Inhibition of Transforming Growth Factor-β-Induced Cataractous Changes in Lens Explants by Ocular Media and α2-Macroglobulin

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Purpose. To investigate the ocular media for the presence of inhibitors of transforming growth factor-β (TGF/β) using a lens epithelial explant system in which TGF/β induces cataractous changes. The effect of α2-macroglobulin, an inhibitor of TGF/β in other systems, also was assessed.

Methods. Explants prepared from 21-day-old rats were cultured with TGF/β2 with and without 50% bovine aqueous or vitreous or α2-macroglobulin. α2-macroglobulin was added to an aqueous concentrate, shown to contain endogenous TGF/β activity by blocking with anti-TGF/β. Explants were monitored by phase-contrast microscopy for 5 days and assessed in terms of capsule wrinkling, spindle-cell formation, blebbing, and cell loss. α2-macroglobulin in the ocular media was assessed by enzyme-linked immunosorbent assay and Western blot analysis.

Results. At 50% strength, neither aqueous nor vitreous demonstrated TGF/β-like activity; however, aqueous partially and vitreous completely prevented cataractous changes induced by 25 and 100 μg/ml TGF/β2, respectively. α2-macroglobulin (50 to 200 μg/ml) also protected against these changes, with complete inhibition of TGF/β2 or aqueous-derived TGF/β activity at the highest concentration. A threefold higher concentration of α2-macroglobulin was detected in vitreous than aqueous.

Conclusions. Both aqueous and vitreous contain molecule(s) that inhibit TGF/β2 activity. α2-macroglobulin has been identified in the ocular media and shown to block cataractous changes induced by TGF/β. Maintaining appropriate levels of α2-macroglobulin or similar molecules in the ocular media may protect lens cells from the damaging effects of TGF/β, and reduced levels may predispose to cataract. Invest Ophthalmol Vis Sci. 1996;37:1509-1519.

Recently, it was shown in this laboratory that transforming growth factor-β (TGF/β) induces cataractous changes in rat lens epithelial explants.1,2 These include the formation of spindle-shaped cells, capsule wrinkling, accumulation of extracellular matrix, and cell death with features of apoptosis. Some or all of these changes are typically found in human subcapsular cataract, both the anterior and posterior forms, and in after-cataract (posterior capsular opacification). In addition, whole rat lenses cultured with TGF/β develop opacities and morphologic features typical of human anterior subcapsular cataracts.3 Two molecular markers for these forms of cataract, α-smooth muscle actin and type I collagen, are induced in lens explants and cultured lenses by TGF/β.2,3 TGF/β is present in the eye. Both the protein4-5 and its mRNA5-8 are expressed in the lens, and TGF/β is found in the ocular media that bathe the lens.9-16 Thus, TGF/β may be involved in the etiology of major forms of cataract, and it is important that mechanisms that regulate its activity and availability to lens cells be elucidated.

In mammals, three isoforms of TGF/β have been identified, TGF/β1, TGF/β2, and TGF/β3.17 TGF/β2 is considered to be the predominant form in the ocular media, and all three isoforms have been localized in the lens.4,9,11,14 In general, TGF/β is synthesized and secreted as a latent complex, composed of the TGFβ
dimer, a latency-associated protein (LAP), and an additional disulphide-linked binding protein.\textsuperscript{17,18} Dissociation of the biologically active 25 kDa TGFβ dimer from this complex occurs at extremes of pH, with heat or urea treatment, or through the action of proteases or thrombospondin, an extracellular matrix protein.\textsuperscript{17,19} On release, the biologic activity of the TGFβ dimer can be inhibited by specific association with a number of proteins, including the serum protein α2-macroglobulin\textsuperscript{20,21} and the proteoglycans decorin and biglycan.\textsuperscript{22} Thus, TGFβ may be present in a biologically inactive form either because it remains associated with the LAP complex or because, after release, it has become bound to some other regulatory molecule.

