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TGFβ in fibroproliferative diseases in the eye

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1. ABSTRACT

Transforming growth factor β (TGFβ) is believed to be the most important ligand in the pathogenesis of fibrotic diseases in the eye. Such ocular fibrotic diseases include scarring in the cornea and conjunctiva, fibrosis in the corneal endothelium, post-cataract surgery fibrosis of the lens capsule, excess scarring the tissue around the extraocular muscles in the strabismus surgery and proliferative vitreoretinopathy. In the proliferative stage of diabetic retinopathy, fibrogenic reaction causes tractional retinal detachment in association with contraction of the tissue. A myofibroblast, the major cellular component in the fibrotic lesions, is derived from both mesenchymal cells (in cornea and conjunctiva) and epithelial cell types (lens or retinal pigment epithelium or corneal endothelium) through epithelial-mesenchymal transition (EMT). The myofibroblasts cause excess accumulation of fibrogenic extracellular matrix with resultant tissue contraction and impaired functions. Although various cytokine signaling pathways are involved in the fibrogenic reaction in tissues, TGFβ/Smad signal is the critical one. Blocking Smad signal by chemical or natural inhibitors or anti-Smad gene introduction effectively suppress fibrogenic reaction; inhibition of both fibroblast-myofibroblast conversion or EMT. Such strategies can be clinically tested.

2. INTRODUCTION

Transforming growth factor β (TGFβ) is believed to be the most important ligand in the pathogenesis of fibrotic diseases like cutaneous and scarring, keloids, and liver and lung fibrosis, *etc*. Three isoforms are known (b1 to b3). Although their effects *in vitro* are similar to those of each other, *in vivo* expression pattern is quite specific to each isoform. TGFβ upregulates fibrogenic cytokines and molecules involved in tissue fibrosis. TGFβ also modulates inflammation and immuniy. The ocular tissue is also susceptible to the fibrotic diseases, which include scarring in the cornea and conjunctiva, post-cataract surgery fibrosis of the lens capsule, fibrosis in the corneal endothelium, excess scarring the tissue around the extraocular muscles in the strabismus surgery and proliferative vitreoretinopathy. In the proliferative stage of diabetic retinopathy, fibrogenic reaction occurs around the neovascularization, that causes tractional retinal detachment in association with contraction of the tissue. The lesions are characterized by appearance of myofibroblasts, the key player of the fibrogenic reactioan, and excess accumulation of extracellular matrix (ECM) with resultant tissue contraction and impaired functions, that are modulated by TGFβ signal. This article reviews the roles of signals activated by TGFβ, its modulators and of myofibroblast in fibrogenic reaction in the eye. Modulation of signal

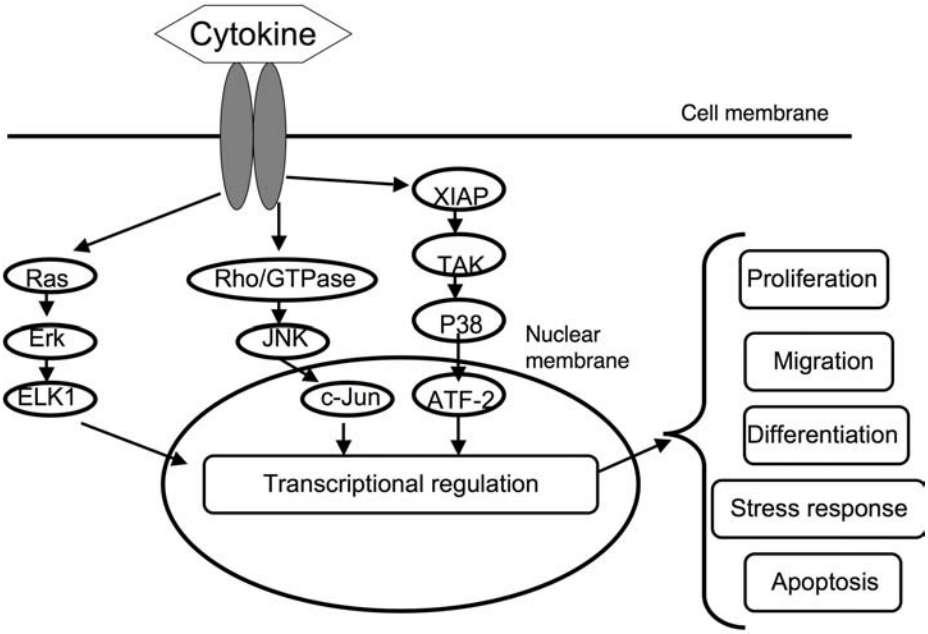

Figure 1. Cytokine signaling cascades. Binding a ligand, including transforming growth factor b to its specific receptor actiovates signaling cascades tof Ras/mitogen-activated protein kinase (MAP kinase or p42/p44), c-Jun N-terminal kinase (JNK) or p38 MAP kinase. The first pathway is mainly involved in cell proliferation regulation, whereas the latter two modulate cell stress responses or cell survival/cell death.

transduction molecules *e.g.,* Smad and mitogen-activated protein kinases, is beneficial and can be an important treatment regiment to prevent or treat these diseases.

3. OVERVIEW OF TGFβ SIGNAL TRANSDUCTION

Binding of a cytokine/growth factor to its specific cell surface receptor is followed by activation of intracellular signaling cascades. The ligand/receptor signaling cascades often converge via mitogen-activated kinases (MAPK) that include three subfamilies, *i. e.,* p42/44 ERK (external regulated kinase, MAP kinase), c-Jun N-terminal kinase (JNK), and p38 MAP kinase (Figure 1). These signaling pathways mediate various biological responses by regulating expression of genes. For example, in general, pP42/44 MAP kinase is involved in cell proliferation regulation whereas JNK and p38 are activated upon cellular stresses and modulate cell survival/cell death or control expression of stress-response genes.

