TGF-β2–Induced Matrix Modification and Cell Transdifferentiation in the Human Lens Capsular Bag

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PURPOSE. To study the role of TGF-β2 in posterior capsule opacification (PCO) and to determine whether CAT-152 (derdelimumab), a fully human monoclonal antibody that neutralizes the effect of TGF-β2, can also provide therapeutic benefit for PCO.

METHODS. In vitro capsular bags were prepared from human donor eyes and maintained in a 5% CO₂ atmosphere at 35°C. To investigate expression of active TGF-β2, capsular bags were incubated in serum-free EMEM for 2, 28, or more than 100 days and analyzed by ELISA (n = 4 at each time point). To study underlying mechanisms, match-pair experiments were also performed, so that the medium was supplemented with 0, 1 or 10 ng/mL TGF-β2 with or without 10 μg/mL CAT-152 (n = 4 in all cases). Ongoing observations were by phase-contrast microscopy. In addition, donor material from patients who had undergone cataract surgery was analyzed. Cellular architecture was examined by fluorescence cytchemistry. Expression of matrix metalloproteinase (MMP)-2 and -9 was assessed by gelatin zymography.

RESULTS. Analysis of capsular bags from donor eyes that had received an intraocular lens (IOL) revealed the presence of endogenous active TGF-β2, matrix wrinkling, and expression of transdifferentiation markers αSMA and fibronectin. When cultured in vitro, donor bags also showed sustained release of MMP-2 and -9. Culture of capsular bags prepared in vitro from whole lenses showed that TGF-β2 (1–10 ng/mL) stimulated transdifferentiation and contraction of the capsular bag, resulting in light scatter. TGF-β2 also induced sustained release of MMP-2 and -9. Active TGF-β2 was detected in these cultures. The human monoclonal anti-TGF-β2 antibody CAT-152 (10 μg/mL) effectively inhibited all TGF-β2-induced effects.

CONCLUSIONS. Addition of TGF-β2 accelerates transdifferentiation and contraction of the capsular bag, resulting in light scatter. CAT-152 inhibited all the effects of TGF-β2 that were examined and therefore has the potential to suppress development of PCO and provide potential therapeutic benefit to cataract patients. (Invest Ophthalmol Vis Sci. 2002;43: 2301–2308)

CATaract is responsible for rendering millions of people blind throughout the world.¹ At present, the only means of treatment is surgical intervention, and this initially restores high quality vision. Unfortunately, between 20% and 30% of patients who undergo cataract surgery, experience a secondary loss of vision over time that necessitates further corrective surgery.² ³ A modern cataract operation generates a lens capsular bag, which comprises a proportion of the anterior and the entire posterior collagen capsule. The bag remains in situ and separates the aqueous and vitreous humors and, in the majority of cases, houses an artificial lens. Despite the rigors of surgery, a significant number of lens epithelial cells remain on the anterior capsule. These cells subsequently grow across all available lenticular surfaces, including the previously cell-free posterior capsule surface. In doing so, the cells encroach on the visual axis and the ensuing cellular and capsular changes that result from growth induce light scatter, diminishing visual quality. The act of surgery causes a wound which, in turn, initiates a wound-healing response. The early phases of this wound-healing process are influenced by the inflammatory response within the eye. This gives rise to elevated proteins in the aqueous humor, although these effects last only days or weeks.⁴ ⁵ PCO, in most cases, does not become clinically relevant until months or years after surgery.² However, the long-term development of PCO may be explained by recent in vitro findings that show lens cells can survive, grow, and maintain metabolic activity in protein-free medium for more than 100 days.⁶ ⁷ Therefore, it is important to understand the relative roles of the paracrine and autocrine systems in the regulation of PCO development. In turn, this information could provide new opportunities and targets for therapies to prevent PCO.

