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A new model of anterior subcapsular cataract: involvement of TGFβ/Smad signaling

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Purpose: To develop a new animal model of anterior subcapsular cataract formation by topical application of alkali to the eye and to examine the role of Transforming growth factorβ/Smad3 (TGFβ/Smad3) signaling in the formation of this cataract model.
Methods: Under anesthesia, one eye of adult Wistar rats (n=142) was subjected to alkali burn by topical application of 1 N NaOH. The eye was then histologically examined at specific time intervals. Immunohistochemistry with a battery of antibodies was carried out to examine the epithelial-mesenchymal transition (EMT) in lens epithelium. Enzyme immunoassay was employed to determine the level of growth factors in aqueous humor and lens tissue. Smad3-null mice were also used to examine the role of Smad3 signaling in cataractogenesis in this model.
Results: Two days post-burn of the ocular surface, lens epithelium underwent EMT as evidenced by the upregulation of Snail and α-smooth muscle actin and formed a multilayer of cells beneath the capsule. Smad signaling was found to be activated in EMT-type lens cells. The majority of myofibroblast-type lens cells expressed proliferative cell nuclear antigen (PCNA). The total amount of active TGFβ2, total TGFβ2, and Fibroblast growth factor 2 (FGF2) increased in the aqueous humor and lens. Loss of Smad3 attenuated, but did not completely abolish, EMT in the lens epithelium.
Conclusions: Topical alkali treatment of the ocular surface readily induces an EMT-type anterior subcapsular cataract. Smad3 signaling is involved, but not required, for achievement of EMT in the lens epithelium in this cataract model.

It is well established that lens epithelial cells undergo epithelial-mesenchymal transition (EMT), which results in generation of myofibroblasts and tissue fibrosis in an injured lens including the postoperative lens capsule or in cataracts [1-4]. Although a variety of growth factors are believed to orchestrate lens epithelium EMT [5-7], we have reported that Transforming growth factorβ/Smad3 (TGFβ2/Smad3) signaling is required for this reaction [3,4,8]. Upon injury to the lens, Smad2, Smad3, and Smad4 translocate to the nucleus within 12 h. This phenomenon is abolished by intraocular injection of a neutralizing antibody against TGFβ2 [3]. This is consistent with previous reports that TGFβ2, instead of TGFβ1 or 3, predominates in ocular aqueous humor [9-11]. However, it has been reported that under physiological conditions a certain percentage of this TGFβ2 is in the inactive form and is quickly activated upon external stimuli (i.e., injury) [12,13].

It has been suggested that TGFβ is also involved in cataract development [14-16]. McAvoy and his colleagues reported that a rat lens cultured with TGFβ2 develops a subcapsular cataract with EMT in the epithelium [17]. FGF2 production by the lens epithelium is considered to be involved in its proliferative activity [18,19].

It has been clinically observed that an alkali burn of the ocular surface induces anterior subcapsular cataract formation during the late phase of healing in patients [20]. This notion prompted us to hypothesize that the exposure to an animal eye to alkali might develop an anterior subcapsular cataract which models human cases and might induce EMT via TGFβ/Smad signaling.

In the present study, we successfully developed an anterior subcapsular cataract in rats that contains lens epithelium-derived myofibroblasts. Smad3 signaling is activated in these lens cells in association with upregulation of TGFβ2 activation in the aqueous humor.

Although a number of cytokines and growth factors are believed to be activated in the process of intraocular inflammation, TGFβ2 and FGF reportedly play major roles in regulation of lens cell behaviors (i.e., fibrogenic reaction and cell proliferation) during wound healing [7,8,18,19,21]. We therefore assayed the amount of these two growth factors in lens and aqueous humor in our cataract model.

Finally we showed that loss of Smad3 attenuated, but not completely abolished, this phenomenon in mice, indicating that Smad3 signaling is involved in the development of EMT-associated anterior capsular cataract formation upon exposure of the eye to alkali.

METHODS

Alkali injury in the animal eye: All experiments were performed in accordance with the ARVO Resolution on the Use of Animals in Research and approved by the Committee of Animal Experiments of Wakayama Medical University. Adult

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male Wistar rats (n=142) were anesthetized with diethyl ether inhalation. NaOH (1.0 N, 10 μl) was dropped on the right eye of each rat. After the procedure, animals were sacrificed and eyes were harvested at these time intervals: 1 (n=10), 2 (n=10), 6 (n=10), and 12 (n=10) hours; 1 (n=22), 2 (n=10), and 5 (n=22) days; 1 (n=16) and 3 (n=10) months. Untreated eyes (22) were used as controls. All rat lenses formed anterior subcapsular cataracts by day 2 following alkali exposure. They were fixed in 4% paraformaldehyde and embedded in paraffin. Nine Smad3$^{-/-}$ mice and 9 wild-type littermates were anesthetized with diethyl ether inhalation. NaOH (1.0 N, 3 μl) was applied to the right eye of each mouse. The animals were sacrificed and enucleated at 5 (n=6), 10 (n=6), and 20 (n=6) days. Eyes were fixed in 4% paraformaldehyde and embedded in paraffin.

Histology and immunohistochemistry: Deparaffinized sections cut at 5 μm thickness were stained with hematoxylin and eosin alone or with the following antibodies diluted in PBS: goat polyclonal anti-Snail antibody (sc-10432, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-αSMA antibody (1:100; NeoMarker, Fremont, CA), goat polyclonal anti-Type I collagen antibody (1:100; Southern Biotechnology Associates, Inc. Birmingham, AL), anti-TGFβ1, anti-TGFβ2, and anti-TGFβ3 antibody as previously reported [22,23], goat polyclonal anticonnective tissue growth factor(CTGF) antibody (1:100; Santa Cruz Biotechnology), rabbit polyclonal antiphospho-Smad2 antibody (1:50; Chemicon, Temecula, CA), rabbit polyclonal anti-Smad3 antibody (1:100; Zymed, South San Francisco, CA), rabbit polyclonal antiproliferative cell nuclear antigen (PCNA) antibody (FL-261; 1:100; Santa Cruz Biotechnology). Specimens were washed in phosphate buffered saline (PBS) and allowed to react with peroxidase conjugated polyclonal secondary antibodies (1:200 in PBS; Cappel, Organon-Teknika, West Chester, PA). Specimens were washed again and the reaction was visualized with 3,3'-diaminobenzidine (DAB) using a previously reported technique [3]. Sections were counterstained with methylgreen and mounted in balsam. For proliferative cell nuclear antigen (PCNA) immunostaining color development by DAB was performed with 3% hydrogen peroxide and 3% nickel chloride in order to increase the color contrast.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out on deparaffinized sections cut at 5 μm thickness as previously reported in the literature [24]. In brief, sections were digested with proteinase K (10 μg/ml; Sigma, St. Louis, MO) for 5 min at room temperature. Sections were washed with PBS and treated with 1X TdT buffer containing TdT (Gibco BRL, Carlsbad, CA) and biotinylated dUTP (Boehringer Mannheim, Sandhofer, Mannheim, Germany) for 45 min at 37 °C. After the sections were washed with PBS, they were treated with strept-avidin-peroxidase and DAB. After counterstaining with methylgreen, the sections were embedded. For

