LABORATORY SCIENCE

TGFB-Smad signalling in postoperative human lens epithelial cells

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Aims: To localise Smads3/4 proteins in lens epithelial cells (LECs) of fresh and postoperative human specimens. Smads3/4 are involved in signal transduction between transforming growth factor β (TGFB) cell surface receptors and gene promoters. Nuclear localisation of Smads indicates achievement of endogenous TGFB signalling in cells.

Methods: Three circular sections of the anterior capsule, one lens, and 17 capsules undergoing postoperative healing were studied. Immunohistochemistry was performed for Smads3/4 in paraffin sections of the specimens. The effect of exogenous TGFB2 on Smad3 subcellular localisation was examined in explant cultures of extracted human anterior lens epithelium.

Results: The cytoplasm, but not the nuclei, of LECs of uninjured lenses was immunoreactive for Smads3/4. In contrast, nuclear immunoreactivity for Smads3/4 was detected in LECs during capsule healing. Nuclei positive for Smads3/4 were observed in monolayered LECs adjacent to the regenerated lens fibres of Sommerring’s ring. Interestingly, the nuclei of LECs that were somewhat elongated, and appeared to be differentiating into fibre-like cells, were negative for Smads3/4. Fibroblast-like, spindle-shaped lens cells with nuclear immunoreactivity for nuclear Smads3/4 were occasionally observed in the extracellular matrix accumulated in capsule opacification. Exogenous TGFB induced nuclear translocation of Smad3 in LECs of anterior capsule specimens in explant culture.

Conclusions: This is consistent with TGFB induced Smad signalling being involved in regulating the behaviour of LECs during wound healing after cataract surgery.

MATERIALS AND METHODS

Specimens
All specimens examined had been removed from Japanese patients, with a mean age of 64 years (range 28–80; Table 1). Specimens were obtained at the Wakayama Medical College Hospital, Wakayama, Japan, or were supplied by the IOL Implant Data System Committee of the Japanese Society of Cataract and Refractive Surgery. The specimens were fixed immediately after the removal. They were processed for histological examination as follows after informed consent was obtained. Circular sections of the anterior capsule of cases 1–3 were obtained during cataract surgery. The crystalline lens of case 4 was extracted because of its dislocation and was removed from an enucleated globe. Anterior capsular specimens with anterior subcapsular cataract were excluded in this study. Cases 5–21 were capsules in various stages of postoperative healing obtained during a subsequent operation to treat proliferative vitreoretinopathy, dislocation of the capsular bag with an IOL, or malignant glaucoma. Cases 6 and 8 were postoperative capsules without IOL implantation. Cases 7 and 16 had undergone implantation of an Acrysof IOL (Alcon, Fort Worth, TX, USA) and a silicone IOL, respectively. Other postoperative specimens had a polymethylmethacrylate IOL. Specimens were fixed in 10% formalin and embedded in paraffin as previously reported.

Immunohistochemistry
Deparaffinised sections cut at 5 µm thickness were immunostained with rabbit polyclonal anti-Smad3 antibody (diluted × 100 in phosphate buffered saline (PBS); Zymed, South San Francisco, CA, USA), goat polyclonal antibody against Smad4 (diluted × 200 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-α-SMA antibody (×100, Sigma, Saint Louis, MO, USA) or rabbit polyclonal anti-β-crystallin antibody (McAvoy, 1978, 5 µg/ml in PBS). After washing in...
PBS, specimens were allowed to react with peroxidase conjugated polyclonal secondary antibodies (×200 in PBS; Cappel, Organon-Teknika, West Chester, PA, USA). Reactivity/specificity of the antibodies used here was referred to the previous publication by Flanders et al.25 After another wash, the reaction product was visualised with 3,3’-diaminobenzidine and sections were counterstained with methyl green and mounted in balsam. Negative control staining was performed with rabbit, goat or mouse non-immune IgG as primary antibody, as appropriate.