TGFβ superfamilly includes TGFβ family members, bone morphogenic proteins, or activin, etc. TGFβ family members, *i. e.,* TGFβ1 to β3, utilize the Smad signaling pathway which is specific to the members of TGFβ superfamily, in addition to the other signaling cascades mentioned above. Upon TGFβ binding to its receptor, a pair of transmembrane receptor serine-threonine kinases are activated. Receptor-activated Smad proteins, Smad2 and Smad3, are phosphorylated directly by the TGFβ receptor type I kinase (ALK5). They then partner with the common mediator, Smad4, and translocate to the nucleus where they play a significant role in modulation of expression TGFβ-dependent gene targets (Figure 2). Details of differences between Smad2 and Smad3 were recently investigated by using a gene expression array made of embryonic fibroblasts obtained from embryos lacking either Smad2 or Smad3 (1, 2). For example, TGFβ1-mediated induction of matrix metalloproteinase-2 was selectively dependent on Smad2, whereas induction of c-fos, Smad7, and TGFβ1 autoinduction relied on expression of Smad3. *In vivo*, the roles of Smad2 and Smad3 differ because the lack of Smad2 is lethal for mice at the embryonic stage whereas those lacking Smad3 survive (3, 4, 5, 6). The bone morphogenetic proteins (BMPs), which are members of the TGFβ superfamily, bind to their own receptors and phosphorylate Smads 1, 5, and 8 which then bind to Smad4 for translocation to the nucleus. Smads6/7 are known to be inhibitory Smads, that block phosphorylation of Smads2/3 (4, 7), and thus suppresses Smads2/3-mediated gene expression.

Smad signal is known to be further modulated via cross-talks between other signaling, i. e., MAK kinases. Two types of modulations are known; phosphorylation of Smad middle linker region by MAP kinases and Smad7 induction by MAP kinases. The middle linker region of Smad2 or Smad3 can be phosphorylated by MAP kinase, i.e., MAPK, JNK, or p38MAP kinase following activation of various ligands and/or external stimuli. It is very unlike that TGFβ modulates functions of a cell alone *in vivo*. Under conditions of marked expression of various growth factors/cytokines such as in an injured tissue, it is quite possible that such phosphorylation in the Smad liker region might further modulate Smad -dependent gene expression during the processes of wound healing or tissue fibrosis *in vivo,* as observed in *in vitro* (5, 8). For example, it has been

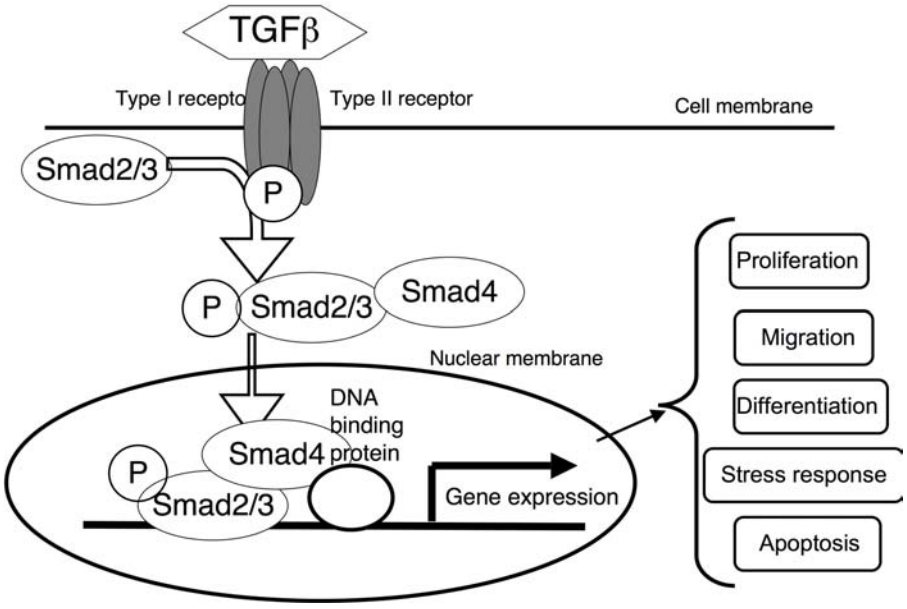

Figure 2. TGFβ/Smad signal. Smad2 or Smad3 is phosphorylated at its C-terminal region upon TGFβ binding to receptor. Phosphorylated Smad2 or Smad3 forms a complex with the common Smad (Smad4) and translocates to the nucleus to bind to gene promoters. Co-factors modulate Smad-dependent gene expression.

reported that the phosphorylation in the Smad linker region by ERK MAP kinase or p38MAP kinase is required for full-activation of Smad function. We reported that inhibition of p38 signal suppresses Smad-dependent gene expression by a reporter assay in a human retinal pigment epithelial cell line, ARPE-19. However, this phenomenon was not observed in primary cultures of human subconjunctival fibroblasts, indicating the cell-specificity of such modulation of Smad-dependent gene expression by Smad linker region phosphorylation (9, 10). Smad signals can also be modulated by cross-talks between non-MAP kinase signals. For example, interferon-γ-activated STAT signal or nuclear factor-κB up-regulates Smad7, counteracting the TGFβ/Smad signal (11, 12, 13, 14).

4. CENTRAL ROLE OF TGFβ IN FIBROGENIC REACTION

Tissue integrity or homeostasis is maintained by a complex interplay of cells and ECM. Following initial tissue repair post-injury, tissues is continuously under remodeling for the restoration of normal structure and function. TGFβ up-regulates fibrogenic ECM genes that are involved in both initial tissue repair and long-term remodeling, but in turn in unfavorable scarring when the activity is disregulated, which often lead to failure of tissue remodeling and dysfunction of tissues due to excess accumulation and contraction of ECM (15, 16). The main cellular component for such ECM expression is the myofibrobasts generated by the effects of TGFβ (as discussed later in Chapter 3). The expression of the majority of the ECM components and enzymes involved in matrix reorganization/maturation mostly depends on Smad3, whereas expression of matrix metalloproteinase-2 is Smad2-dependent. In Smad3-null mice, re-epithelialization is accelerated and fibrosis is reduced during tissue repair in skin (17). However, blocking TbrII (TGFβ type II receptor) by dominant negative expression in fibroblasts in a transgenic mouse model instead resulted in a paradoxical systemic tissue fibrosis (18). The exact reason for this discrepancy is to be uncovered, lacking Smad3 means other TGFβ-derived signals, i. e., MAP kinase, p38 and JNK are maintained, being different from blocking the ligand's signals at the receptor level. Although various cell types are capable of expression of TGFβ , the main source in local healing tissue is believed to be a macrophage that also express various inflammatory/fibrogenic growth factors/cytokines, For example, in the progression of hepatic fibrosis, myofibroblasts mainly generated from hepatic stellate cells (minorly from hepatocytes and bone marrow-derived cells) express fibrogenic growth factors/cytokines including TGFβ.