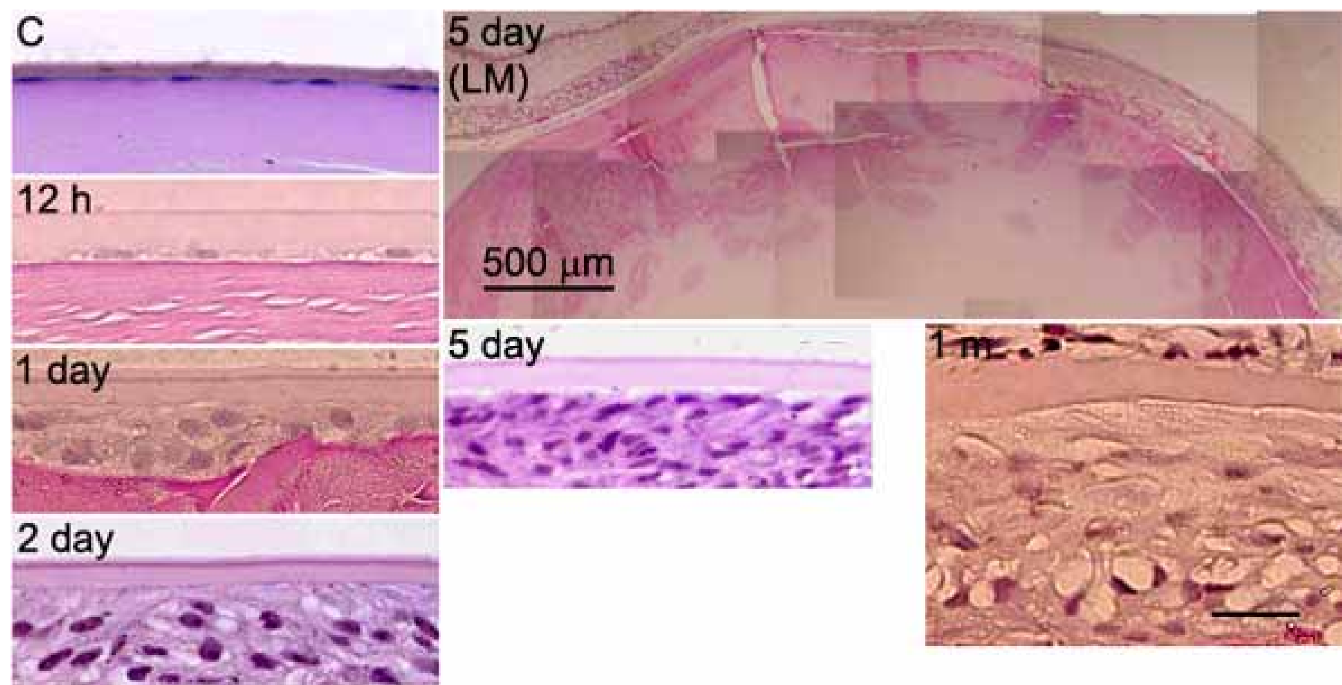


Figure 1. Alkali injury to the ocular surface induces anterior subcapsular cataract. At 12 h, anterior lens epithelial cells partially disappeared, as compared to the control (C) tissue. At day 1, lens epithelial cells formed multiple layers beneath the anterior capsule. At day 2, the layers of spindle-shaped fibroblastic cells were observed beneath the anterior capsule and became prominent after day 5. At 1 month (1 m), fibrous tissue was observed beneath the anterior capsule. The tissues were stained with hematoxylin and eosin. The bar in the low magnification (LM) 5 day image represents 500 μm; the bar in 1 m represents 10 μm and applies to all other images.

