Inducers of Cross-Linked Actin Networks in Trabecular Meshwork Cells

Steven O'Reilly,1,2 Natalie Pollock,1,2 Laura Currie,1 Luminita Paraoan,1 Abbot F. Clark,3,4 and Ian Grierson1,5

PURPOSE. It is well established that the unusual actin arrangements known as cross-linked actin networks (CLANs) can be induced by dexamethasone (DEX) in trabecular meshwork (TM) cells. Recent work reporting their presence in elderly glaucomatous and nonglaucomatous tissue, however, has highlighted the presence of other inducers. In this study, the authors sought to identify CLAN induction agents that may be present within and around the outflow system.

METHODS. Studies were conducted on confluent bovine TM (BTM) cells in culture, and actin was stained with Alexa Fluor 488 phalloidin to identify CLANs in the target cells. The CLAN-inducing potential of aqueous humor was expanded and included investigation of transforming growth factor-beta 2 (TGF-β2). The effect of decorin and fetal calf serum (FCS) on BTM cell cytoskeleton was also investigated, and all were compared with DEX with an exposure period of up to 7 days.

RESULTS. CLAN numbers were increased after 7 days of exposure to TGF-β2 (45%), aqueous humor (37%), and decorin (69%). Even FCS had some modest CLAN-inducing ability (reaching 12%) in BTM cells. Neutralization of TGF-β2 reduced CLAN incidence in aqueous humor conditions to baseline (12%) levels. Blocking TGF-β2 receptors reduced CLAN formation in TM cells by 25% to 30%, whereas the inhibition of Smad3 negated CLAN incidence.

CONCLUSIONS. In this study, the authors identified TGF-β2 as a CLAN-inducing component present in aqueous humor. Decorin was also implicated as another CLAN-inducing agent, and it was confirmed that FCS has CLAN-inducing properties.

The trabecular meshwork (TM) exhibits marked contractile properties, and TM contraction is associated with reduced aqueous humor drainage through the outflow system. Clearly, the smooth muscle-like properties of TM cells are of considerable importance both in health and potentially in glaucoma, when the outflow system’s drainage capacity is often compromised. Studies of the TM cell cytoskeleton and particularly its contractile actinomyosin component have been conducted both in vitro culture monolayers and in the tissue itself. However, we still have much to learn about the cellular and subcellular organization and the role of actin and actin-associated proteins in a range of essential functional activities undertaken by TM cells, including phagocytosis, migration, adhesion, stretch, and contraction. Attempts to modulate the cytoskeleton of TM cells therapeutically have not yet reached the patient, but there has been considerable success in experimental studies. Therefore, the principle of cytoskeletal modification as a potential treatment is well established.

A plethora of cytoskeletal active agents promote aqueous humor outflow, but others, including corticosteroids, can have the opposite effect to the point of producing elevated intraocular pressure and sometimes permanent glaucoma. The normal distribution pattern of actin in the cytoplasm of TM cells is bundles of actin filaments forming stress fibers both in vitro and in situ. Corticosteroids, such as dexamethasone (DEX), stimulate the formation of cross-linked actin networks (CLANs) in TM cells both in vitro and in situ. CLANs have been defined as polygonal arrangements of actin composed of hubs and spokes that form platelike structures in the cytoplasm.

Recent observations have shown that CLANs develop without corticosteroid exposure in substantial numbers in confluent cultures of glaucomatous TM cells, and an association has recently been made between CLANs and β3 integrin signaling. In addition, CLANs are abundant in the TM in situ taken from elderly donors with and without glaucoma. We have shown recently that bovine aqueous humor has CLAN-promoting activity (likely a protein between 5 and 30 kDa), though the exact aqueous humor component and mechanism that led to CLAN development were unclear.

In the present study we set out to identify components of bovine aqueous humor responsible for promoting CLAN formation in bovine TM cells. In the course of this work, we identified transforming growth factor beta 2 (TGF-β2) as associated with CLAN formation. TGF-β2 is a multifunctional cytokine capable of inducing various intracellular pathways, and it is present in the TM and aqueous humor, where it is thought to have a key role in both healthy and glaucomatous tissue.