**Explant culture experiment**

In order to define whether nuclear translocation of Smad3 is induced by TGFβ2, we cultured the anterior capsular sections excised during cataract surgery in the presence and absence of TGFβ2. An anterior capsule was put into the Eagle’s medium (Gibco BRL, Life Technologies Inc, Gaithersberg, MD, USA) in the presence or absence of the active form of porcine TGFβ2 (1.0 ng/ml, R & D system, Minneapolis, MN, USA) immediately after extraction. We have confirmed that this TGFβ2 is effective to human cells in vitro by determining collagen I upregulation in human subconjunctival fibroblast culture. Altogether 15 specimens were studied, the ages ranged from 58 to 80 years of age and the mean age was 67.7 (SD 6.31) years; the ratio of male to female specimens was 8 to 7. Capsu- 

**RESULTS**

As shown in Table 1 we obtained specimens for analysis from patients at the time of cataract surgery (cases 1–4) and at various times after cataract surgery (cases 5–21). This allowed us to analyse the distribution of TGFβ signalling molecules, the Smads, in relation to postoperative cellular changes. In particular, we examined Smads 3/4 localisation patterns in cells of the epithelium, Sommerring’s ring and the ECM accumulated next to the implanted lens optic. In general, we noted that, beginning at 10 days after surgery, indications of lenticular fibre regeneration (Sommerring’s ring) could be observed in the peripheral capsular bag. Fibroblast-like spindle-shaped lens cells were commonly observed in areas of fibrous opacification of the capsule in the specimens obtained at or beyond 0.65 year after initial surgery. Histology was similar to that previously reported.11

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**Table 1 Summary of the cases and the results**

<table>
<thead>
<tr>
<th>Case No</th>
<th>Age*</th>
<th>Sex</th>
<th>Duration</th>
<th>Cause†</th>
<th>Smad3</th>
<th>Smad4</th>
<th>Presence/absence of nuclear Smads3/4 positive cells</th>
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<tr>
<td></td>
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<td>Epithelial-type cells adjacent to regenerated lens fibres in Sommerring’s ring</td>
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<td></td>
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<tr>
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<td>F</td>
<td>–</td>
<td>CCC‡</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>2</td>
<td>72</td>
<td>M</td>
<td>–</td>
<td>CCC</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>M</td>
<td>–</td>
<td>CCC</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>4</td>
<td>72</td>
<td>F</td>
<td>–</td>
<td>Lens dislocation</td>
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<td>–</td>
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<td>M</td>
<td>6 days</td>
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<td>NE</td>
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<td>6</td>
<td>64</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>77</td>
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<td>+</td>
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<td>–</td>
<td>NE</td>
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<tr>
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<td>68</td>
<td>M</td>
<td>9 y</td>
<td>IOL dislocation</td>
<td>–</td>
<td>–</td>
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<tr>
<td>21</td>
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<td>M</td>
<td>10 y</td>
<td>IOL dislocation</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Age at the removal of the intraocular lens (IOL); † duration between implantation and explantation of the IOL; y = year(s); ‡ CCC = continuous circular capsulorhexis; PVR = IOL removal to obtain the better observation of the fundus during vitrectomy for proliferative vitreoretinopathy; capsulotomy = obtained by anterior capsulotomy during cataract surgery; – = negative; + = positive (regardless the incidence of positive cells); NE = not examined.

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**Figure 1 Intracellular immunolocalisation of Smad3 in human lens epithelial cells on the capsular specimen obtained during cataract surgery (A, case 2) or extracted 10 days after cataract surgery (B; case 6). Lens epithelial cells on the capsular specimen, obtained during first cataract surgery are immunoreactive for Smad3 in the nuclei (A, case 6). Lens epithelial cells on the capsular specimen obtained during first cataract surgery are immunoreactive for Smad3 in the cytoplasm, but not in the nuclei. On the other hand, lens cells 10 days postoperatively show nuclear immunoreactivity for Smad3 with a weak reaction in the cytoplasm. Indirect immunostaining. Bar, 10 μm.**
Epithelial cells and cells adjacent to Sommerring’s ring

To determine if there was any evidence of TGF\(\beta\) signalling through the Smad pathway as a result of cataract surgery, we compared the intracellular localisation of Smads in epithelial cells excised at the time of cataract surgery with epithelial cells in specimens collected postoperatively. Smad3, a TGF\(\beta\) signalling Smad, was detected in the nuclei of epithelial-shaped lens cells as early as 10 days postoperatively, whereas it was negative in the nuclei of freshly isolated lens epithelia (Fig 1).