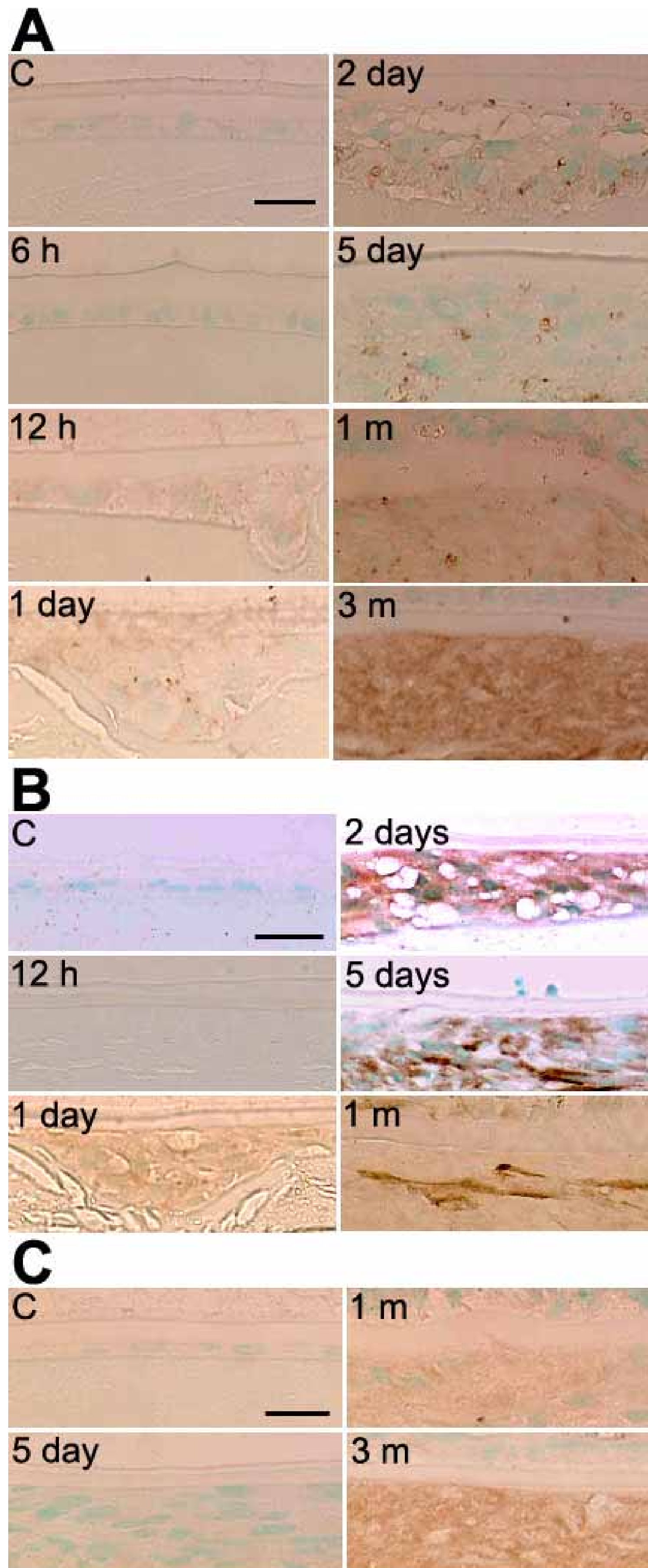


Figure 2. Expression pattern of epithelial mesenchymal transition (EMT) markers in rat lenses following alkali injury. **A**: Immunolocalization of Snail in rat lenses following alkali injury ("C" indicates control tissue). Snail protein is detected weakly in lens epithelial cells at 12 h. Its expression increased in EMT lens cells at the later times until 3 months. **B**: Immunolocalization of αSMA in rat lenses following alkali injury ("C" indicates control tissue). The lens epithelial cells that have formed multiple layers beneath the anterior capsule weakly express αSMA at day 1. At day 2, elongated cells multilayered beneath the anterior lens capsule are markedly labeled with the antibody. **C**: Immunolocalization of collagen type I in rat lenses following alkali injury ("C" indicates control tissue). Collagen type I is weakly detected in EMT lens cells at one month and strongly positive at three months. Nuclei were counterstained with methylgreen. The bars in the control images represent 10 μm.

a negative control, specimens were stained without biotinylated-dUTP. For a positive control, the sections were treated with 10 μg/ml of DNase I for 10 min and then examined.

Active TGFβ2 level in injured rat lens and aqueous humor: The lens and aqueous humor were obtained from alkali injured rat eyes 1 and 5 days post-burn. Each lens was sonicated with an ultrasound tissue homogenizer in PBS (1 ml/mg of tissue). All specimens were stored at -80 °C until measurement of TGFβ2 content. The amount of TGFβ2 in each specimen was measured by using an enzyme-immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Both the active and inactive forms of TGFβ2 were assayed by following a previously reported procedure [25].

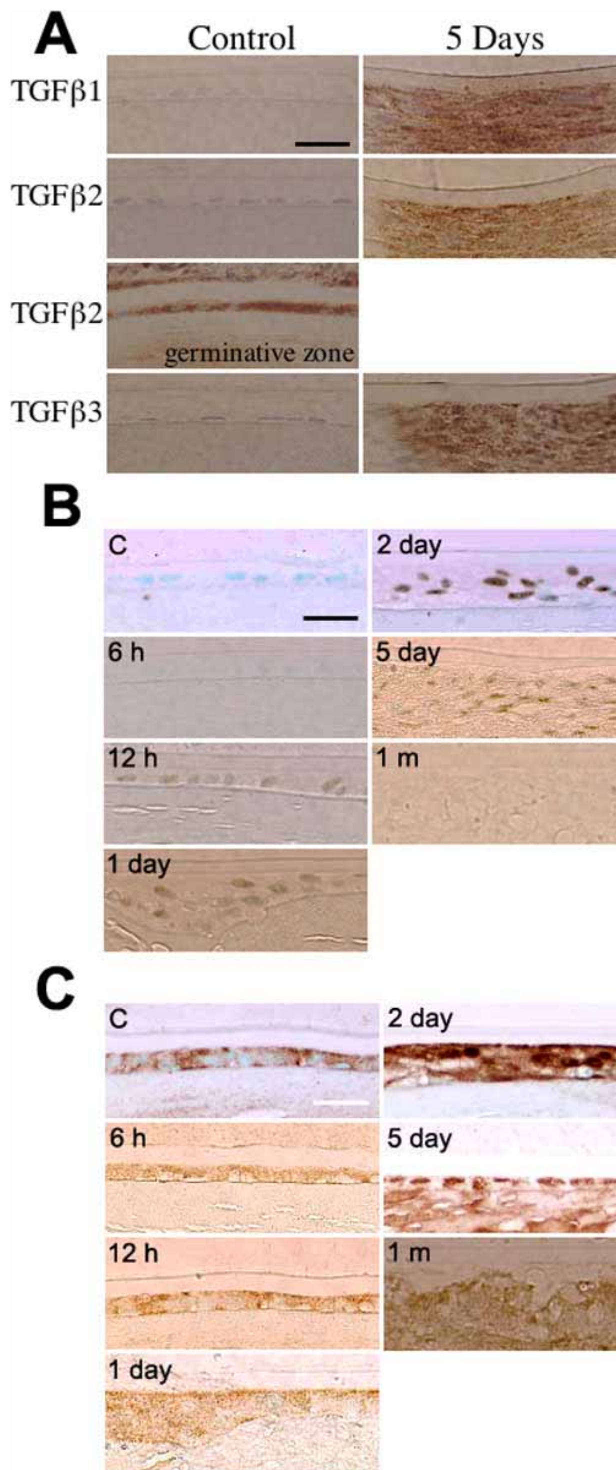
FGF2 level in injured rat lens and aqueous humor: The lens and aqueous humor were obtained from alkali injured rat eyes 1 and 5 days, and 1 month post-burn. Each lens was sonicated with an ultrasound tissue homogenizer in phosphate-buffered saline (PBS) 1 ml/mg of tissue. All the specimens were stored at -80 °C until measurement of FGF2 content. The amount of FGF2 in each specimen was measured by using an enzyme-immunoassay kit (R&D Systems) according to the manufacturer's protocol. FGF2 were assayed as previously reported [19].

Smad3 knockout mouse: To examine the role of Smad3 signaling in the formation of the anterior subcapsular cataract upon an alkali exposure, we used Smad3-null mice. The crystalline lens of Smad3-null mice exhibits no structural abnormalities as previously reported (data not shown) [8]. Deparaffinized sections were cut at 5 μm thickness. Histology and immunofluorescent microscopy for αSMA, collagen VI, phospho-Smad2 and

Smad3 were performed as previously reported in the literature [26].