In the present study we focused on TGF-β and, in particular, the TGF-β2 isoform. This important family of growth factors is composed of homodimeric polypeptides that regulate many aspects of cellular function, including cell growth, differentiation, inflammation, and wound healing.⁶ ⁷ ⁸ Although five members of the TGF-β family have currently been identified, only TGF-β isoforms 1, 2, and 3 have been detected in mammals.¹³ TGF-β exists in both latent and active forms. Active TGF-β is likely to be released from the latent form through degradation of prosegments. This process may be performed by proteases, such as plasmin and the cathepsins.¹⁴ TGF-β initiates its response through three major high-affinity cell surface receptors.¹⁴ In addition, the activity of TGF-β can be regulated by proteins in surrounding fluids, such as the humors of the eye, which contain, for example, α₂-macroglobulin. This particular protein has a high affinity for free active TGF-β and may serve as a regulator in the eye.¹⁵ ¹⁶

TGF-β isoforms have been detected in mouse, rat, and human lens cells.¹⁷ ²⁰ Gordon-Thomson et al.¹⁸ performed an investigation using rat lens tissue and probed for both protein and mRNA of TGF-β isoforms 1, 2, and 3 using immunofluorescence and in situ hybridization, respectively. Although all three isoforms of the protein were identified in the lens, only mRNA for TGF-β1 and -β2 were at detectable levels. Regarding human lens cells, TGF-β1 and -β2 have been detected in primary cultures by using RT-PCR techniques and in situ hybrid-
ization. To begin to understand the regulation and putative roles of TGF-β in lens cells, it is necessary to know the expression of receptors. Of the three major TGF-β receptor types, type I and II have been detected in chick lens cells, by Western blot techniques and, in addition, RT-PCR techniques, in a human lens cell line, primary cells, and native epithelium.

Although TGF-β is likely to play an important role in the normal lens, it is becoming increasingly obvious that it can greatly influence transdifferentiation, which is proposed to be involved in pathologic conditions of the lens. This is perhaps best illustrated in the formation of subcapsular plaques. In an elegant study, it was reported that cultured rat lenses generate anterior capsular plaques in response to TGF-β exposure. In that study the effects of TGF-β1, -β2, and -β3 were investigated, and the results demonstrated that TGF-β2 and -β3 had a 10-fold greater potency to induce opacity than TGF-β1. Further reports of rabbit and bovine lens culture models have shown that after exposure to TGF-β1, increased expression levels of fibronectin and α-smooth muscle actin (α-SMA) were observed. These proteins both serve as markers of transdifferentiation. Similarly, immunohistochemical analysis of tissue obtained at autopsy has shown that in eyes in which PCO had developed, there was expression of α-SMA, fibronectin, and collagen type I. The transdifferentiation of lens epithelial cells to fibroblast-like cells is also proposed to increase contractility, and this phenomenon, in addition to plaque formation, could be integral to the pathogenesis of posterior capsule opacification.

Although TGF-β has been studied extensively in the lens in recent years, this work has largely focused on the development and the formation of anterior subcapsular cataract, which comprise only a small proportion of total cataracts. However, the functional role of TGF-β in the formation of PCO, which affects a large proportion of cataract patients, has not been investigated in the same detail. In the present study, we deemed it necessary to test the hypothesis that TGF-β2, which is the major isoform within the eye, plays a critical role in both the short- and long-term development of PCO. To substantiate this idea, it is necessary to show first that TGF-β2 is present in human capsular bags and secondly that addition of TGF-β2 to the medium can induce the matrix and cellular changes exhibited in vivo. Furthermore, with the advent of novel fully human monoclonal antibodies, it is possible to treat patients with antibody therapy. One such antibody, the anti-TGF-β2 neutralizing antibody, CAT-152 (lerdelimumab; Cambridge Antibody Technology, Melbourn, UK) is in clinical trials for glaucoma filtration surgery. Using the human capsular bag model, we tested the ability of CAT-152 to prevent the possible paracrine and autocrine effects of TGF-β2.