5. GENERATION OF MYOFIBROBLASTS BY TGF®

Besides excess accumulation of ECM, a fibrotic lesion is characterized by the presence of myofibroblasts, the kay player in fibrogenic reaction, in association with persistence of inflammation, both of which must decline for the healing process to be complete and the restoration of normal tissue functions (Figure 3) (19, 20, 21, 22, 23). This cell type is derived from both activated fibroblasts and epithelial cell types. Myofibroblasts are usually derived from fibroblasts that are activated by various cytokines upon tissue injury. Expression of α-smooth muscle actin (α SMA), the hallmark for fibroblast-myofibroblast conversion, is mediated by Smad2 (24, 25, 26). αSMA produces a contractile force in a scarred tissue (27). Some

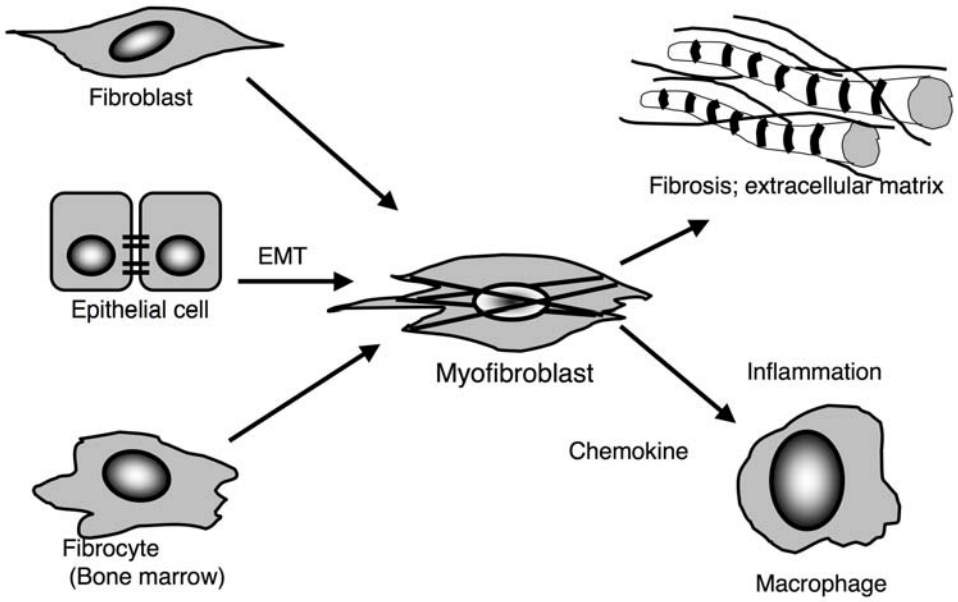

Figure 3. A myofibroblast is the main player in the process of tissue fibrosis. A myofibroblast is derived form either a fibroblast, an epithelial cell or a bone-marrow derived cell (a fibrocyte), and exerts a central role in cell repopulation, inflammation and extracellular matrix reconstruction in the process of formation of a fibrotic lesion. The process of production of a myofibroblast from an epithelial cell is called as "Epithelial-mesenchymal transition, EMT".

reports suggest that circulating bone marrow derived cell types (so-called fibrocytes) could differentiate and become myofibroblasts in a local healing tissue, but this is still a controversial issue (28, 29, 30). Recent study showed that contraction of myofibroblasts further activates TGFβ at the site of cell-matrix contact. Another source of myofibroblasts in a fibrotic lesion is EMT as described in Section 6.

6. EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

The process of transdifferentiation in which an epithelial cell changes its phenotype to a (myo)fibroblast is called an EMT (31, 32) (Figure 3), and is observed in the process of interstitial renal fibrosis (retinal tubular epithelial cells) (33), of pulmonary fibrosis (type II alveolar epithelial cells) (29), of liver fibrosis (34), or in specific ocular tissues. Like mesothelium corneal endothelium also undergo EMT, although it is not of a real epithelial cell type. Acquisition of motility and metastasis in neoplastic epithelial cells (cancer cells) is also considered to be the result of EMT (35, 36). Transcrption factors involved in EMT includes ZEB (*Sip1/δEF1*), bHLH (*E47/Twist*) and *Snail1/2* (37, 38). Expression of these molecules seems to be strictly regulated by various signaling pathways, *i. e.,* Smads, p42/p44 ERK or p38MAP kinase, and nuclear factor-κB (NF-κB), *etc*. Expression of *Snail*, the master transcription factor involved in an early step of the EMT is an important step in the process of tissue fibrosis, and is controlled by Smad3 signaling (39). Dependency of EMT on *Snail1* or *Snail2 (Slug),* another Snail member transcription factor, seems to be cell-type specific. These transcription factors are up-regulated by TGFβ and directly suppress E-cadherin expression which is essential in the maintenance of epithelial phenotype.

7. OUTLINE OF OCULAR FIBROTIC DISORDERS

The eye is a unique tissue composed of surface ectodermal tissues and neuronal tissues (Figure 4). The former includes cornea and conjunctiva that exhibit a structure similar to skin except for the avascularity of the cornea. However, The essence of fibrotic diseases in the eye is quite similar to that seen in fibrotic disorders in other tissues of the human body. The diseases include scarring in the cornea and conjunctiva, the fibrosis in the lens capsule post-cataract surgery, or proliferative vitreoretinopathy (PVR), which is characterized excess scarring tissue formed on the detached retina (40) (Figure 5). In proliferative stage of diabetic retinopathy, fibrogenic reaction adjacent to the retinal neovascularization has a significant role in the development of tractional retinal detachment. Excess accumulation of ECM and appearance of myofibroblasts, in association with inflammatory cells, are observed in all the cases in the eye. Myofibroblasts are derived from mesenchymal cells, *i. e.,* subconjnctival fibroblasts or keratocytes (corneal fibroblasts), or epithelial cell types, *i. e.,* lens or retinal pigment epithelium in each specific injured site. Although it has long been believed that the main component of proliferative diabetic retinopathy is retinal neovascularization, the fibrogenic process that occurs around such new vessels causes a traction force and the resultant detachment of the retina making this condition a fibrotic disease as well. In an adult human eye, TGFβ2 predominates in the aqueous humor. The importance of TGFβ2 in ocular physiology is also demonstrated in embryonic development; by the