In the current study, the ocular media were assessed for the presence of inhibitors of TGFβ\textsubscript{2} using a lens epithelial explant system in which TGFβ induces cataractous changes. In addition, this system was used to investigate the effect of α2-macroglobulin, which has been detected in human aqueous.\textsuperscript{23}

**MATERIALS AND METHODS**

All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Recombinant human TGFβ\textsubscript{2} was obtained from Genzyme (Cambridge, MA) and stored at −70°C. Bovine serum α2-macroglobulin (Boehringer-Manheim, Darmstadt, Germany) was dissolved in distilled water (10 mg/ml) and stored at −70°C before use. A rabbit polyclonal antibody against human α2-macroglobulin was obtained from Sigma (St. Louis, MO). A pan-specific TGFβ antibody (rabbit immunoglobulin G [IgG] fraction) that recognizes all mammalian isoforms (BDA 47; R & D Systems, Minneapolis, MN) was used in cultures at a final concentration of 50 μg/ml. Nonimmune IgG was prepared from rabbit serum by standard protein A-Sepharose chromatography.

**Preparation and Culture of Lens Epithelial Explants**

Explants were prepared from 21-day-old Wistar rats by a standard method.\textsuperscript{1-24} Briefly, lenses were removed and incubated in culture medium (medium 199 containing bovine serum albumin and antibiotics) for approximately 15 minutes. Epithelia and the associated capsule were peeled from fibers and pinned out in culture dishes containing 2 ml medium with the cellular surface uppermost. The whole epithelium was used, and each dish contained two explants. At the start of the experiment, that is, 1 to 3 hours after preparation of the explants, medium was replaced with fresh culture medium (final volume, 1 ml/dish), which included ocular media and/or α2-macroglobulin or antibody as required by the experiment. TGFβ\textsubscript{2} was diluted in culture medium and added immediately (10 μl/dish), as required. Explants were cultured for up to 5 days with daily monitoring by phase-contrast microscopy.

Note that the “untrimmed” explants used in this study contained cells derived from both the germinative and central regions of the lens epithelium. No differences were observed in the responses of cells from these two regions.

**Preparation of Samples of Ocular Media**

Aqueous and vitreous were collected from freshly slaughtered cattle, as described previously,\textsuperscript{25} transported to the laboratory on ice, and used the same day. In some experiments, samples were diluted with an equal volume of sterile culture medium, using repeated passage through a 19-gauge needle to ensure thorough mixing. These mixtures, designated 50% strength aqueous or vitreous, were equilibrated at 37°C for 30 minutes in 5% CO\textsubscript{2}-air before use. Alternatively, 14 ml portions of aqueous were concentrated by ultrafiltration at 4°C through a YM 30 membrane (Amicon, Beverly, MA) to give a concentrate equivalent to 4 ml original aqueous per milliliter. “Double-strength” aqueous was prepared by mixing this concentrate with an equal volume of culture medium and supplementing with bovine serum albumin and antibiotics at the usual final concentrations. In addition, a sample of human aqueous was obtained from a 35-year-old man who died as a result of trauma and was stored at −70°C for enzyme-linked immunosorbent assay (ELISA).

**α2-Macroglobulin Enzyme-Linked Immunosorbent Assay**

Ocular media or bovine serum were diluted with 0.1 M carbonate buffer, pH 9.6, and were added (2 to 10 μl) to Immulon 4 multi-well plates (Dynatech, West Sussex, UK) containing carbonate buffer (100 μl final volume). Bovine serum α2-macroglobulin was used as standard. After overnight incubation at 4°C, the wells were washed (three times) with phosphate-buffered saline−0.05% Tween 20 and incubated for 1 hour at 37°C in α2-macroglobulin antibody diluted 1:2000 in wash buffer. Bound antibody was detected using a horseradish peroxidase-conjugated secondary antibody and an appropriate substrate.\textsuperscript{26}