METHODS

In Vitro Capsular Bag Model

The use of human tissue in the study was in accordance with the provisions of the Declaration of Helsinki. The model previously described by Liu et al. was used. After removal of corneoscleral discs for transplantation purposes, human donor eyes obtained from the East Anglian Eye Bank were used to perform a sham cataract operation. The resultant capsular bag was then dissected from the zonules and secured with a sterile 35-mm polymethylmethacrylate Petri dish. Eight entomology pins (D1; Watkins and Doncaster Ltd., Kent, UK) were inserted through the edge of the capsule to retain its circular shape. Capsular bags to be analyzed for active TGF-β2 were maintained in EMEM (Sigma, Poole, UK) alone for 2, 28, and more than 100 days (n > 4 for each time point). They were then placed in homogenizing buffer (composition in millimolar: phosphate 6, KCl 100, NaCl 225, EGTA 1, EDTA 1, mercaptoethanol 10, N-ethylmaleimide 10, phenylmethylsulfonyl fluoride [PMSF] 0.2, E64 0.005, and 1% [vol/vol] Tween 20 [pH 7.4]) for ELISA analysis. In certain cases, experiments were performed on match pairs of capsular bags that were maintained in EMEM (Sigma) alone, 1 ng/mL TGF-β2, or 10 ng/mL TGF-β2 (n = 4 pairs in all cases). In these studies, supplements of either 10 μg/mL CAT-152 or 10 μg/mL CAT-001 (null control IgG4 antibody) were added. CAT-152 is a human IgG4 antibody that selectively neutralizes TGF-β2. Its chemical and pharmacologic characterization has been reported. The match-pair experiments were performed over a 28-day period, at which time end-point analysis was performed with immunocytochemistry. Incubation was at 35°C in a 5% CO2 atmosphere. The media were sampled and replaced every 2 to 4 days. Ongoing observations were performed using phase-contrast microscopy.

Ex Vivo Specimens

Seven donor eyes possessed capsular bags that had been generated by cataract surgery and contained intraocular lenses (IOLs). The capsular bags were dissected from the zonules, and four were immediately placed in homogenizing buffer (composition in millimolar: phosphate 6, KCl 100, NaCl 225, EGTA 1, EDTA 1, mercaptoethanol 10, N-ethylmaleimide 10, PMSF 0.2, E64 0.005, and 1% [vol/vol] Tween 20 [pH 7.4]) for ELISA analysis. All specimens showed signs of cell growth and development of PCO. Two preparations were cultured in EMEM for 8 days. The medium was sampled and replaced every 2 days and was analyzed for expression of matrix metalloproteinase (MMP). In one extremely rare case a capsular bag generated at cataract surgery 1 month before the donor’s death was received and after dissection was processed immediately for immunocytochemical evaluation.

Immunocytochemistry

Washes were performed three times for 5 minutes each in PBS containing BSA and Nonidet (0.02% and 0.05%). The pinned capsules were fixed for 30 minutes in 4% formaldehyde in PBS and permeabilized in PBS containing 0.5% Triton X-100, also for 30 minutes. Nonstructural sites were blocked with normal goat serum (1:50 in 1% BSA-PBS). Anti-α-SMA and anti-fibronectin were diluted 1:100 and applied for 60 minutes at 35°C, followed by a wash. α-SMA (Sigma) and fibronectin (Sigma) were visualized with fluorescence-conjugated secondary antibody (ALEXA 488; Molecular Probes, Leiden, The Netherlands). The Factin cytoskeleton was stained with Texas red-X-phalloidin (Molecular Probes) for 10 minutes, and cell nuclei with DAPI at 1 mg/mL for 10 minutes, all at room temperature. The stained preparations were washed again washed exchanged, floated onto microscope slides, and mounted in mounting medium (Vectorshield; Vector Laboratories, Peterborough, UK). Images were viewed with a fluorescence microscope (Eclipse E800; Nikon) with a cooled charge-coupled device (CCD) camera (Princeton Instruments, Ltd., Marlbo, UK) and image analysis software (MetaMorph; Universal Imaging Corp., West Chester, PA).

Estimation of Active TGF-β2

Homogenates of noncultured ex vivo specimens, together with capsular bags that had been maintained in serum-free EMEM for 2, 28, and more than 100 days were probed for active TGF-β2 with a commercially available ELISA kit for human TGF-β2 (R&D Systems, Abingdon, UK). The optical density of each well was determined with a multwell plate reader (Victor; EG&G Wallac, Cambridge, UK). The optical density of each well was determined with a multwell plate reader (Victor; EG&G Wallac, Cambridge, UK). The readings from the standard series were plotted with logarithmic axes and the data from the samples were then applied to the graph to provide the estimated level of growth factor in picograms per milliliter. This value was then used to determine levels per bag.