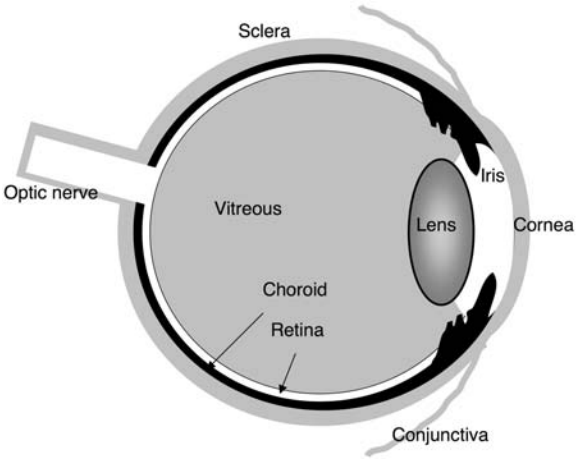

Figure 4. Schematic structure of a human eye.

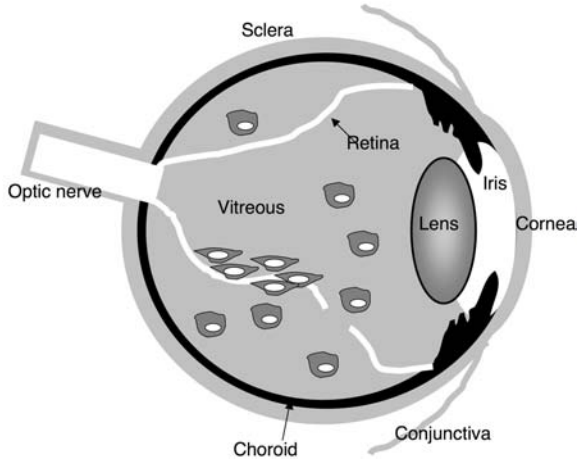

Figure 5. Proliferative vitreoretinopathy**.** Myofibroblasts (black arrows), mainly derived from dispersed retinal pigment epithelium (white arrows) through epithelial-mesenchymal transition, grow on the detached retina, producing a fibrous lesion. The fibrous plaques reduce flexibility of the detached retina

observation that embryos of a TGFβ2-null mouse, but not TGFβ1 or TGFβ3-null mouse, have multiple ocular abnormalities, *i. e. ,* loss of corneal endothelium and the anterior chamber, as well as corneal stroma and retinal and vitreous hypercellularity, all of which are due to impaired immigration of neural crest cells (41). Ocular surface epithelium express TGFβ1. 2. and 3. The cells inside the eye also express all the TGFβ members during disease process, i. e., inflammation or tissue repair. TGFβ also upregulates other growth factors/cytokines, e. g., connective tissue growth factor or fibroblast growth factor, involved in tissue fibrosis.

8. FIBROSIS/SCARRING IN OCULAR SURFACE, I. E., CORNEA AND CONJUNCTIVA

The ocular surface, i. e., cornea and conjunctiva, is covered with stratified epithelium and underlying connective tissue. The serve as the outercoat of the eye structure that blocks interfering by external stimuli of chemical components, microbial attack or mechanical trauma. Their most prominent difference are the lack of blood vessels and its transparency of the cornea. Ocular surface scarring diseases include Stevens-Johnson's syndrome or post-alkali burn scarring etc. In the majority of these diseases the components of the disease process include inflammation, fibroblast activation and ECM accumulation (42, 43). Once the tissues are injured, mesenchymal cells and infiltrated inflammatory cells, i. e., macrophages, secrete various growth factors/ cytokines including TGFβ, one of the most important fibrogenic growth factors. Although TGFβ is critical in the maintenance of the tissue integrity, it also promotes fibrogenic reaction in the healing subepithelial tissues.

The avascularity, transparency and the regular curvature are all essential for proper light refraction and, therefore, vision. Although the it lacks vasculature, the main components involved in tissue repair of the cornea are quite similar to those of skin; stratified epithelium and a collagenous matrix containing mesenchymal cells (corneal fibroblasts) lying beneath it. An organized ECM structure of collagen fibers of types I, III, and V and proteoglycans among the fibers is essential to the maintenance of its transparency and the regular shape. Transparency of the cornea is reduced by stromal fibrosis/scarring, leading to the impairment of the patients' vision (39, 44). Such severe corneal fibrosis could be surgically treated by using transplantation of an epithelial cell sheet grown on a membrane (45). Although conjuncitva is much vasculalrized, the behaviors of epithelial cells and mesenchymal cells (fibroblasts) in a healing, injured, conjunctiva are similar to those seen in a healing cornea. Scarring of conjunctiva potentially causes problems of reduction in filtration efficacy following glaucoma filtering surgery (46). Topical mitomycin C is applied to local tissue after trabeculectomy in order to suppress excess proliferation of subconjunctival fibroblasts. Although it is markedly effective in the majority of cases, we do encounter patients for whom such effectiveness is limited (47). Similarly to the wound healing process in cornea, the TGFβ family has three isoforms, β1, β2 and β3, are bel;ieved to have important roles in wound healing in conjunctiva. A neutralizing antibody against TGFβ2 was tested to try to suppress excess fibrosis/scarring in filtering blebs, but no significant effect was observed (48). Although aqueous humor contains abundant TGFβ2 (49, 50), TGFβ1 and β2 are also expressed in local cells (conjunctival epithelium and fibroblasts) in the filtering bleb tissue (13, 49, 51), that might account for the failure of the trial of TGFβ 2 antibody for suppression of conjunctival scarring.