At 2.7 years postoperatively, in the area of the peripheral capsular bag, cuboidal, epithelial-like lens cells were positive for nuclear Smad3. Adjacent cells that were somewhat elongated, lacked immunoreactivity for Smad3 in their nuclei (Fig 2). The cuboidal cells between the regenerated fibre-like cells and residual capsule appeared morphologically similar to lens epithelial cells by light microscopy, whereas the elongated cells looked like epithelial cells undergoing the transition into fibres. These epithelial and fibre-like phenotypes were consistent with the distribution of fibre specific \(\beta\)-crystallin; cuboidal cells were negative for \(\beta\)-crystallin, whereas both denucleated lenticular fibres and elongated cells containing nuclei were immunoreactive for \(\beta\)-crystallin (Fig 3). Five years postoperatively, the cuboidal, epithelial-like lens cells maintained their nuclear Smad3 immunoreactivity and a similar loss of nuclearly localised Smad3, as described above, was evident in the differentiating fibre-like cells (Fig 4).

Smad4 is the member of Smad family of proteins which works in both TGF\(\beta\) and BMP signal transduction pathways. Similar to smad3, Smad4 was detected in the cytoplasm, but not in the nuclei, of lens epithelial cells lining the inner surface of the uninjured anterior lens capsule (Table 1, Fig 5A). Postoperatively, Smad4 was located in the nuclei of cuboidal lens epithelial cells between the anterior and posterior capsules in the peripheral capsular bag and in lens epithelial cells adjacent to the regenerated lenticular structure.

Figure 2 Immunolocalisation of Smad3 in lens cells in Sommerring’s ring 2.7 years postoperatively (case 12). Lens epithelial cells beneath the capsule (arrowheads) are positive for nuclear Smad3. (A) and (B) Higher magnification pictures of boxed areas a and b, respectively, show the progressive disappearance of nuclear Smad3 in elongating lens cells (arrows) as they differentiate into the fibre-like cells. Indirect immunostaining. Bar, 20 \(\mu\)m.

Figure 3 Immunolocalisation of \(\beta\)-crystallin in Sommerring’s ring 2.7 years postoperatively (case 12). (A) Enlarged/elongated lens cells forming the Sommerring’s ring structure are immunostained for \(\beta\)-crystallin, whereas monolayered epithelial-shaped lens cells are unstained; (B) and (C) are the high magnification pictures of boxed areas b and c, respectively. Asterisks in (B) show immunostained enlarged/elongated cells reactive for fibre specific \(\beta\)-crystallin. Arrows in (C) show a monolayer of unstained lens cells. Indirect immunostaining. Bar, 60 \(\mu\)m (A); 20 \(\mu\)m (B and C).

Figure 4 Immunolocalisation of Smad3 in lens cells in Sommerring’s ring 5 years postoperatively (case 16). Lens epithelial cells beneath the anterior capsule (boxed area a) are positive for nuclear Smad3, but somewhat elongated lens cells in the equator (boxed area b) do not show nuclear immunoreactivity. The asterisk indicates the position of the haptic loop of the intraocular lens. Indirect immunostaining. Bar, 50 \(\mu\)m; inserts bar, 10 \(\mu\)m.

Figure 5 Intracellular immunolocalisation of Smad4 in lens epithelial cells following cataract surgery. (A) (Case 1), epithelial cells (arrows) in an uninjured lens show Smad4 immunoreactivity in the cytoplasm. In (B) a specimen obtained 6 days after cataract surgery (case 5), some of lens epithelial cells show Smad4 in the nuclei (arrowheads), whereas others do not. (C) Nuclei of the lens epithelial cells (arrowheads) in the closed capsular bag 1 year postoperatively exhibit nuclear Smad4 immunoreactivity (case 10). AC = anterior capsule. Indirect immunostaining. Bar, 20 \(\mu\)m (A) and (B), and 50 \(\mu\)m (C).