Zymographic Analyses

All reagents were from Sigma (Poole, UK) unless otherwise stated. Gelatinolytic activity of culture media was analyzed by electrophoresis under nonreducing conditions on a 7% SDS-polyacrylamide gel con-
taining 0.5 mg/mL denatured collagen type I (gelatin; Sigma) as previously described by Gavrilovic et al.35 After electrophoresis, gels were washed twice in 2.5% Triton-X 100 for 15 minutes and then incubated for 16 hours at 37° C in 100 mM Tris-HCl (pH 7.9), 30 mM CaCl₂, and 0.02% azide. The gels were stained with Coomassie brilliant blue (Sigma), and the images were captured with a gel scanner.

RESULTS

Assessment of an Ex Vivo Capsular Bag 1 Month after Surgery

Phase-contrast microscopy revealed that within 1 month, a surprising degree of wrinkling of the posterior capsule had taken place (Fig. 1A). Furthermore, this matrix contraction was associated with large numbers of cells that had progressed onto the posterior capsule in association with matrix contraction. Fluorescence micrographs illustrating several layers of nuclei, some of which exhibit a spindle shape (arrows) and are oriented along capsular wrinkles. Fluorescence micrograph showing Factin distribution in cells growing across the posterior capsule (PC) and also those growing on the outer surface of the anterior capsule (AC). A higher-magnification fluorescent micrograph demonstrating the Factin organization of cells residing on the posterior capsule (AC). Fluorescence micrograph showing α-SMA distribution in cells growing across the posterior capsule and also those growing on the outer surface of the anterior capsule (AC). A higher-magnification fluorescent micrograph demonstrating the α-SMA organization of cells residing on the posterior capsule in association with matrix contraction.

TGF-β2 Detection

The levels of active TGF-β2 in capsular bag preparations are presented in Figure 2. Analysis of capsular bag homogenates showed detectable TGF-β2 at all stages of culture in protein-free medium. Levels at day 28 were significantly higher than at day 2, but were not significantly different from levels detected after more than 100 days of culture (t-test: P < 0.05). Furthermore, in cases in which the donor had undergone a cataract operation, analysis of the capsular bag revealed TGF-β2 to be present at levels that were not significantly different from long-term capsular bag cultures.

Observations of In Vitro Capsular Bags

After the generation of the capsular bags, cell regrowth was observed on the anterior capsule within the first few days of culture, and by day 8, cells were growing on the previously cell-free posterior capsule (Fig. 3). Although all the preparations showed evidence of growth in serum-free medium, no

FIGURE 1. Examination of a capsular bag removed from a donor eye that had undergone cataract surgery 32 days before the time of death. (A) A phase-contrast micrograph shows wrinkling of the posterior capsule and the associated cellular morphology. (B) Fluorescence micrograph illustrating several layers of nuclei, some of which exhibit a spindle shape (arrows) and are oriented along capsular wrinkles. (C) Fluorescence micrograph showing Factin distribution in cells growing across the posterior capsule (PC) and also those growing on the outer surface of the anterior capsule (AC). (D) A higher-magnification fluorescent micrograph demonstrating the Factin organization of cells residing on the posterior capsule (AC). (E) Fluorescence micrograph showing α-SMA distribution in cells growing across the posterior capsule and also those growing on the outer surface of the anterior capsule (AC). (F) A higher-magnification fluorescent micrograph demonstrating the α-SMA organization of cells residing on the posterior capsule in association with matrix contraction.
The presence and absence of CAT-152 (data not shown). However, the degree of αSMA and fibronectin expression was enhanced by TGF-β2 in a dose-dependent manner (Figs. 5E, 5F, 5H, 5I). CAT-152 suppressed TGF-β2-induced expression of αSMA and fibronectin, and the levels were similar to those observed in serum-free conditions (Figs. 5K, 5L).