9. STRATEGIES TO PREVENT EXCESS FIBROGENIC REACTION IN OCULAR SURFACE TISSUE BY TARGETING TGFβ SIGNALS

Blocking unfavorable cytokine activity, *i. e.,* TGFβ, has therapeutic effects on fibrogenic or scarring diseases. Gene introducion is one of the effeicient routes for blocking signaling cascades in the cytoplasm, while

other protein-based agents might not be effective. It has been reported that blocking type II TGFβ receptor by sysntemic expression of soluble receptor by adenoviral gene expression in muscles suppressed scarring and neovascularization in a healing rat cornea post-alkali burn (52). Blocking p38MAP kinase also suppresses fibrogenic reaction in ocular fibroblasts independently Smad signal. TGFβ-activated p38MAP kinase is critical for the migratory activity of corneal epithelial cells during tissue repair (53). Therefore, it is beneficial to maintain p38 signaling for cell migration, but to specifically block signals that yielded unfavorable scarring and development of neovascularization in a healing cornea. TGFβ/Smad signal is suitable for this purpose. However, this strategy might potentially impair the healing of conjunctival epithelium, in that epithelial migration is regulated by TGFβ/p38 signaling. We showed that deletion of Smad3 gene or Smad7 overexpression had a similar anti-profibrogenic/proinflammatory effect in the healing of an alkali-burned mouse cornea or of mechanically injured mouse conjunctiva. (54). Smad7 not only blocked TGFβ/Smad signaling in local corneal (myo)fibroblasts, but also inhibiting macrophage recruitment in the inflamed tissue, resulting in a reduction of local macrophage-derived growth factors. Smad7 gene transfer attenuates the fibrogenic reaction in a healing mouse cornea and conjunctiva, suggesting that blocking TGFβ/Smad signal might have a therapeutic potential in the prevention of excess scarring in these tissues in humans.

Such anti-Smad signal strategy includes gene transfer of BMP-7, Id2/3, or The peroxisome proliferator-activated receptor (PPAR)g. PPARg is a member of PPAR family (β, δ, or γ), which are involved in modulation of adipose metabolism, and inflammatory cell function, as well as behaviors of non-inflammatory cells, *i. e.,* fibrogenic reaction or cell proliferation during wound healing (55, 56, 57). PPARγ is a nuclear receptor for ligands of 15-deoxy-d12, 14-prostaglandin J2 (15d-PGJ2), thiazolidinedione, *etc*, although 15d-PGJ2 also has PPARg-independent actions. Like other tissues, gene transfer of PPARγ blocks injury-induced fibrogenic reaction in mouse cornea and conjunctiva as well as in cultured fibroblasts (58).

Moreover, we also showed that over-expressed Smad7 does not inhibit phosphorylation of the p65 subunit (RelA) of NF-κB (59), a signal transmitter related to inflammation, but does block its nuclear translocation (9). This might explains why Smad7 gene transfer is more effective in the suppression of inflammation and excess fibrogenic reaction in an alkali-burned cornea as compared with Smad3 gene deletion. It was confirmed that inhibiting NF-κB by a peptide inhibitor, SN50, produces a therapeutic effect on alkali-burned corneas in mice (9). The mechanism of action of SN50 include suppression of the inflammatory response and also acceleration of epithelial cell proliferation through over-activation of TNFα/JNK signal (9).

Other growth factors/cytokines are known to further modulate TGFβ's action in tissue. For example, TNFα antagonizes TGFβ effects *in vivo* in healing tissues. In TNFα-null mice, inflammation, tissue scarring and stromal neovascularization all were less severe in the earlier phase of healing, but later, such events were more marked in association with over-expression of inflammation/fibrosis-related growth factors in cornea and other tissues of TNFα-null mice (60, 61, 62, 63). Experiments in these reports revealed that TNFα expressed in macrophages, but not resident mesenchymal cells, antagonizes fibrogenic effects by TGFβ.

10. LENS EPITHELIUM EMT AND EYE DISEASES

Crystalline lens is a transparent tissue which reflect light properly as a "lens". Different from real "lens" it is not solid and change its thickness by contraction of ciliary muscle to obtain the best focus. Lens tissue is formed by the invagination of surface ectoderm on the optic cup during embryonic development. Thus, the crystalline lens has its basement membrane structure in the most outer portion and the lens epithelial cells line the inner surface of the anterior capsule. This basement membrane is called as lens capsule.

Cataract is a group of diseases of loss of transparency in the lens tissue caused by various systemic or local problems as well as just ageing. The types of cataract is identified by the location of opacification in the lens tissue. Anterior subcapsular type of cataract shows fibrous plaque of opacity just beneath the anterior lens capsule. The opaque tissue consists of myofibroblasts and accumulation of fibrous CEM inside the lens capsule. Because the lens tissue contains epithelial cells and lens fiber cells, the myofibroblasts seen inside the capsule must be originated from epithelial cells through EMT.

In modern cataract surgery, an artificial intraocular lens (IOL) made of a hard or soft plastic material is implanted in the residual bag of the lens capsule after removing the opaque lens content. Lens epithelial cells start to proliferate and to migrate toward the behind the IOL and then transform into the myofibroblast through the process of EMT. Aftercataract (secondary cataract or post-operative capsular opacification), the most common complication, is caused by myofibroblast generation through EMT of lens epithelial cells and subsequent accumulation of fibrous ECM components, *i. e.,* collagen types, proteoglycans and basement membrane components on the inner surface of the residual lens capsule (64, 65, 66, 67) (Figure 6). It is to be noted that post-injury lens tissue, including capsular opacification, contains significant myofibroblasts, although there are no local fibroblasts in this tissue, indicating that all the myofibroblasts are derived from the lens epithelium, different from other EMT-related tissue fibrosis, i. e., renal interstitial fibrosis or pulmonary fibrosis, both of them contains myofibroblasts derived form local fibroblasts and epithelial cells (or, in part, bone marrow-derived cells). Lens fiber regeneration also contributes to the development of secondary cataracts by forming Sommerring's rig and Elschnig's pearls (66, 67). One of the most critical growth factors involved in the

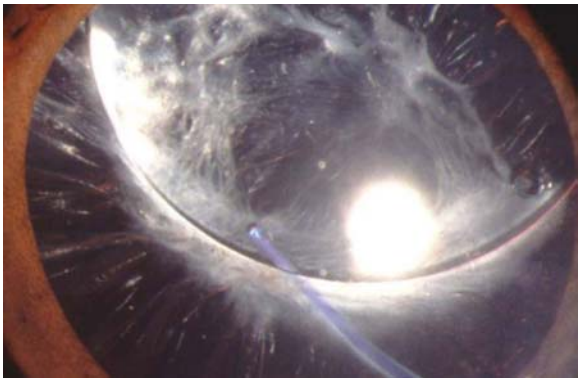

Figure 6. Fibrous opacification of the posterior capsule resulting in decentration of the intraocular lens.