**Western Blot Analysis**

Samples of fresh aqueous or vitreous (1 ml) were lyophilized and resuspended in 40 or 100 μl, respectively, of sodium dodecyl sulfate sample buffer (0.06 M Tris-HCl, 10% [vol/vol] glycerol, 2% sodium dodecyl sulfate, 5% [vol/vol] β-mercaptoethanol, and 0.1% [wt/vol] bromophenol blue, pH 6.8) and heated at 90°C to 100°C for 3 minutes. Using a PhastSystem (Pharmacia, Uppsala, Sweden), 4 μl samples were run on 4% to 15% gradient gels, and proteins were trans-
ferred to an Immobilon-P membrane (Millipore, Bedford, MA) at 1 mA/cm² for 25 minutes. The membrane was blocked (1% skim milk powder in phosphate-buffered saline) for 1 hour at room temperature, and immunoreactive bands were detected using α2-macroglobulin antibody (diluted 1:100) with a hors eradish peroxidase-conjugated secondary antibody (diluted 1:200; Silenus, Hawthorn, Australia) and diaminobenzidine tetrahydrochloride (Sigma) as substrate. Skim milk powder (1%) in phosphate-buffered saline was used as diluent for all antibodies. Controls, in which nonimmune IgG replaced the specific antibody, were included routinely and always showed no reactivity.

RESULTS

TGFβ2 was used in this study because it is considered to be the predominant form of TGFβ in both aqueous and vitreous.5,11,12

TGFβ2 Dose–Response Analysis

Explants were cultured with concentrations of TGFβ2 ranging from 25 to 100 pg/ml and monitored carefully by phase-contrast microscopy for 5 days. The changes induced by culturing with TGFβ2 are illustrated in Figure 1. Explants cultured without added TGFβ2 (controls) remained as a normal epithelial monolayer throughout the culture period (Fig. 1A). The only change observed was slight wrinkling of the capsule, which occurred in most explants but did not usually become apparent until 4 to 5 days of culture. In TGFβ2-treated explants, changes first were detected on day 2 and became more pronounced by day 3. These included the appearance of a latticework of fine wrinkles in the capsule and rows of elongated cells, some of which were spindle shaped (Figs. 1B, 1C). In addition, by day 3, some cells were covered with numerous blebs (Fig. 1C). As discussed previously, the latter is a characteristic of cell death by apoptosis.1,27 By day 4, blebbing had increased, and patches of bare capsule were visible where cells had been lost; spindle cells were still distinguishable (Fig. 1D). At 5 days, only a few cells remained on the capsule. All these TGFβ2-induced responses have been discussed previously in relation to cataract.1,3

A similar progression of morphologic changes was induced at all concentrations of TGFβ2 investigated, that is, capsule wrinkling and spindle-cell formation, blebbing, and cell loss. All explants were graded for each of these changes according to the relative response observed with the various concentrations of TGFβ2 used, taking the response observed at the highest concentration of TGFβ2 as the upper limit (Table 1). All responses were concentration dependent, although this became less evident as culture progressed. The highest concentration of TGFβ2 used, 100 pg/ml, appeared to be near saturating.

Inhibitory Effects of Ocular Media on TGFβ2 Activity

The system for semiquantitatively grading explant responses to TGFβ2 was then used in assessing the ocular media for the presence of inhibitors of TGFβ activity. Lens epithelial explants were cultured with TGFβ2 in the presence and absence of 50% aqueous or vitreous. Changes in the explants were assessed after 3 days of culture because any inhibitory effects would be detected most readily at this time (see Table 1). Because TGFβ2-induced cell loss does not become a major feature until after 3 days of culture (Table 1), apoptosis was assessed in terms of the proportion of cells exhibiting blebbing in these experiments (see Fig. 1C). The grading system was found to give a reliable indication of between-treatment differences, provided that explants in all treatment groups were processed in parallel.

Explants cultured with 25 to 100 pg/ml TGFβ2 showed expected morphologic changes by 3 days, including capsule wrinkling and formation of spindle-shaped cells with some blebbing (Fig. 2A; Table 2A). At all concentrations of TGFβ2 investigated, aqueous appeared partially to inhibit the changes induced by TGFβ2 (Fig. 2B; Table 2B), whereas vitreous completely blocked them (Fig. 2C; Table 2C).