RESULTS

Histology of alkali-injured rat lenses: Figure 1 shows the histology of lenses at various intervals following alkali injury. At 12 h, anterior lens epithelial cells partially disappeared. At day 1, lens epithelial cells formed multiple layers beneath the anterior capsule. At day 2, the layers of spindle-shaped fibroblastic cells were observed beneath the anterior capsule, and



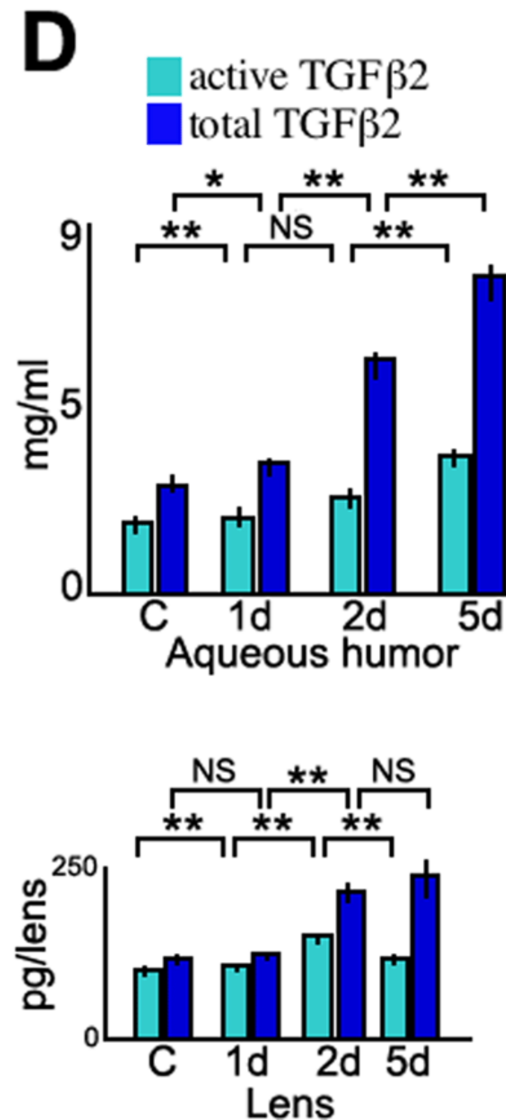


Figure 3. Transforming growth factor β (TGFβ) expression and its signaling in lens or aqueous humor of an alkali-burned rat eye. **A**: Immunolocalization of TGFβ1, 2, and 3 in rat lenses at day 5 following alkali injury. TGFβ1 and TGFβ3 are not detected in the uninjured lens and at day 5. TGFβ2 was detected in only the germinative zone of lens epithelial cells in the uninjured lens. At day 5, epithelial mesenchymal transition (EMT) lens cells express TGFβ1, TGFβ2, and TGFβ3. **B**: Immunolocalization of phospho-Smad2 in rat lenses following alkali injury. At 12 h, phospho-Smad2 protein is weakly detected in the nuclei in the lens epithelial cells and then is detected in the nuclei of the majority of elongated cells in the cell multilayer of the cataractous area through days 1-5. It is no longer detected in such elongated cells in the cataractous region at 1 month. **C**: Immunolocalization of Smad3 in rat lenses following alkali injury. Smad3 protein is detected in the cytoplasm of uninjured lens epithelial cells at 6 h to day 1. At days 2 and 5, Smad3 protein is detected in the nuclei of fibroblastic cells as well as in their cytoplasm. It is, however, detected in the cytoplasm, but not the nuclei at 1 month. Nuclei were counterstained with methylgreen (**A**-**C**). The bars represent 10 μm. **D**: TGFβ2 levels in injured rat lens and aqueous humor. Active and total TGFβ levels were assayed using ELISA. The asterisk indicates a $p<0.05$ and the double asterisk indicates a $p<0.01$; nonsignificant comparisons are marked "NS".

they became prominent after day 5. At 1 month, fibrous tissue was observed beneath the anterior capsule (Figure 1). These findings strongly suggested that the lens epithelial cells in an eye with an alkali burn had undergone EMT. Thus, we examined the expression of EMT markers as follows.

Immunohistochemistry for Snail, αSMA, or collagen I: Snail is shown to be an immediate-early Smad3-dependent gene target of TGFβ in fibroblasts [27]. Our immunostaining detected weak expression of Snail in lens epithelial cells at 12 h. The expression gradually increased in lens cells (Figure 2A).