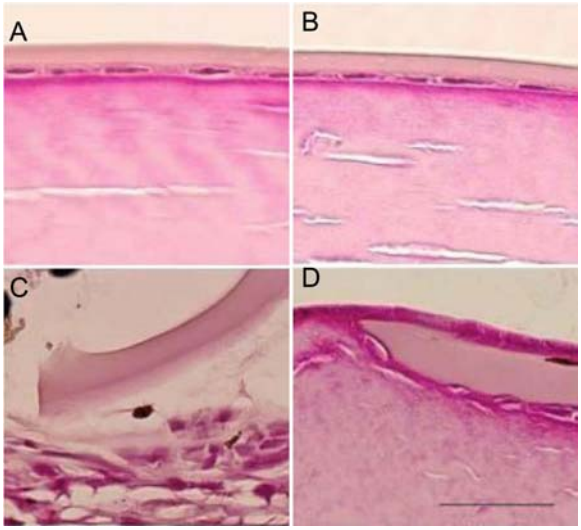

Figure 7. Light microscopic histology of lens of Smad3-knockout mice. Uninjured lenses of both $Smad3^{+/+}$ (A) and $Smad3^{-/-}$ (B) mice are similar without morphological abnormality. At week 8 post-puncture injury the cells form a multilayer of elongated fibroblast-like cells which are generated through epithelial-mesenchymal transition in a wild type mice (C), whereas such tissue is not observed in a $Smad3^{-/-}$ injured lens (D). Bar, 50 mm.

development of secondary cataracts is reportedly TGFβ (67, 68, 69).

Lens epithelial cell EMT in post-operative secondary cataracts is mimicked by puncture of the lens in animals (70). In the mouse model producing by this procedure, Smad signal is activated in lens epithelial cells adjacent to the wound in 12 hrs. Smad4 nuclear translocation is readily blocked by intraocular administration of anti-TGFβ2 neutralizing antibody, indicating that TGFβ2 is the TGFβ family member that activates lens epithelium post-injury in mice. Then the cells begin to express αSMA, the hallmark of EMT, mRNA and protein at 3 and 5 days, respectively when the cells exhibit a fibroblastic appearance. This injury-induced EMT in lens epithelium is indeed blocked by lacking Smad3 in mice (51) (Figure 7). However, non-Smad signals can transmit EMT signals when cells receive strong stimuli, such as adenoviral over-expression of the active form of TGFβ1 in lens epithelium or marked intraocular inflammation induced by ocular alkali exposure (39, 71, 72, 73). This indicates that the signal toward EMT can bypass Smad3 in such circumstances, although the signal is not fully transmitted (74). Other signals such as Rho-kinase, PI3-kinase or SRC are also reported to modulate the process of EMT in lens cells (75, 76, 77, 78).

Although Smads2/3 are phosphorylated at their C-terminal regions and middle linker regions, the roles of the phosphorylation of the middle linker region in the progress of EMT is to be uncovered. Our preliminary data showed that human lens epithelial cells of an uninjured lens are negative for phospho-Smad2 at both C-terminal and middle liner regions. While epithelial-shaped lens cells in a post-operative healing lens are labeled for C-terminally phosphorylated Smad2, but not for middle linker phosphorylation, post-EMT myofibroblast-like lens cellsare labeled for both (Saika S, *et al.* unpublished data, 2008).

ECM components also modulate cell behaviors. It is well-known that the process of conversion of a fibroblast to a myofibroblast, driven by TGFβ/Smad2 or Smad3 signaling (71, 79), is further modulated by scaffolds of ECM components. For example, β1 integrin-mediated cell binding to ED domain-positive fibronectin, but not vitronectin, is essential to the fibroblast-myofibroblast conversion (20, 22). EMT is also modulated by ECM components. Lumican or osteopontin is required for EMT of lens epithelial cells in mice. Lumican is a core protein component of keratan sulfate proteoglycan in corneal stroma, as well as being located in various connective tissues as a glycoprotein (80). Lumican begins to be expressed in lens epithelium of humans and mice prior to EMT, and loss of lumican impairs EMT in mice, suggesting a role of lumican for providing the scaffold that is required for the EMT process (81, 83). Studies in osteopontin-null mice showed that loss of osteopontin delayed activation of Smad2/3 and subsequent injury-induced EMT in the lens epithelium (83, 84). Our unpublished data further show that loss of osteopontin impairs activation of TGFβ1/Smad2/3 and p38 MAP kinase in cultured ocular fibroblasts (Fujita N, *et al.*, Unpublished data, 2008). Osteopontin seems to be required for full-activation of TGFβ signaling and might also be a target of prevention of scaring/fibrosis. Vitronectin and collagen type I also promote lens epithelial cell EMT (85).

Prevention of secondary cataract formation is necessary to maintain good vision for patients and also for proper examination of the ocular fundus of these patients. The latter is critical in patients with retinal diseases, *i. e.,* diabetic retinopathy or various macular diseases. Mechanical approaches for prevention of secondary cataract formation has provided a satisfactory outcome; t is well-established that the sharp-angled edge of the optic portion of an intraocular lens blocks migration of residual

lens epithelial cells toward the inner surface of the posterior capsule. However drugs or surface improvement/modification of an intraocular lens that are effective in prevention of development of secondary cataracts must be clinically tested, although some are reported to have a potential *in vitro* or in animals (86, 87, 88). Studies by employing strategies of gene knockout or gene trnsfer described above indicate that anti-Smad strategies will be inhibitory to unfavorable EMT. This point will be discussed later in this issue.