Note that regardless of whether TGFβ2 was included, explants cultured with vitreous were not identical to controls with respect to the arrangement of cells in the monolayer (see Figs. 2C, 2E). The morphologic changes observed with vitreous are indicative of cell migration and are typically observed in explants undergoing early stages of fiber differentiation in response to fibroblast growth factor (FGF). As shown previously, under these conditions, vitreous contains sufficient FGF to induce a fiber differentiation response.25,28

Cells in explants cultured with 50% aqueous, in the absence of TGFβ2, retained an epithelial morphology throughout the culture period (see ref. 28). In all explants cultured with aqueous beyond 3 days, regardless of whether TGFβ2 was present, deep folds developed across the capsule, resulting in progressive shrinking and compaction of the explant (not shown; see ref. 28).

Detection of α2-Macroglobulin in the Ocular Media

α2-macroglobulin, a known inhibitor of TGFβ in some systems,26 was detected in the ocular media by ELISA (Fig. 3). Using a commercial preparation of bovine serum α2-macroglobulin as standard, aqueous and vitreous were shown to contain approximately 5 and 15 μg/ml, respectively. The concentration of α2-macro-
FIGURE 1. Morphologic effects of transforming growth factor-β (TGF-β) on lens epithelial explants. In explants with no added TGF/β2 (controls), the cells retained an epithelial morphology (A, cultured for 4 days). After 3 days culture with 50 pg/ml TGF/β2 (B,C), distinctive features were a fine network of wrinkles in the capsule (B, arrow) and rows of elongated cells, many of which were spindle shaped (C, arrows). Some cells were covered with small blebs (C, arrowhead). After 4 days of culture with TGF/β2 (D), there were patches of capsule devoid of cells, and most of the remaining cells were covered with small blebs. Wrinkles showed up clearly on bare patches of capsule (arrow). Bars = 50 μm; A, C, and D are at the same magnification.

globulin in bovine serum, assayed in parallel, was found to be 3.5 mg/ml. In addition, a sample of human aqueous was found to contain 5 μg/ml. Western blot analysis of aqueous under reducing conditions, using the same antibody, showed a distinct band of reactivity at 180 kDa (Fig. 4), a value similar to that reported previously for α2-macroglobulin.29 Although vitreous, which contains extracellular matrix proteins, was less amenable than aqueous to Western blotting, at maximum protein load a faint band was detectable at approximately the same molecular weight (data not shown).

Inhibitory Effect of α2-Macroglobulin on TGFβ2 Activity
To determine whether α2-macroglobulin can act as an inhibitor of the effects of TGF/β on lens cells, explants were cultured with TGFβ2 in the presence and absence of α2-macroglobulin. TGFβ2 was used at a concentration of 25 pg/ml, and α2-macroglobulin was used at a concentration of 50 to 200 μg/ml. At all concentrations of α2-macroglobulin, substantial inhibition of capsule wrinkling, formation of spindle-shaped cells, and cellular blebbing was observed, but its effects were greatest at 200 μg/ml, a concentration that virtually abolished TGFβ2 activity (Fig. 2D; Table 3). Controls in which α2-macroglobulin was replaced with another serum protein, IgG (100 μg/ml), showed no inhibition of TGFβ2 activity (data not shown).

Effect of α2-Macroglobulin on Endogenous TGFβ Activity in an Aqueous Concentrate
Although cells in explants cultured with 50% aqueous did not show any evidence of the changes typically
### TABLE 1. Effect of TGFβ2 on Lens Epithelial Explants: Dose-Response Analysis

<table>
<thead>
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<th>Response</th>
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<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
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<tbody>
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<td>Day 2*</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wrinkling</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Spindle cells</td>
<td></td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
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<tr>
<td>Cell loss</td>
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<td></td>
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<td>Day 3</td>
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<td>Spindle cells</td>
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<td></td>
</tr>
<tr>
<td>Cell loss</td>
<td></td>
<td></td>
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</table>

Six explants were assessed at each concentration of TGFβ2. Three categories of response illustrated in Figure 1 were assessed, namely, wrinkling of the lens capsule, formation of spindle-shaped cells, and cell loss. Generally, the number of pluses indicates the proportion of the explant surface exhibiting that particular feature. A dash indicates no change. Throughout the culture period, control explants cultured without TGFβ2 remained as epithelial monolayers and showed none of the changes observed in TGFβ2-treated explants.