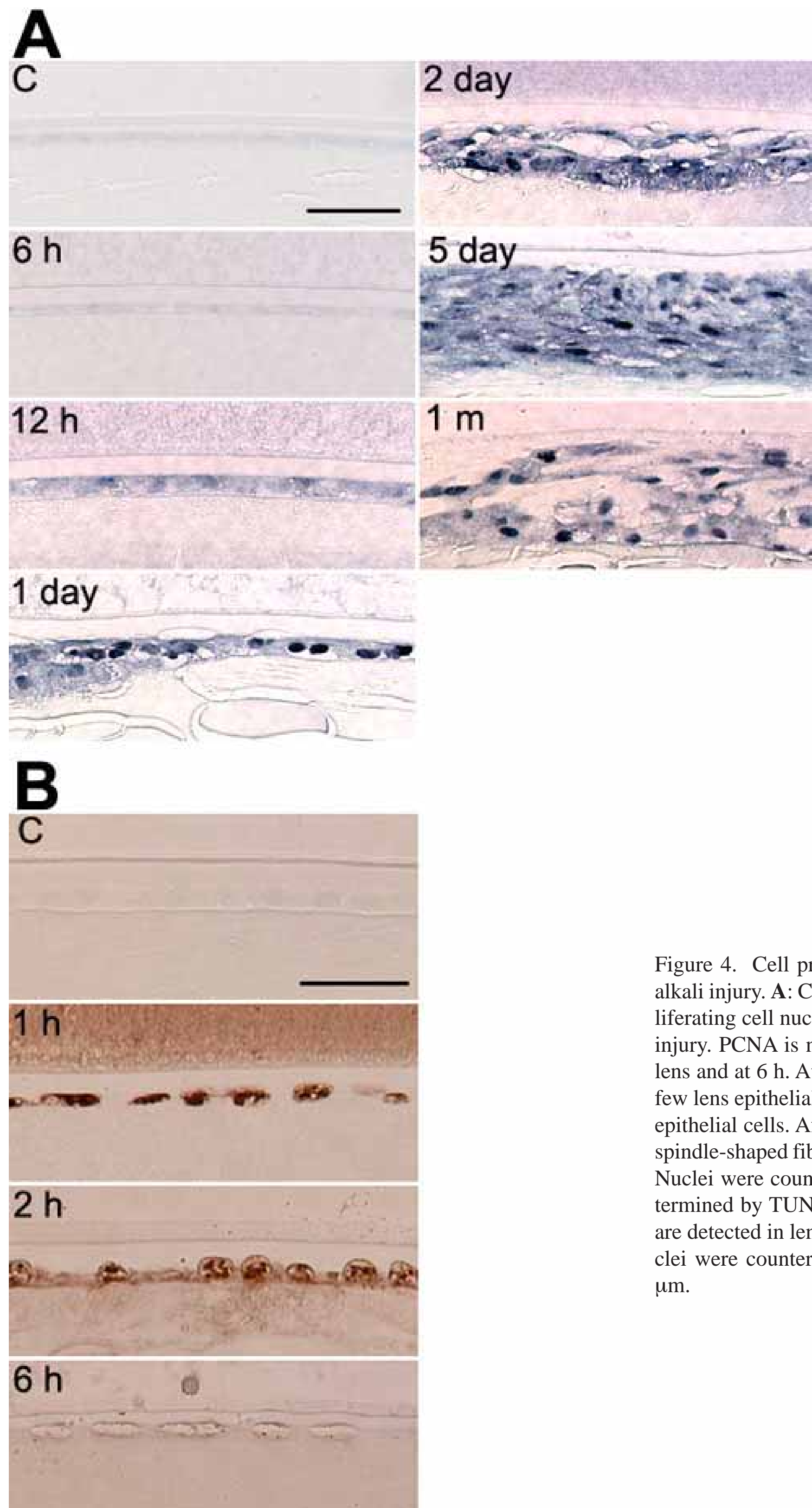


Figure 4. Cell proliferation and cell death in rat lenses following alkali injury. **A**: Cell proliferation as evaluated by expression of proliferating cell nuclear antigen (PCNA) in rat lenses following alkali injury. PCNA is not detected in the central region in the uninjured lens and at 6 h. At 12 h, PCNA is weakly positive in the nuclei of a few lens epithelial cells. At day 1, PCNA was detected in many lens epithelial cells. After two days, PCNA was positive in the layers of spindle-shaped fibroblastic cells and was also detected at later times. Nuclei were counterstained with methylgreen. **B**: Cell death as determined by TUNEL staining. From 1-2 h, TUNEL-positive nuclei are detected in lens epithelial cells. At 6 h, they are not present. Nuclei were counterstained with methylgreen. The bars represent 10 μm.

αSMA is a hallmark of myofibroblast generation and EMT in lens epithelial cells [15,28]. Although the epithelial cells in an uninjured lens were not labeled with the anti-αSMA antibody, the cells weakly expressed αSMA in day 1. At day 2, elongated multilayers of cells beneath the anterior lens capsule were markedly labeled with the antibody (Figure 2B). Collagen type I is the major extracellular matrix component in human anterior capsular cataracts and is known to be upregulated during EMT of lens cells [29,30]. Although collagen type I was not detected in uninjured lens, it was weakly detected in lens cells which had undergone EMT at 1 month and strongly positive at 3 months (Figure 2C).

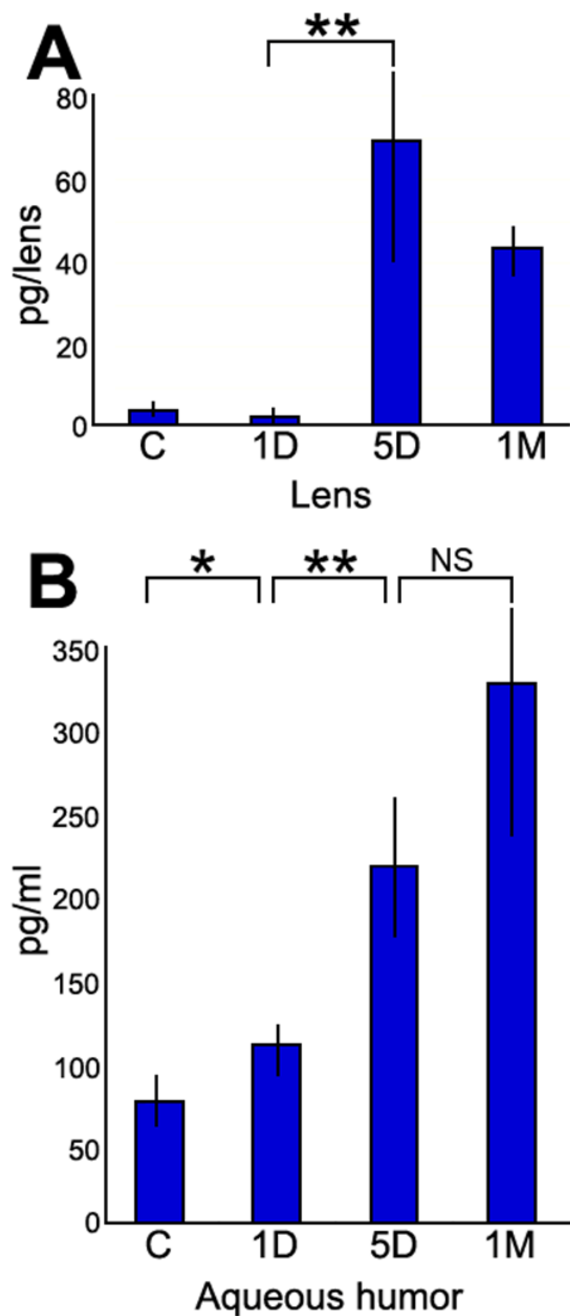


Figure 5. Fibroblast growth factor 2 (FGF2) levels in injured rat lens and aqueous humor. FGF2 levels were assayed using ELISA. The asterisk indicates a $p<0.05$ and the double asterisk indicates a $p<0.01$; a nonsignificant comparison is marked “NS”.

Expression of TGFβs and connective tissue growth factor (CTGF) in lens epithelium: We examined the expression of TGFβ1, TGFβ2, and TGFβ3 at day 5. TGFβ1 and TGFβ3 were not detected in the uninjured lens. TGFβ2 was detected in only the germinative zone of lens epithelial cells in the uninjured lens. At day 5, EMT lens cells expressed TGFβ1, TGFβ2, and TGFβ3 (Figure 3A). Although connective tissue growth factor (CTGF) was not detected in uninjured lens, it was weakly detected in lens cells that had undergone EMT at 1 month and strongly positive at 3 months (data not shown).

Expression pattern of phospho-Smad2 and Smad3: Although phospho-Smad2 protein was not detected in uninjured lens, at 12 h, it was weakly detected in the nuclei in the lens epithelial cells and also detected in the nuclei of the majority of elongated cells in the cell multilayer of the cataractous area through days 1-5. It was no longer detected in such elongated cells in the cataractous region at 1 month (Figure 3B). Smad3 protein was detected in the cytoplasm of uninjured lens epithelial cells. At 2 and 5 days, Smad3 proteins were detected in the nuclei and cytoplasm of fibroblastic cells. It was, however, detected in the cytoplasm, but not the nuclei, at 1 month (Figure 3C).