11. RETINAL PIGMENT EPITHELIUM EMT AND RETINAL FIBRSIS (PVR)

During embryonic development, both neural retina and retinal pigment epithelium are of origin of neural ectoderm. RPE cells are normally located to the cell layer external to the retina. Adhesion between photoreceptor cells and pigment epithelium are quite loose and retina could be detached at this adhesion with various diseases. Vitreous gel detaches from the inner surface of the retina with aging. Contracted gel will produce a traction force where vitreous gel adheres to the retina, causing a break in the retina. In rhegmatogenous retinal detachment liquefied vitreous fluid or aqueous humor influxes beneath the retina through a break in the peripheral retina and the overlying retina is detached from the pigment epithelium. Once neuronal retina is detached, pigment epithelial cells (RPEs) begun to be dispersed in subretinal fluid. PVR is a disease caused by the formation of fibrotic tissue on the detached retina, which reduces the flexibility of the retina and may potentially make it difficult to reattach to the retina (89). Cultured RPE cells undergo transformation to fibroblast-like cells through the process of EMT, proliferate and produce extracellular matrix components in response to exposure to exogenous TGFβ . A similar phenomenon might occur on the detached retina, participating in this fibrotic sequelae of PVR. The RPE is the most critical contributor to the development of fibrous tissue on the retina, although various other cell types including Muller glia cells are involved in the fibrotic reaction of the detached retina (90, 91, 92). Such floating RPEs further didstributed in the vitreous cavity through the detachment-causing retinal break.

Like EMT in lens epithelium in the formation of secondary cataracts, TGFβ is likely a key player in the development of PVR, although various other growth factors, including platelet-derived growth factor, hepatocyte growth factor, and activin, are all reportedly involved in its pathogenesis (93 - 99). The concentration of TGFβ 2 in the vitreous humor of the eye correlates with the severity of the PVR, supporting its importance in the pathogenesis (93, 100). Similar to other cell types, *i. e.,* lens epithelial cells, EMT of RPE cells is also suppressed by the loss of Smad3 *in vivo*, resulting in the attenuation of development of PVR. We then confirmed that Smad7 gene introduction suppresses PVR in mice (101 - 104).

12. CORNEAL ENDOTHELIUM EMT

In the physiological condition, corneal endothelial cells exhibit a limited activity of cell proliferation. Thus, defect in the endothelium is mainly covered by cell migration and cell enlargement. However, the cells also cause fibrogenic reaction in an injured cornea. In the normal condition, endothelium keeps epithelial/endothelial produces ECM components o its basement membrane and Descemet's membrane. However, in pathological condictions, *i. e.,* infection or injury, the endotheliual cells begin to express fibrogenic components. For example, endothelium of syphilic keratitis exhibit a fibroblastic appearance with accumulation of fibrous collagens which are not normally expressed by endothelium between the cell layer and Descemet's membrane. A cryo-burned rabbit cornea also forms a retrocorneal fibrous tissue between the cell layer and Descemet's membrane (105). This alteration of cell phenotype to a fibrogenic type is considered to a process similar to EMT.

13. PROLIFERATIVE DIABETIC RETINOPATHY

Diabetes mellitus causes diabetic retinopathy which damage the retina and eventually patients' vision. The disease is stages as three; simple or preproliferative retinopathy and proliferative stage retinopathy (106). The main phenomenon in the non-proliferative stage includes damage of the pericytes and obstruction of the retinal microvasculature. Along with spreading of the area of the retina with microvascular obstruction, ischemic retinal tissue expresses various cytokines/growth factors, i.e., vascular endothelial growth factor (VEGF) involved in new vessel formation (107). Neovascularization grows on the posterior surface of the vitreous from the retinal vessels. Then, fibrogenic reaction occurs in cells (such as pericytes) around naked new vessels to form "fibrovascular" proliferative tissue, contraction of which potentally cause tractional retinal detachment (108). TGFβ is also belived to be involved in the phenomenon for VEGF induces TGFβ expression. Myofibroblasts are observed in such proliferative tissue (109).

14. BLOCKING EMT BY INTRODUCTION OF ANTI-SMAD GENES SUPPRESSES EMT-RELATED OCULAR DISEASES

We have used adenovirus as a gene transfer vector, especially that driven by the Cre/LoxP system to effectively overexpress exogenous genes in tissue. A benefit of ocular tissues is it is easy to apply adenoviral gene transfer via local administration (or infection) avoiding serious systemic side effects. The advantages of the Cre/Lox system include the following points; (1) cell-toxic gene-expressing viral vector can be grown and harvested in 293 cells and (2) cell-type specific promoter-derived Cre makes it possible to deliver a gene to the specific cell type *in vivo*. Inagaki *et al* (2005) reported transfer of a gene to hepatic stellate cells *in vivo* in a rat liver during experimental liver fibrosis by using the Cre/LoxP system adenovirus-mediated gene transfer that utilizes the promoter of type I collagen α2 gene (110). We also successfully introduced the gene for green fluorescent protein by using the same system in human cultured subconjunctival

fibroblasts and fibroblasts in a mouse eye post-incision injury in the conjunctiva (111). Recent evidence suggests that adenoviral vector-mediated ocular gene transfer is a viable approach for the treatment of ocular disorders in a human trial for the treatment of age-related macular degeneration (112). Adenoviral vector-mediated gene transfer of pigment epithelium-derived factor exhibited possibility of anti-angiogenic activity that may last for several months after a single intravitreous injection (113). Loss of Smad3 markedly impairs injury-induced EMT in lens epithelium in mice. This indicates that anti-Smad signal strategies might prevent unfavorable EMT. For example, over-expressed Smad7 blocks injury-induced lens epithelium EMT in mice, indicating that strategies that attenuate Smad3 signal might have a therapeutic potential to prevent development of secondary cataracts (67). The same strategy of Smad7 overexpression blocks EMT of retinal pigment epithelial cells *in vitro* and also suppresses development of PVR post-retinal detachment in mice. Similarly, Our unpublished data showed that the development of the retrocorneal fibrosis in a rat eye post-alkali burn was effectively prevented by prior treatment of the endothelium with Smad7 adenoviral gene introduction; Smad7 over-expression blocked expression of phosphorylated Smad2, αSMA and fibrous collagen types in the healing endothelium (114). Such endothelial mesenchymal transition-related tissue fibrosis is observed in pressure-induced cardiac fibrosis or peritoneal subendothelial fibrosis in patients with peritoneal hemodyalisis.