* No changes were observed in any of the explants before day 2 of culture.
† At 3 days of culture, there was minimal cell loss at all concentrations of TGFβ2; explants were therefore assessed in terms of cell blebbing, which precedes cell loss. Beyond 3 days of culture, spindle cell formation could not be assessed because of extensive cell loss at all concentrations of TGFβ2.

induced by TGFβ (see above), it was found that such changes were induced by double-strength aqueous (see Materials and Methods). Capsule wrinkling, formation of spindle-shaped cells, and cellular blebbing were all evident (Figs. 5A, 5B). To determine whether these responses were the result of endogenous TGFβ activity, a pan-specific neutralizing TGFβ-antibody was added to the explants with the double-strength aqueous. The antibody completely inhibited all three TGFβ-induced responses (Fig. 5C), although cells in the monolayer were slightly different from controls (see Fig. 2E) in that they showed some evidence of migratory behavior (see Fig. 2C). The latter response was probably caused by FGF, which is present in small amounts in aqueous.25 Nonimmune IgG had no effect on the aqueous-induced changes (not shown). Adding back the filtrate removed from the aqueous concentrate during processing did not alter the effects of the aqueous concentrate (nor TGFβ2) on lens cells (not shown), indicating that the observed endogenous TGFβ-like activity did not result from the removal of a low molecular weight inhibitor during ultrafiltration.

Thus, concentrating aqueous provided a source of aqueous-derived TGFβ activity for use in testing the effects of α2-macroglobulin. It was important to determine whether α2-macroglobulin inhibited aqueous-derived TGFβ, that is, the form(s) of TGFβ potentially available to the lens in situ, because interaction with α2-macroglobulin may vary with TGFβ isoform.30 Explants were cultured with double-strength aqueous in the presence and absence of α2-macroglobulin at 50 to 200 μg/ml. At 50 μg/ml, α2-macroglobulin substantially inhibited the effects of the aqueous-derived TGFβ, and at 200 μg/ml, virtually complete inhibition was observed (Fig. 5D; Table 4).

The folding and shriveling noted in explants cultured for more than 3 days with 50% aqueous were observed in explants cultured with double-strength aqueous. Interestingly, this shriveling was blocked completely by the inclusion of TGFβ-antibody with double-strength aqueous and was blocked partially or almost completely by 100 or 200 μg/ml α2-macroglobulin, respectively (not shown). The significance of these findings is not clear at present.

### DISCUSSION

It has been shown in this laboratory that TGFβ1 and TGFβ2 induce cataract-like changes in lens epithelial cell explants and whole cultured lenses1-5 and that TGFβ2 is the more potent isoform.31 The current study supports these findings and establishes that such changes are induced in explants by concentrations of TGFβ2 as low as 25 pg/ml (Table 1). Both TGFβ1 and TGFβ2 are present in the ocular media (TGFβ2 is the predominant form; see Introduction), and various levels of TGFβ biological activity have been reported...
FIGURE 2. The effect of ocular media on transforming growth factor-β2 (TGF-β2) activity. Explants were cultured for 3 days with (A to D) or without (E) TGF-β2. Various potential inhibitors were included with the TGF-β2: aqueous (B), vitreous (C), or α2-macroglobulin (D). (A) Explants cultured with 25 pg/ml TGF-β2 exhibited distinctive morphologic changes. Rows of elongated (often spindle-shaped) cells were conspicuous (arrow). (B) In the presence of 50% aqueous, the responses induced by 25 pg/ml TGF-β2 were much weaker than in A. Some cell elongation was evident, and there were a few groups of spindle-shaped cells (arrow). (C) When 50% vitreous was included with 100 pg/ml TGF-β2, no characteristic TGF-β-induced responses were observed, but cells showed some evidence of cellular reorganization. (D) When 200 μg/ml α2-macroglobulin was included with 25 pg/ml TGF-β2, the cells retained an epithelial morphology, as in explants cultured in control medium (E). Bar = 50 μm; all figures are at the same magnification.

