in myofibroblasts (Hinz et al., 2010, 2012; Klingberg et al., 2013). Myofibroblast is also the cell type involved in unfavorable disorganized ECM accumulation, that leads to stromal scarring/fibrosis and opacification.

3. Growth factors involved in corneal keratocyte-myofibroblast transformation

The fibrogenic process is stimulated by inflammatory cell-derived growth factors or cytokines, especially, the fibrogenic cytokine, transforming growth factor β (TGFβ) (Roberts et al., 2006; Schuppan and Kim., 2013). TGFβ is believed to be the most important growth factor involved in keratocyte-myofibroblast conversion, although other factors further modulate the process (Singh et al., 2014). Upregulating αSMA and ECM gene expression are involved in tissue fibrosis/scarring. TGFβ family consists of three isoforms (β1 − β3); each has a similar biological activity, although each of them is expressed in a distinct spacial and temporal pattern under strict gene expression regulation. Besides *de novo* expressed TGFβ, TGFβ translocates to an uninjured tissue as a latent form and is quickly activated by various stimuli. A detailed description of various mechanisms of activation can be found in publications, which in some cases are mediated by various proteins, cathepsins, plasmin, calpain, thrombospondin, matrix metalloproteinases and integrins (Nishimura, 2009; Worthington et al., 2011). Activated TGFβ, after dissociation from latency-associated peptides, binds to cell surface receptor composed of types I (ALK5) and II receptor peptides. Blocking unfavorable TGFβ signaling could be a potential strategy for prevention or treatment of fibrogenic or scarring diseases (Roberts et al., 2003; Saika et al., 2008a,b; 2010; Akhurst and Hata. 2012; Yanagita, 2012). Besides TGFβ, Interleukin-4 (IL-4), IL-6, IL-13, platelet-derived growth factor and others reportedly positively modulate keratocyte-myofiboblast transformation (Singh et al., 2014; Wynn, 2011).

TGFβ signaling is involved in various aspects of cell behaviors in the tissue repair process in corneal layers, *i. e.,* epithelium, stroma and endothelium. Three TGFβ family members are all detected in injured corneal tissue although there are some differences in their localization (Saika, 2004). In uninjured mouse cornea inactive form of TGFβ1 is mainly detected in the epithelium, but active form of TGFβ1 is not observed in both epithelium and stroma. Subsequent to injury, the healing stroma contains the active TGFβ1, but in the epithelium only the inactive form is present. TGFβ is also chemoattracttant to macrophages (discussed below). Infiltrating inflammatory cells, *i. e.,* macrophages, are considered to be one of the major TGFβ sources in an injured cornea besides resident corneal cells. Although TGFβ is essential to the restoration of structure and function of cornea, disregulated TGFβ signaling accelerates keratocyte-myofibroblast transformation, the main event in the formation of corneal stromal scarring/fibrosis similar to scar tissue formation in other internal organs, *i. e.,* liver or pancreas. Blocking type II TGFβ receptor activation by systemic soluble receptor expression by intramuscular administration of adenoviral vector suppressed scarring and neovascularization in a healing rat cornea post-alkali burn (Sakamoto et al., 2000), clearly indicating the central role of TGFβ in corneal stromal scarring.

Although macrophages are believed to the major source of TGFβ in a healing tissue, it was recently reported that macrophages in an inflammed tissue are classified into two subsets, M1 and M2 macrophages (Wynn et al., 2013). Once a tissue is injured, peak M1 macrophage pro-inflammatory infiltration reportedly precedes to the appearance of M2 anti-inflammatory/pro-fibrotic macrophages. M1 macrophages are induced by interferon-γ or TNFα and TGFβ and IL-4/13 elicit increase in M2 macrophages. M2 macrophages are capable of activating fibroblasts through increases in TGFβ and PDGF expression to undergo myofibroblast transdifferentiation. M2 macrophages also produce factors that induce myofibroblast apoptosis and affect convergent fibrogenic process. Further study is needed to delineate how M1/M2 expression of

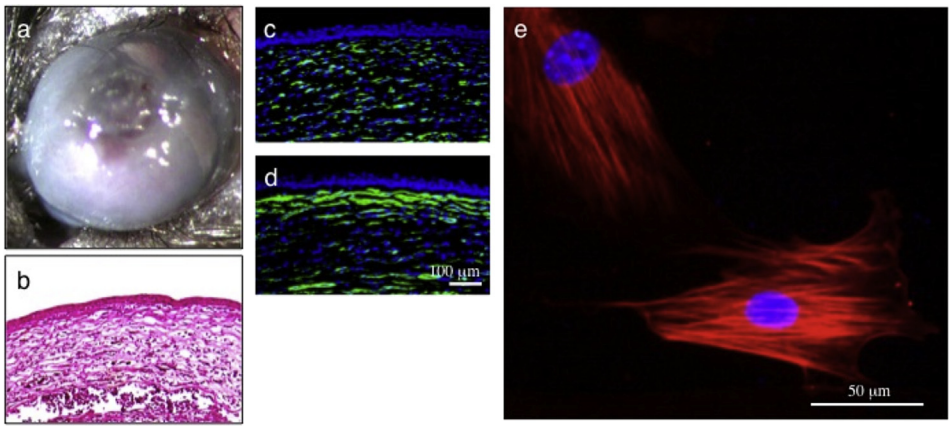

Fig. 1. Distribution of macrophages and myofibroblasts in an alkali-burned healing mouse cornea at day 7. A cornea of C57BL/6 mouse was exposure to 1.0 N NaOH as previously reported (Saika et al., 2005b) (a). At day 7 the healing cornea was histologically examined by using hematoxylin and eosin staining (b), immunohistochemistry for macrophages (F4/80 antigen, c) or myofibrobalsts (α-smooth muscle actin) (d). The healing cornea contains abundant macrophages and myofibroblasts. Bar 100 μm. Frame e shows intracelluylar distribution of αSMA in cultured ocular fibroblasts from C57BL/6 mouse with the exposure to 1.0 ng/ml of transforming growth factor β1. Bar 50 μm.