Active TGFβ2 level in injured rat lens and aqueous humor: Since expression of Smad2/3 strongly suggested that TGFβ2 in the eye might be activated, we examined the amount of active and inactive TGFβ2 in aqueous humor and lens tissue in an alkali-burned rat eye. The concentration of active TGFβ2 (ng/ml) in the aqueous humor was 1.77 in an uninjured eye and 1.86, 2.29, or 3.41 at day 1, 2, or 5 post-alkali burn, respectively. The concentration of total TGFβ 2 (ng/ml) in the aqueous was 2.67 in an uninjured eye and was 3.26, 5.86 or 7.81 at day 1, 2, or 5 post-burn, respectively. Active TGFβ 2 (pg/lens) was 103 in an uninjured eye and was 111, 153, or 122 at day 1, 2 or 5 post-burn, respectively. The amount of total TGFβ2 (pg/lens) was 122 in an uninjured eye and was 126, 218, or 238 at day 1, 2, or 5 post-treatment, respectively (Figure 3D). The amount of both active and total TGFβ2 in the aqueous humor slightly increased at day 1 and then increased up to 1.3 fold and 2.2 fold at day 2, and 1.9 fold and 2.9 fold at day 5, respectively. In lens tissue also, the amount of both active and total TGFβ2 slightly increased at day 1 and then increased up to 1.5 fold and 1.8 fold at day 2, and 1.2 fold and 2.0 fold at day 5, respectively. The amount of active TGFβ2 in the lens tissue was maximum at day 2.

Cell proliferation and cell death: To detect cell proliferation in multilayered lens cells, we immunostained the specimens for PCNA. PCNA was weakly detected in the germinative zone in the uninjured lens (data not shown). At 12 h, PCNA was weakly positive in the nuclei of a few lens epithelial cells while at day 1, PCNA was detected in many cells. After two days, PCNA was positive in the layers of spindle-shaped fibroblastic cells and this activity persisted (Figure 4A).

TUNEL-positive cells were not detected in uninjured control lens. From 1-2 h post-alkali treatment, TUNEL-positive

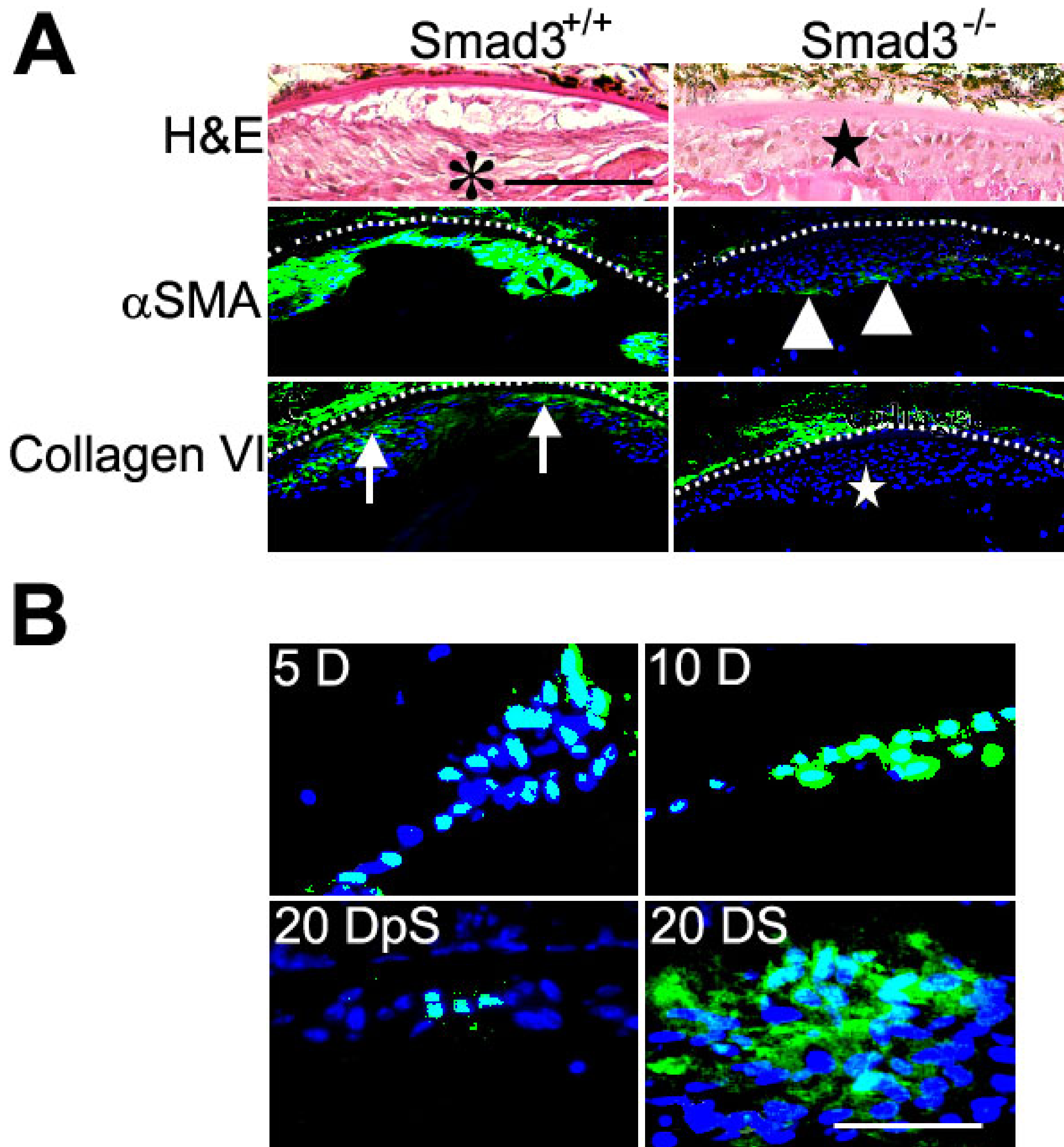


Figure 6. Development of alkali burn-induced subcapsular cataract is attenuated, but not completely blocked, by loss of Smad3. **A**: Histology and immunolocalization of αSMA and collagen type VI in Smad3$^{-/-}$ mouse lens at 20 days following alkali injury. Hematoxylin and eosin staining revealed lens epithelial cells are elongated, fibroblast-like in their shape (asterisk) in Smad3$^{+/+}$ mice, while they are relatively ovoid and epithelial-like in morphology (star) in Smad3$^{-/-}$ mice at day 20 post-alkali exposure. αSMA is markedly expressed in almost all the cells (asterisk) beneath the anterior capsule in a Smad3$^{+/+}$ lens, whereas faint, but positive, αSMA immunoreactivity is detected in a Smad3-null lens. In a Smad3-null lens αSMA-positive lens epithelial cells are mainly detected on the border between the cell mass and lens cortex (arrowheads). Type VI collagen is detected in the lens of a Smad3$^{+/+}$ mouse (arrows), but not in a Smad3$^{-/-}$ lens (star). Nuclei were counterstained with DAPI and the scale bar represents 50 μm. **B**: Immunolocalization of phospho-Smad2 and Smad3 in mouse lens following alkali injury. Phospho-Smad2 is detected in the nuclei of lens epithelial cells at day 5 and 10. The number of positive phospho-Smad2 nuclei seems less at day 20 as compared with earlier times. Smad3 is located in the cytoplasm and nuclei of lens cells in the cell multilayer at day 20. Smad3 and phsopho-Smad2 are not detected in the nuclei of epithelium of an uninjured lens of either Smad3$^{+/+}$ or Smad3$^{-/-}$ mice (not shown). Nuclei were counterstained with DAPI. The bar represents 20 μm.