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Smad4 was also detected in most cell nuclei of cuboidal lens cells in the capsular bag, but not in the nuclei of the somewhat elongated lens cells that appear to be undergoing differentiation into lenticular fibre-like cells (Fig 6).

Lens epithelial cells in specimens obtained 6.2, 8, and 10 years postoperatively or later did not show positive immunoreactivity for nuclear Smads (Table 1).

**Fibroblast-like lens cells amid extracellular matrix (ECM) accumulation**

Fibroblastic, spindle-shaped lens cells with nuclear immunoreactivity for Smads3/4 were observed in regions of ECM accumulation (Fig 7A–C). Although cells with unstained nuclei were also present amid matrix, the majority of the fibroblast-like lens cells there were positive for αSMA as previously reported (Fig 8). In specimens with an IOL, which was associated with ECM accumulation, however, cells with nuclear Smad3 (not illustrated) and Smad4 (Fig 7D, E) were prominent in the fibrous tissue adjacent to the optic portion of the IOL, while most elongated lens cells within the ECM accumulation did not stain for nuclear Smads3/4. Fibroblastic lens cells in specimens obtained 8 and 10 years postoperatively or later did not show positive immunoreactivity for nuclear Smads (Table 1).

**Smad3 translocation in lens epithelial cells cultured with exogenous TGFβ2**

To determine if TGFβ induces nuclear translocation of Smads in vitro, we cultured anterior capsule specimens in the...
presence or absence of this growth factor. In the absence of TGFβ, Smad3 protein was not detected in the nuclei of lens epithelial cells after 30 minutes' incubation (A). Cytoplasmic immunoreactivity was weak after 1 hour (C) and weak after 4 hour (E) incubation periods. The cells in the presence of TGFβ at 30 minutes showed faint nuclear Smad3 (B), whereas those at 1 hour exhibited marked Smad3 nuclear immunoreactivity (arrowheads, D). After 4 hours, weak nuclear immunoreactivity was detected in some cells (arrowheads), but not in others (F). Indirect immunostaining. Bar, 10 µm.

Controls
No specific immunoreactvity was observed in each negative control when non-immune serum was substituted for the primary antibody (not illustrated).

DISCUSSION
Results from the present study indicate that human lens epithelial cells are regulated by endogenous TGFβ during postoperative healing. Nuclear localisation of Smads3/4 was demonstrated in postoperative lenses, while these Smads were not detected in the nuclei of lens epithelial cells after 30 minutes, 1 hour, or 4 hours culture (Fig 9A, 9C, 9E). Cytoplasmic immunoreactivity was weak, or absent, after 30 minutes but was particularly strong after 1 hour culture (Fig 9C). In specimens cultured with TGFβ nuclear localisation of Smad3 was evident. Little reactivity was detected after 30 minutes (Fig 9B); however, after 1 hour with TGFβ there was strong nuclear reactivity (Fig 9D). After 4 hours with TGFβ, Smad3 was absent from many nuclei and only weakly detected in others (Fig 9F). Nuclear immunoreactivity was also absent after 3 hours in TGFβ treated specimens (not shown).

No specific immunoreactvity was observed in each negative control when non-immune serum was substituted for the primary antibody (not illustrated).

TGFβ, Smad3 protein was not detected in the nuclei of lens epithelial cells after 30 minutes, 1 hour, or 4 hours culture (Fig 9A, 9C, 9E). Cytoplasmic immunoreactivity was weak, or absent, after 30 minutes but was particularly strong after 1 hour culture (Fig 9C). In specimens cultured with TGFβ nuclear localisation of Smad3 was evident. Little reactivity was detected after 30 minutes (Fig 9B); however, after 1 hour with TGFβ there was strong nuclear reactivity (Fig 9D). After 4 hours with TGFβ, Smad3 was absent from many nuclei and only weakly detected in others (Fig 9F). Nuclear immunoreactivity was also absent after 3 hours in TGFβ treated specimens (not shown).