Expression of MMP

Under serum-free conditions, there was a high level of MMP-2 and a significant level of MMP-9 within 2 days of culture (Fig. 6A). These declined to near-undetectable levels by day 8. Supplementing the medium with TGF-β2 induced expression of both MMP-2 and -9 to a consistently high level by day 8 (Fig. 6A). However, the induction of both MMP-2 and -9 was evident sooner with 10 ng/mL TGF-β2. TGF-β2 neutralization by CAT-152 negated the large increases induced by addition of TGF-β2, but did not ablate the transient increase immediately after surgery (Fig. 6A). Analysis of the medium from cultured ex vivo capsular bags also showed high levels of MMP at day 2 (Fig. 6B), and this expression was maintained at similar levels throughout the culture period (Fig. 6B).
DISCUSSION

The results obtained in this study illustrate the dynamic events that take place after cataract surgery. Examination of a capsular bag, removed from a donor 1 month after a cataract operation, provided an important insight into the cellular events taking place and provided an indication of the time-scale of these events. The wrinkling of the capsule was severe, but localized near the capsulorrhexis. In this specimen, an IOL had been inserted and would therefore place sites of tension where the IOL was in apposition with the capsule. The IOL is also thought to serve as a barrier restricting cell movement, at least in the first few months of culture. This marked wrinkling was associated with cells lying either perpendicular to or along the fold. Furthermore, the actin cytoskeleton within the cells not only consisted of very long stress fibers, but was also aligned either perpendicular or along the wrinkle. These findings are similar to previous results on capsular bags in vitro, which have shown that in association with matrix wrinkles, both cell nuclei and F-actin, visualized with immunocytochemistry, are oriented in this manner. In the ex vivo preparation, αSMA was highly expressed, showing that transdifferentiation had occurred. Although several studies have shown that αSMA is expressed in human capsular bag specimens obtained several months and years after surgery, this study shows that transdifferentiation events can occur in vivo within 1 month, which is also the standard period of our experimental studies. Although the ex vivo specimen provides valuable information concerning the phenotypic markers of PCO, it does not yield an insight into the underlying causes of these events. Therefore, the objective of the present in vitro studies was to elucidate mechanisms that give rise to these phenotypic markers.

The in vitro capsule series of experiments produced clear-cut evidence that TGF-β2 can greatly affect lens cells within the capsular bag and that this can occur by both autocrine and paracrine mechanisms. Under serum-free conditions, wrinkles were seen at day 28 in some cases (Fig. 5). However,
these discontinuities in an otherwise homogeneous monolayer only became evident once the previously cell-free posterior capsule was totally covered. Expression of the transdifferentiation marker αSMA, which is not normally present in the intact lens, was also observed in some cells at day 28. However, fibronectin expression was negligible. Although addition of CAT-152 did not show marked differences in expression of either of these markers when compared with the serum-free control, the antibody inhibited postconfluence formation of wrinkles on the central posterior capsule. Elucidation of these apparently subtle differences may necessitate further biochemical or molecular techniques. It should be noted that active TGF-β2 was also detected in the capsular bag throughout incubation, with a peak in the initial 28 days. These findings therefore imply that TGF-β2 is a potential endogenous regulator of matrix contraction.

A possible paracrine role for TGF-β2 is much easier to establish. Notably, unlike serum-free cultures, wrinkling of the capsule was observed as the cells were growing across the posterior capsule. Once a confluent cover of the posterior capsule was achieved, wrinkles could be seen across the entire posterior capsule and were considerably greater than the serum-free control. Accompanying the matrix changes were significant increases in αSMA and fibronectin levels, and it should be noted that the expression pattern was also found in the ex vivo specimen analyzed 1 month after cataract surgery. Previous work using the capsular bag model has shown that maintenance in fetal calf serum (10%) could further stimulate growth across the posterior capsule. However, this increased movement is not associated with contraction of the matrix, suggesting that wrinkle formation is not simply the result of cellular movement. As in serum-free cultures, serum-stimulated cultures began to wrinkle once complete cover is achieved.

In serum, it should be noted that the major isoform is TGF-β1. This isoform is 10 times less potent than TGF-β2 at instigating transdifferentiation and formation of anterior subcapsular cataract. Therefore, the levels of TGF-β1 in serum may be insufficient to mediate changes.

The major isoform within the eye is TGF-β2, of which most is in the latent form. The levels of TGF-β2 reported within human aqueous humor, detected by ELISA, range from 0.491 to 1.48 ng/mL (total) and 0.182 to 0.283 ng/mL (active). However, after cataract surgery or other injuries to the eye, the levels of active TGF-β2 can increase dramatically. The peak of these levels is likely to occur within the first few days after injury. Protein flare measurements also show postsurgical peaks followed by a gradual decline returning to baseline months after surgery. The work presented shows that TGF-β2 can induce dramatic changes to the cells and underlying capsule that are similar to those observed at similar time points in vivo.