In aqueous or vitreous from humans and other species. In the current study, lens explants cultured with bovine aqueous at 50% strength showed none of the cataractous changes characteristically induced by TGFβ (Table 2). However, a double-strength concentrate of aqueous exhibited TGFβ-like activity, as indicated by capsule wrinkling, spindle-cell formation, and cell blebbing in lens explants (Figs. 5A, 5B). All these changes were abolished completely by a pan-specific antibody against TGFβ (Fig. 5C) previously shown to block the effects of exogenous TGFβ2 on lens explants, providing confirmation that they were, in fact, mediated by a TGFβ isoform(s). The TGFβ activity of the aqueous concentrate was equivalent to that observed using 25 to 50 pg/ml TGFβ2 (see Tables 1 and 4). This result is consistent with a previous study of bovine aqueous, which, using a different bioassay, also showed a low level of TGFβ activity. The fact that TGFβ activity was observed only in double-strength aqueous in this study may simply be a concentration effect, although it is possible that at least some of this...
TABLE 2. Inhibition of TGF/β2-induced Changes in Lens Epithelial Explants by Ocular Media

<table>
<thead>
<tr>
<th>Response</th>
<th>TGF/β2 (pg/ml)</th>
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<td>0 25 50 100</td>
</tr>
<tr>
<td>No addition</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td>Wrinkling</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td>Spindle cells</td>
<td>++ + +</td>
</tr>
<tr>
<td>Blebbing</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>With aqueous</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td>Wrinkling</td>
<td>++ + + + +</td>
</tr>
<tr>
<td>Spindle cells</td>
<td>+ + + +</td>
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<tr>
<td>Blebbing</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>With vitreous</td>
<td>++ ++ ++ +</td>
</tr>
<tr>
<td>Wrinkling</td>
<td>++ + + + +</td>
</tr>
<tr>
<td>Spindle cells</td>
<td>+ + + + +</td>
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<tr>
<td>Blebbing</td>
<td>+ + + + +</td>
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</tbody>
</table>

Explants were cultured for 3 days with or without TGF/β2, as indicated, in culture medium (no addition) or in aqueous or vitreous diluted to 50% normal strength with culture medium. For each treatment, assessments are based on observations of 7 to 12 explants at each concentration of TGF/β2. The number of pluses indicates the degree of response observed in the explants in the categories indicated. Blebbing is the cellular change that precedes and is associated with cell loss in TGF/β2-treated explants. nd = not determined. For further details, see Table 1.

activity arises by proteolytic activation of TGFβ during processing because proteases are known to be present in aqueous.25 Other studies suggest that human aqueous may contain relatively high levels of TGFβ activity, but it should be noted that only samples from diseased, generally cataractous, eyes have been analyzed.9,11,12,14-16 Thus, it is not clear what level of TGFβ stimulation lens cells normally receive in situ. Nevertheless, the results of the current study show that aqueous contains TGFβ, which, under certain conditions, can induce cataractous changes in lens cells. It is, therefore, important to understand the mechanisms that regulate TGFβ activity in the lens environment.

There are two known mechanisms for suppressing TGFβ activity. One general mechanism is to maintain TGFβ in its latent form, that is, complexed with LAP. Alternatively, once released, the active TGFβ dimer may be inhibited by association with a variety of biologic molecules (see Introduction). One such molecule is α2-macroglobulin, a 720 kDa serum protein whose primary function is thought to be the inactivation and clearance of proteases from serum and extracellular matrix. 