Figure 9  Nuclear translocation of Smad3 in the epithelium of human lens anterior capsule specimens cultured in the presence of exogenous TGFβ2. In controls, cultured in the absence of TGFβs, Smad3 immunoreactivity was weak, or absent, from the cytoplasm of lens epithelial cells after 30 minutes' incubation (A). Cytoplasmic immunoreactivity was very strong after 1 hour (C) and weak after 4 hour (E) incubation periods. The cells in the presence of TGFβ2 at 30 minutes showed faint nuclear Smad3 (B), whereas those at 1 hour exhibited marked Smad3 nuclear immunoreactivity (arrowheads, D). After 4 hours, weak nuclear immunoreactivity was detected in some cells (arrowheads), but not in others (F). Indirect immunostaining. Bar, 10 µm.

No specific immunoreactvity was observed in each negative control when non-immune serum was substituted for the primary antibody (not illustrated).

DISCUSSION
Results from the present study indicate that human lens epithelial cells are regulated by endogenous TGFβ during postoperative healing. Nuclear localisation of Smads3/4 was demonstrated in postoperative lenses, while these Smads were not detected in the nuclei of lens epithelial cells of uninjured lenses or in the cells of anterior capsule freshly obtained during ocular surgery. It has long been hypothesised that TGFβs might influence postoperative lens cell behaviour. This is because it was shown that aqueous humour collected at the time of surgery contains abundant TGFβ2, although the ratio of active/total TGFβ in aqueous humour is reportedly altered during the relatively earlier phase of healing interval following cataract surgery. Moreover, we have shown that TGFβ2, but not TGFβ1 and TGFβ3, is involved in ocular morphogenesis in mice. A similar phenomenon was observed by us in injured mouse lenses; in vivo neutralisation of TGFβ2, but not TGFβ1 and TGFβ3, by exogenous antibodies inhibits nuclear translocation of Smad4 during lens wound healing in mice, indicating that endogenous TGFβ2 utilises the Smad signalling pathway during healing of the mouse lens epithelium.

In Sommerring's ring of the peripheral capsular bag, lens epithelial cells located between regenerated lenticular fibre-like cells and lens capsule were found to be positive for nuclear Smads3/4. This finding indicates that TGFβ signals are modulating these postoperative lens cells. Although these cells were attached to fibre-like cells and were epithelial-like in morphology similar to the epithelial cells in uninjured lenses, the presence of nuclear Smads3/4 positive lens cells indicates that they may be physiologically and transcriptionally different. Interestingly, the nuclei of elongated, β-crystallin positive, lens fibre-like cells, lacked nuclear immunoreactivity for Smads3/4. This finding might indicate that the TGFβ-Smad signalling is not essential to the differentiation of these fibre-like cells during wound healing. Although there is evidence that TGFβ signalling is required for events in normal fibre differentiation in mice, as yet there are no indications of whether this process is mediated by Smads. TGFβ is known to activate other signalling pathways; for example, the JNK pathway, rather than the Smad proteins, is involved in the response of muscle cells to TGFβ.

TGFβ upregulates expression of ECM components in various cell types including lens cells. We detected accumulation of collagen types, laminin, and fibronectin in human opacified lens capsules with an IOL in the previous study and also in injured mouse lenses in a late phase of repair (Saika et al, data submitted). These ECM components are likely to be accumulated by the fibroblast-like lens cells...
induced by TGFβ. In the present series of human specimens, some fibroblast-like lens cells in the accumulated ECM were positive for nuclear Smad3/4, although many were negative. There was some variation among specimens, in particular, nuclear Smad3/4 positive cells were more common in one specimen extracted at a relatively early phase of healing (0.75 year postoperatively; case 9, Fig 7A). Interestingly, lens cells attached to the IOL were more likely to stain for nuclear Smad4, while many of those within the ECM were unstained (Fig 7C, D). This suggests that attachment to a foreign body, such as an IOL, could influence susceptibility to TGFβ and fibrotic changes. On the other hand, such as an IOL, could influence susceptibility to TGFβ (Fig 7C, D). This suggests that attachment to a foreign body, Smad4, while many of those within the ECM were unstained year postoperatively; case 9, Fig 7A). Interestingly, lens cells specimen extracted at a relatively early phase of healing (0.75 year postoperatively; case 9, Fig 7A). Interestingly, lens cells

REFERENCES