Bone morphogenic protein-7 (BMP-7) or pigment epithelium-derived factor is known to be anti-TGFβ cytokines. BMP-7 is a member of the TGFβ superfamily, and counteracts TGFβ/Smads2/3 signals by induction of expression of inhibitors of differentiation 2 and 3 (Id2 and Id3), both of which block Smads2/3 phosphorylation. Gene transfer of BMP-7 by topical administration of an adenoviral vector suppresses fibrogenic and inflammatory reactions in an alkali-burned cornea in mice (113). Exogenous BMP-7 or overexpression of Id2/3 is also reported to suppress EMT in cultured renal or lens epithelial cells (71, 115, 116, 117). Together these findings suggest the potential of BMP-7 or Id2/3 to inhibit the lens cell fibrogenic reaction in the formation of secondary cataracts *in vivo* (54). We indeed showed that adenoviral gene transfer to an injured mouse lens induced up-regulation of Id2/3 and that gene transfer of BMP-7, Id2 or Id3 attenuated EMT in mouse lens epithelial cells *in vivo*.

TGFβ -related non-Smad signal also involved in fibrogenic reaction and EMT in a injured tissue. p38MAP kinase modulates Smad signal. We reported that SB202190, a p38MAP kinase inhibitor, suppresses fibrogenic gene expression and reduces reporter gene expression using a Smad-dependent promoter and fibrogenic gene expression in human retinal pigment epithelial cell line. *In vivo* adenoviral gene transfer of dominant-negative p38MAP kinase suppresses the fibrotic reaction by retinal pigment epithelium in an experimental mouse PVR model (118). Activation of Smads by phosphorylation at its middle linker region by MAPK is also a potential target of inhibition of TGFβ signaling. It has been reported that p38MAP kinase is involved in EMT in cultured cells. However, another report shows that Smads and p38 MAP kinase independently regulate collagen I α1 mRNA in hepatic stellate cells (119), indicating that cell-type dependency of the role of p38MAP kinase in the modulation of Smad signal.

15. TARGETING TGFβ/SMAD SIGNALING FOR PREVENTION/TREATMENT OF OCULAR TISSUE FIBROSIS BY NON-GENE TRANSFER STRATEGIES

Recently, blocking antibodies against specific growth factors/cytokines have been tested for their therapeutic effects on cancers, neovascularization-related diseases, or fibrotic/inflammatory diseases. For example, a neutralizing antibody against vascular endothelial growth factor is effective in suppression of progression of colon cancer and also neovascularization in diabetic retinopathy or age-related macular degeneration. An antibody against TNFα suppresses not only excess inflammation in joints of patients with rheumatoid arthritis, but also is effective in the treatment of persistent uveitis in Bahcet's disease. On the other hand, trials to access anti-fibrogenic effects of a neutralizing anti-TGFβ2 antibody, CAT-152, exhibited limited effectiveness. (120). Although TGFβ2 predominates in the aqueous humor, other TGFβ family members, *i. e.,* TGFβ1 and TGFβ3, could be involved in the fibrogenic response by the cells in ocular tissues, This might account for the failure of the trial of CAT-152 in suppression of conjunctival fibrosis. From this point of view, it is quite reasonable to try to prevent tissue fibrosis by blocking signaling pathways that are common to TGFβ family members. Indeed, we showed here the effectiveness of blocking TGFβ/Smad signaling by adenoviral gene transfer techniques in the prevention/treatment of ocular fibrotic diseases is described. Results suggest there is also potential efficacy of blocking Smad signaling by other strategies, i. e., chemical inhibitors or natural compounds that have anti-Smad effect. For example, systemic administration of inhibitors of ALK5 effectively suppresses not only metastasis of experimental neoplasm transplanted in a muse tissue but also fibrogenic reaction and unfavorable tissue fibrosis in animals (121, 122, 123).

Natural chemicals that are capable of affecting Smad signal are also candidates to treat fibrogenic disorders. It has also been reported that components of herbal medicines have anti-fibrogenic/inflammatory effects in other parts of body, i. e., liver, kidney, or lung. For example, tetrandrine, the major component of an herbal medicine, Boui, also blocks fibrogenic reaction of cultured subconjunctival fibroblasts via a mechanism, at least in part, of blocking Smads2/3 signal by up-regulation of Smad7, the inhibitory Smad, like halofuginene (113). Because tetrandrine has suppressive effects on the progress of experimental pulmonary fibrosis in animals, it also might have similar effects on ocular fibrotic diseases. halofiginone also reportedly up-regulates Smad7 (120). We reported that components of an herbal medicine, Inchin-Ko-Tou, genipin, and emodin have anti-

fibrogenic/inflammatory effects *in vitro* in cultured ocular cells or *in vivo* on the healing of an alkali-burned cornea in mice. Genipin suppresses the fibrogenic reaction in various cultured cells*, i. e*., α-TN4 mouse lens epithelial cell line or human subconjunctival fibroblasts, suggesting their potential anti-fibrotic effects on secondary cataract or post-trabeculectomy filtering bleb scarring. Emodin also has an anti-fibrogenic/inflammatory effects (125, 126, 127). We showed that emodin reduced expression of growth factors/cytokines and ECM components in cultured subconjunctival fibroblasts, new vessel formation by cultured vascular endothelial cells, and also had a therapeutic effect on healing of an alkali-burned mouse cornea, including suppression of expression of inflammatory cytokines and reduction of stromal scarring and neovascularization.

16. SUMMARY AND PERSPECTIVE

We showed that the each part of the eye is susceptible to the fibrotic diseases, that are characterized by the appearance of myofibroblasts and accumulation of ECM. The process of generation of myofibroblasts from either fibroblasts of epithelial cell types are mediated by the growth factors including TGFβ, one of the most potent factors involved in tissue fibrosis. Blocking the signal activated by TGFβ is one of the powerful tool to prevent or treat the diseases.

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Abbreviations: α SMA: α-smooth muscle actin; BMP: bone morphogenetic proteins; EMT: epithelial-mesenchymal transition; ECM: extracellular matrix; JNK; c-Jun N-terminal kinase (JNK); MAPK: mitogen-activated kinases; NF-κB: nuclear factor-κB; TGFβ: transforming growth factor β; PPAR: peroxisome proliferator-activated receptor; PVR: proliferative vitreoretinopathy ; TNFα: tumor necrosis factor a; RPE: retinal pigment epithelium; VEGF: vascular endothelial growth factor

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