TABLE 3. Inhibition of TGF/β2 Activity by α2-Macroglobulin

<table>
<thead>
<tr>
<th>Response</th>
<th>α2-macroglobulin (μg/ml)</th>
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<td>0 50 100 200</td>
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<tr>
<td>Wrinkling</td>
<td>+ + + + + + +</td>
</tr>
<tr>
<td>Spindle cells</td>
<td>+ + + + + + +</td>
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<tr>
<td>Blebbing</td>
<td>+ + + + + + +</td>
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Lens epithelial explants were cultured for 3 days with 25 pg/ml TGF/β2 and α2-macroglobulin at the concentrations indicated. The number of pluses indicates the degree of response observed in the categories indicated. For further details, see Tables 1 and 2.
FIGURE 5. The effect of α2-macroglobulin on endogenous transforming growth factor-β (TGF/β) activity in an aqueous concentrate. Explants were cultured for 3 days. When used at double strength (see Materials and Methods), aqueous induced distinctive TGF/β-like changes, including capsule wrinkling (A, arrow), formation of spindle-shaped cells (B, arrow), and cell blebbing (arrowhead). The TGF/β-like activity of the aqueous concentrate was abolished by an antibody against TGF/β (C) or by α2-macroglobulin at a concentration of 200 µg/ml (D). Bars = 50 µm; B to D are at the same magnification.

cellular fluids. In addition, α2-macroglobulin has been shown to bind a number of cytokines, including TGF/β, FGF, and PDGF, with highest affinity for TGF/β. Binding kinetics vary with the conformational state of the α2-macroglobulin. In serum, which exhibits negligible TGF/β activity, virtually all TGF/β is associated with α2-macroglobulin.

TGF/β is present in the ocular media in two pools, one designated active and the other latent. Active TGF/β has been assessed by bioassay in terms of the ability of TGF/β to inhibit proliferation in cell cultures or by ELISA using an antibody claimed to recognize specifically the free TGF/β dimer. Estimates of the proportions of the two pools vary, with marked differences between species, and levels of active TGF/β have been shown to vary in certain diseases of the eye. The general assumption appears to be that latent TGF/β in the ocular media is still complexed with LAP. It should be noted, however, that the methods used in these studies to identify the active and latent TGF/β pools may not differentiate between TGF/β associated with LAP or bound to other regulatory proteins. For example, in one study in which ELISA was used to assess the TGF/β pools in aqueous and vitreous, it is clear that TGF/β would have been characterized as latent, whether complexed with LAP or associated with α2-macroglobulin. Other treatments, e.g., heat and low pH, are nonspecific and can cause conformational changes that lead to release or altered presentation of TGF/β whether it is associated with LAP or with α2-macroglobulin. For example, after heat treatment, TGF/β associated with α2-macroglobulin can be detected by a TGF/β antibody against the free 25 kDa dimer.

A major finding of the current study is that aqueous and vitreous contain inhibitors of TGF/β, as indicated by their ability to inhibit exogenous TGF/β2 (Table 2; Figs. 2B, 2C). It is possible that some of the TGF/β normally present in the ocular media appears to be latent because it is associated with an inhibitory
Interestingly, the vitreous, which contained more a2-macroglobulin than the aqueous, also showed more TGFβ inhibitory activity (Table 2; Fig. 2). Vitreous at 50% strength completely inhibited as much as 100 pg/ml TGFβ2, whereas aqueous at the same concentration only partially inhibited 25 pg/ml. Although it is likely that the strong inhibitory activity of the vitreous is caused in part by its a2-macroglobulin content, other potential inhibitors of TGFβ activity may be present as well. The vitreous, an extracellular matrix, contains large amounts of hyaluronic acid.58 recently reported to be an inhibitor of TGFβ,30 and extracellular matrices generally contain other molecules that inhibit and/or bind TGFβ.17,40 In addition, the vitreous contains substantial amounts of the lens fiber-differentiating factor, FGF,25 which also may modulate at least some of the effects of TGFβ2, either directly or by inducing early fiber differentiation.4

The source of a2-macroglobulin in the ocular media has not yet been established. Studies of ocular media proteins indicate that some serum proteins leak through the ciliary body capillaries and diffuse through the irido-stroma into the aqueous.36,41 Serum proteins also diffuse into the vitreous.38,42 Proteins of relatively high molecular weight have been detected in both ocular media.36,41,42 Thus, a2-macroglobulin may enter the aqueous and vitreous along with other serum proteins. Alternatively, at least some may be synthesized locally. The cornea is one possible source because both a2-macroglobulin and its mRNA have been detected in the corneal endothelium.43