nuclei were detected in lens epithelial cells, but they were not present at 6 h. (Figure 4B).

FGF2 level in injured rat lens and aqueous humor: Increases in cell proliferation in lens epithelium post-alkali burn suggested that some growth factor(s) capable of inducing proliferation might be upregulated. Based on previous literature, FGF2 is a likely candidate to promote cell proliferation in an injured lens [19]. We therefore assayed FGF2 levels in the eye by using an enzyme immunoassay. The results showed that the concentration of FGF2 (pg/ml) in the aqueous humor was 79 in an uninjured eye and 113, 220, or 330 at day 1, 5, or 1 month post-alkali burn, respectively. FGF2 (pg/lens) was 4.79 in an uninjured eye and was 3.31, 69.43, or 43.8 at day 1, 5, or 1 month post-burn, respectively (Figure 5).

Smad3 knockout mouse: We took advantage of Smad3-knockout mice to examine the role of Smad3 signaling in EMT of lens epithelium upon ocular alkali burn. Hematoxylin and eosin staining showed that lens epithelial cells were elongated and fibroblast-like (asterisk) in Smad3$^{+/+}$ mice, while they were relatively ovoid and epithelial-like in morphology (star) in Smad3$^{-/-}$ mice at day 20 post-alkali-treatment. These findings suggested that loss of Smad3 might perturb EMT in lens epithelium. We use immunohistochemistry to further examine this hypothesis. αSMA, the hallmark of lens epithelium EMT was markedly expressed in almost all the cells (asterisk) beneath the anterior capsule in a Smad3$^{+/+}$ lens, whereas faint αSMA immunoreactivity was detected in a Smad3-null lens. In a Smad3-null lens αSMA-positive epithelial cells were mainly detected in the border area between the cell mass and lens cortex (arrowheads). Expression of collagen type VI was then examined to evaluate the fibrotic reaction of the cells. Type VI collagen was detected in the lens of a Smad3$^{+/+}$ mouse (arrows), but not in a Smad3$^{-/-}$ lens (Figure 6A, star).

Phospho-Smad2 was not observed in the epithelium of an uninjured lens of either Smad3$^{+/+}$ or Smad3$^{-/-}$ mice (data not shown), while it was detected in the nuclei of Smad3$^{+/+}$ (data not shown) and Smad3$^{-/-}$ mouse lens epithelial cells at day 5 and 10. The amount of nuclear phospho-Smad2 appeared to decrease by day 20 as compared with earlier times (Figure 6B). There did not seem to be a difference in the expression pattern of phospho-Smad2 in lens epithelium between Smad3$^{+/+}$ and Smad3$^{-/-}$ mice. Smad3 was not detected in an uninjured lens epithelium of a Smad3$^{+/+}$ mouse (data not shown), whereas it was located in both the cytoplasm and nuclei of wild-type mouse lens cells in the cell multilayer during healing (Figure 6B). Smad3 protein was not detected in Smad3$^{-/-}$ mouse lens (data not shown).

DISCUSSION

Our study showed exposure of the cornea to alkali resulted in the formation of a multilayer of cells in the lens epithelium. The cells exhibited an elongated morphology and were labeled with anti-αSMA antibody and anti-Snail antibody, indicating that the cells had undergone EMT. This structure was consistent with the characteristics of anterior subcapsular cataracts of human patients. We also observed the appearance of TUNEL-positive cells inside the lens capsule prior to EMT, but the significance of this finding is unclear. Alkali treatment also affected other ocular tissues including cornea. The findings in the burned cornea in rat were similar to those seen in our previous mouse experiments [31,32], including epithelial defect, regeneration of conjunctival epithelium, and stromal inflammation. In later phases, such as one month, stromal neovascularization was developed in association with the reduction of inflammatory cell invasion.

A variety of animal models of cataract (e.g., cataracts induced by feeding an overdose of galactose or selenium) have been reported [33,34]. However, these models were nuclear cataracts or opacification of the lens cortex and did not exhibit EMT of lens epithelium. This is also the case in the inheritable cataract model or in cataracts induced by knockout of specific genes responsible for lens homeostasis in animals. Srinivasan et al. [35] reported that ectopic overexpression of the active form of TGFβ1 in the mouse lens epithelium results in the formation of an anterior subcapsular cataract that consists of EMT-type lens epithelium. West-Mays employed adenoviral gene introduction of active TGFβ1 to induce an EMT-type cataract (personal communication, May, 2005). We have also produced an anterior subcapsular cataract model in a CMV-promoter-driven Smad3-overexpressing transgenic mouse (Muragaki Y, Ooshima A, Saika S, personal communication, 2004). Although these models clearly demonstrate that TGFβ signaling is sufficient to induce an anterior subcapsular cataract, generation of a transgenic mouse model is difficult. In the present study, alkali burn to the rat eye resulted in Smad signaling activation, Snail induction, and finally expression of αSMA in lens epithelium, leading to EMT. Furthermore, loss of Smad3 attenuated lens epithelium EMT upon alkali exposure to the ocular surface, indicating TGFβ/Smad3 signaling is involved in the induction of the EMT-type anterior subcapsular cataract. In a previous paper, we reported that loss of Smad3 abolished injury-induced EMT of lens epithelium by using a puncture model [8]. However, in the present alkali-burn cataract model, the EMT of the lens epithelium was not completely abolished, although it was strongly attenuated. The exact reason for this discrepancy is currently unknown, but a possible explanation involves differences in the level and duration of TGFβ stimuli in the lens epithelium. A puncture injury in the anterior capsule minimally affects the tissue outside the injury site, and tissue inflammation is subtle and disappears quickly, whereas an alkali burn in the cornea is associated with severe inflammation in the anterior chamber, which lasts for a longer period post-burn. Thus, it is possible that activation of a high level of TGFβ for a longer interval might allow the bypass of Smad3 signaling by Smad2 or other non-Smad-signals to subsequently regulate the initiation of EMT. The duration of Smad nuclear translocation supports this; in our previous puncture model Smad2/3 nuclear translocation lasted 48 h, whereas it was still observed at day 5 in the present alkali burn cataract model. Indeed, transgenic overexpression of active TGFβ1 in the Smad3-null background, results in development of EMT-type cataracts (Judy West-Mays, personal communication, May, 2005), further supporting our hypothesis. However, signaling regulation of lens epithelium

EMT may be even more complicated, since it has been reported that conditional deletion of TGFβ receptor type II in lens epithelium by using Pax6 promoter-driven Cre recombinase does not affect lens epithelium EMT upon experimental cataract surgery [36].