We have also identified important extracellular events that occur in parallel and are likely to influence the fundamental structural events. To investigate the mechanisms behind TGF-β2-induced wrinkling and perhaps transdifferentiation, we examined the expression of MMPs that degrade matrix. Knowledge of MMPs in the lens is limited. We have found that medium from the cultured intact porcine lens shows evidence of MMP production only when the lens is stressed (e.g., by H₂O₂), and significant levels are also obtained when capsular bags are cultured that were generated from previously unstressed lenses. In the present experiments, levels of MMP-2 were transiently increased in human capsular bags after the operation. This transient increase can be greatly prolonged by the addition of TGF-β2, and production of MMP-2 and -9 in cultured chick lens annular pad cells can also be stimulated by TGF-β2.

In the capsular bag experiments, production of MMP-2 and -9 at day 2 was insensitive to CAT-152, suggesting that this production resulted from other stress-activated mechanisms. By day 8, the levels of MMP-2 and -9 in the medium were negligible. Addition of TGF-β2 induced a marked increase of both MMPs. This induction of MMP was prevented by CAT-152. The data observed with cultured ex vivo material showed an interesting pattern of expression. As was the case with all other cultures, MMP-2 and, to a lesser extent, MMP-9 were clearly identified. By day 8, the levels of MMP-2 and -9 at day 2 were insensitive to CAT-152, suggesting that this production resulted from other stress-activated mechanisms. It is likely that both sources contribute, but it is interesting to examine the endogenous capacity of lens cells to regulate their own functions. We have reported in this laboratory that lens cells maintained on the native capsule remain metabolically active for more than 1 year in EMEM without additional serum or protein supplements. Furthermore, we have detected both basic FGF and hepatocyte growth factor (HGF) in capsular bag homogenates after long-term culture. In the present study, we report that TGF-β2 was also detected in its active form within serum-free cultured capsular bags and demonstrate that the addition of CAT-152 to serum-free cultures inhibited wrinkling. These results show that TGF-β2 is capable of initiating matrix modifi-
cation, which is functionally relevant to PCO. Therefore, it is feasible that the endogenous synthesis of TGF-β2, along with other growth factors, could influence the persistent progression of PCO once the inflammatory events have ceased.

It is likely that two distinct elements contribute to development of PCO. The first component is the large influx of growth factors and proteins into the ocular media, in response to surgical trauma. This phase induces considerable change to the capsular bag, but in most cases does not impair vision significantly; however, it could create a platform for a second slower, but persistent, phase of change to the capsular bag. We propose that this second phase could be mediated by basal levels of proteins in the aqueous humor and by autocrine mechanisms, which in a proportion of cases could lead to a severe loss of visual quality or blindness.

In this study, TGF-β was continually maintained at a constant concentration throughout culture. Future studies might investigate whether a short pulse of high TGF-β levels is sufficient to give rise to transdifferentiation and wrinkling events sometime later. Exposure of the cells to TGF-β2 could initiate several feedback systems that could give rise to the changes. Therefore TGF-β2 could be a putative biological switch that can potentiate development of PCO. One protein that could be produced in response to TGF-β2 is connective tissue growth factor (CTGF). Moreover, immunohistochemical examination of PCO tissue obtained at autopsy has detected CTGF, which notably is absent from the normal lens.

Using the in vitro capsular bag model, we have shown that TGF-β2 probably plays an important role in development of PCO. Therefore, the advent of the novel human monoclonal anti-TGF-β2 antibody CAT-152, which is shown to negate TGF-β2-induced effects in our system, could be of potential therapeutic benefit to cataract patients in the future. These findings may also have a wider significance, because TGF-β and the processes of transdifferentiation have also been implicated in some forms of subcapsular cataracts and indeed in fibrotic diseases in general, and hence human monoclonal antibodies selected for neutralization of TGF-β2, such as CAT-152, may also have a therapeutic role in these conditions.

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References