When considering a potential inhibitory role for a2-macroglobulin in the eye, another factor to be taken into account is its clearance from the ocular media. Effective clearance may be important because, under certain conditions, a2-macroglobulin-TGFβ complexes can dissociate, releasing active TGFβ.20 Generally, removal of aqueous occurs continuously and fairly rapidly through the trabecular and uveoscleral outflow pathways. It has been shown that a toxin–immunoglobulin conjugate added to aqueous is flushed almost completely into the serum within 4 hours of injection.37 Furthermore, some exchange of vitreous and aqueous is known to occur.44 Hence, a2-macroglobulin and other proteins may be cleared through the aqueous outflow pathway. In serum, a specific mechanism exists for ensuring rapid removal of a2-macroglobulin.21 It is not clear whether there is a comparable specific mechanism for clearing a2-macroglobulin from the ocular media.

In summary, a significant finding of the current study is that the ocular media contain molecule(s) that inhibit TGFβ2 activity. Moreover, a2-macroglobulin has been identified in the ocular media and has been shown to block cataractous changes induced by TGFβ derived from the aqueous. Maintaining appropriate levels of inhibitory molecules, such as a2-macroglobulin, in the ocular media may protect lens cells from the damaging effects of TGFβ, and reduced lev-

### TABLE 4. Effect of α2-Macroglobulin on Endogenous TGFβ Activity in an Aqueous Concentrate

<table>
<thead>
<tr>
<th>Response</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wrinkling</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td>Spindle cells</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Blebbing</td>
<td>++</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Lens epithelial explants were cultured for 3 days with double-strength aqueous concentrate (see Materials and Methods) and α2-macroglobulin at the concentrations indicated. The number of pluses indicates the degree of response observed in the categories indicated. tr = trace. A trace of wrinkling also was observed in control explants in this experiment. For further details, see Tables 1 and 2.

α2-macroglobulin was detected in bovine aqueous and vitreous by ELISA, at apparent concentrations of 5 μg/ml and 15 μg/ml, respectively (Fig. 3). On the basis of a dot blot assay, it had been reported in human aqueous at a concentration of 1 to 5 μg/ml,25 comparable to the amount detected in the current study. In another study of human aqueous, it was not detected, but the limit of detection was <200 μg/ml.14 Estimates of α2-macroglobulin concentrations in the ocular media generally should be interpreted with caution. For example, ELISA results depend on the characteristics of the antibodies and standards used, and the TGFβ inhibitory activity of the α2-macroglobulin in the ocular media may be different from that of commercial preparations of α2-macroglobulin.

Nevertheless, it is clear that α2-macroglobulin is present, and therefore may be involved, in suppressing at least some of the TGFβ activity of the ocular media. Interestingly, the vitreous, which contained more α2-macroglobulin than the aqueous, also showed more
els may predispose to cataract. In the eye, certain conditions probably favor the release of active TGFβ. For example, after eye surgery, TGFβ may be activated by low pH and by the release of proteases. Under such conditions, α2-macroglobulin potentially offers two levels of protection to lens cells: Not only does it inhibit TGFβ activity, but it has also the capacity to bind and inactivate the proteases that release the active dimer. TGFβ is now known to be involved in the etiology of fibrotic diseases in a variety of nonocular tissues, and a protective role for α2-macroglobulin in cytokine-mediated disease has been proposed. The findings of the current study support the hypothesis that inhibitory molecules in the ocular media, including α2-macroglobulin, play a role in protecting the eye against diseases such as subcapsular cataract.

**Key Words**

α2-macroglobulin, cataract models, lens epithelium, ocular media, transforming growth factor-beta inhibition.

**Acknowledgments**

The authors thank Roland Smith for assistance with photography; the F. C. Nichols Abattoirs, Woy Woy (New South Wales), for supplying bovine ocular media; and the Lions New South Wales Eye Bank for supplying human aqueous.

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