The findings that Smad2 and Smad3 are activated in our cataract model as well as Smad3-null mice not forming this type of cataract clearly demonstrate that TGFβ or activin/Smad signaling is involved in an alkali burn-induced EMT-type cataract. Our unpublished observation that Smad3 overexpression resulted in the development of an EMT-type cataract in mice indicates that a certain percentage of TGFβ in the eye lens or aqueous humor is in the active form, but it is not sufficient to drive the Smad3 cascade to induce EMT in the lens epithelium. We therefore examined the proportion of active to inactive TGFβ2 in aqueous humor and lens tissue in an alkali-burned rat eye. The amount of both active TGFβ2 and total TGFβ2 in the aqueous humor slightly increased at day 1 and then increased up to 1.3 fold and 2.2 fold at day 2, and 1.9 fold and 2.9 fold at day 5, respectively. In lens tissue also, the amount of both active TGFβ2 and total TGFβ2 slightly increased at day 1 and then increased further up to 1.5 fold and 1.8 fold at day 2, and 1.2 fold and 2.0 fold at day 5, respectively. Although the proportion of active/latent TGFβ2 in aqueous humor or lens decreased in our model, the amount of active TGFβ2 was upregulated concomitantly with the amount of total TGFβ2 there. We do not know which was more critical for induction of EMT in lens cells post-alkali burn, active TGFβ2 in aqueous humor or lens tissue. Inflammation in the corneal stroma and the anterior chamber upon alkali exposure to the cornea may lead to the greater increase of TGFβ2 in the aqueous humor as compared to the lens tissue. Ohta et al. [37] reported that total TGFβ levels were increased as early as 6 h after induction of experimental autoimmune uveitis (EAU) in mice, and peaked at 12 h at about three times the level of controls and remained elevated through 24 h. Both active TGFβ1 and active TGFβ2 levels rose after EAU induction [37]. The significant role of inflammation in the increase of total TGFβ2 in the aqueous humor might be further supported by our unpublished finding that an injury to the anterior capsule through the cornea with minimal inflammation in the anterior chamber did not increase the total TGFβ2, but decreased it in the lens, although the proportion of active form/latent form was increased. While the amount of active TGFβs was not significantly increased at day 1, phospho-Smad2 was readily detected at 12 h. Besides activation of the ligand, there might be other mechanism(s) which increased the cells' susceptibility to TGFβ. Active TGFβ2 in the lens tissue exhibited its peak at day 2, at the time when the level of expression of phospho-Smad2 was also at its maximum.

In our previous study using a lens injury model, Smad2/3 are both activated by 12 h post-injury [3]. However in the present alkali burn model, Smad2 was phosphorylated in 12 h as in our previous paper, whereas Smad3 translocated to the nuclei of the epithelium by day 2. This suggests that there might be a differential mechanism of activation between Smad2 and Smad3 in lens epithelium.

It is well known that TGFβ signaling secondarily induces expression of other growth factors/cytokines [38,39]. In cell culture, TGFβ2 upregulates FGF2 (unpublished data) and also in vivo intraocular FGF2 levels are dramatically increased along with the activation of TGFβ/Smad signal following lens injury [19]. In our alkali burn-induced cataract model, the majority of the EMT-derived myofibroblastic lens cells were labeled with anti-PCNA antibody, indicating the cells had increased proliferative activity even though TGFβ/Smad signaling in general suppresses cell proliferation [40]. We therefore hypothesized and demonstrated that the amount of FGF2 in the lens and aqueous humor of the burned eye was markedly increased. FGF2 enhances cell proliferation of lens epithelium [18,19]. We have shown that proliferation during healing of a puncture injury in the lens was less in FGF2-null mice as compared with wild-type littermates [19]. Rat lens epithelial cells in the cataractous lens of an alkali-burned eye also exhibited an increase of cell proliferation along with EMT, concomitant with the increased amount of FGF2 in the lens and aqueous humor. These findings suggest that increased cell proliferation in this cataract model might be attributed to the increased FGF2 expression. Moreover, FGF2 is involved in extracellular matrix expression, suggesting cooperative role of FGF2 in TGFβ-induced fibrosis in the lens [41]. TGFβ also upregulates TGFβ itself or CTGF, which are both involved in expression and deposition of extracellular matrix (i.e., collagen, fibronectin, hyaluronan, fibrillin, osteopontin, and lumican) in tissue fibrosis including lens epithelial cells. However, it is known that many cell proliferation-promoting growth factors are upregulated in the diseased eye. We could not define the roles of such non-TGFβ growth factors in the development of cataracts in the present model.

TGFβ is also known to induce apoptosis in cells. We determined the presence of cell death in the lens by using the TUNEL method and showed TUNEL-positive cells were mainly observed beneath the anterior capsule from 1-2 h and no longer seen at 6 h when lens epithelial cells partially disappeared in this region. It seems to take longer for TGFβ to induce apoptosis in lens epithelial cells. For example, Maruno et al. [42] reported TGFβ-induced apoptosis in rat lens epithelial explants after two days and in the intact lens after five days, and Lee et al. [43] reported TGFβ-induced apoptosis in the human lens epithelial cell line HLE B-3 cells after 72 h. Thus the cell death detected in our study might be attributed to other events related to alkali exposure. Nevertheless, there is a possibility that the newly formed acellular space that appears post-alkali exposure might provide a space for cell migration, which could be one of the factors responsible for the induction of EMT.

Our study introduced a new cataract model, the anterior subcapsular type with EMT in lens epithelium, induced by topical alkali exposure to the ocular surface. We also reported here that TGFβ/Smad3 signal is required for the development of this cataract. However this finding does not exclude the possibility that other signaling cascades derived from not only TGFβ but also other cytokines may play a role. Further detailed study is needed to clarify the entire mechanism of in-

duction of EMT in lens epithelial cells. This cataract model is also believed to be useful to test putative anticataract drugs.

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