Further to this, we have identified decorin as another CLAN-inducing agent. Decorin is a common constituent of the extracellular matrix (ECM) and has been found in the TM and the juxtacanalicular tissue. Changes in ECM composition have been linked to age-related and glaucomatous changes in the outflow system, and decorin has been identified as one of several genes found to be upregulated after exposure to DEX. This small molecule proteoglycan has a strong binding affinity for collagen (it was named from this) and several growth
factors, including TGF-β2. In addition, FCS itself seems to have limited CLAN-inducing properties, particularly in bovine TM (BTM) cells.

**MATERIALS AND METHODS**

**Tissue Culture**

We used cultured BTM cells as our main target cell in this investigation. Our long experience with BTM cultures has shown them to have a very reproducible and robust CLAN response to DEX. In addition, we have shown BTM cells to be remarkably resilient to FCS-reduced and FCS-free conditions. Bovine eyes obtained from a local abattoir, with a postmortem interval of no more than 24 hours, were dissected as described previously to provide BTM strips for primary cell growth. BTM cells were cultured in accordance with a previously described protocol. All cultured BTM cells were used after the third passage because the cells exhibited a stable morphology and showed contact inhibition at confluence, thus forming a stable monolayer. BTM cells between passages 3 and 12 (as used in this study) showed no significant alteration in cell morphology, nor did CLAN incidence vary to a significant level. Cells grown from at least three animals were used in each experiment throughout this work to account for any potential individual variations.

Human TM (HTM) primary cultures were used in some of our investigations. HTM cells were obtained from eight donors and were used between passages 4 and 9. One human normal transformed cell line, HTM5 (provided by Iok-Hou Pang, Alcon, Fort Worth, TX), was used between passages 4 and 9. One human normal transformed cell line, HTM5 (provided by Iok-Hou Pang, Alcon, Fort Worth, TX), was used between passages 4 and 9. These cells were cultured identically to BTM cells, as adapted from the protocol described by Pang et al., and were used between passages 10 and 20.

All cells were grown in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS), L-glutamine, penicillin, streptomycin, and fungizone (Sigma, St. Louis, MO). TM cells were seeded onto chamber slides (Nunc Lab Tek; VWR International, LLC, Radnor, PA) and were cultured until confluent. The growth medium was removed, and cells were treated accordingly; this point was set as time 0. In all experiments run for 7 days, fresh medium (specific to the experiment) was added at day 3. Each experimental condition was run alongside cells in DMEM containing either 1% or 10% FCS.

**TGF-β2 Experiments**

Based on the known physiological levels of TGF-β2 in human aqueous humor and on data from preliminary dose-response curves, recombinant human TGF-β2 (Invitrogen, Paisley, UK) at a concentration of 2 ng/mL in DMEM supplemented with 1% FCS was used as our optimum CLAN-inducing medium.

To negate the effects of TGF-β2-induced CLAN formation in the TM cells, we added TGF-β2-neutralizing (polycyclonal goat IgG) antibody (R&D Systems, Minneapolis, MN) at concentrations ranging from 1.6 to 6.4 μg/mL in DMEM containing 1% FCS in combination with TGF-β2 administration.

In separate experiments, BTM cells were treated with both TGF-β2 and one of the following TGF-β receptor antagonists: TGF-βR1 LY-364947 20 μM (Tocris Bioscience, Ellisville, MO); TGF-βR activin receptor-like kinase 5 (Alk-5) inhibitor SB-431542 10 μM; Smad-3 inhibitor, a downstream target of TGF-β2 signaling, SIS-3 25 μM (Sigma-Aldrich). In each experiment, the individual reagents (at specific concentrations) and 2 ng/mL TGF-β2 were added simultaneously to confluent BTM cells with DMEM containing 1% FCS for 7 days.

**Aqueous Humor Experiments**

Aqueous humor (500–800 μL) removed from bovine eyes (<5 hours postmortem) was diluted 1:1 with DMEM containing 1% FCS. Previous work within our laboratory showed that bovine aqueous humor alone was inadequate for optimal BTM cell survival beyond 3 to 5 days. Optimization experiments indicated that the addition of 1% FCS improved cell survival while minimizing the influence of potential CLAN-inducing factors in FCS.

The TGF-β2-neutralizing antibody at a concentration of 1.6 μg/mL was added to the BTM cells with the aqueous humor diluted 1:1 in DMEM containing 1% FCS. The TGF-β2-neutralizing antibody was also added to DMEM containing 1% FCS, and an inappropriate IgG (goat) control (Abcam, Cambridge, MA) was also added to BTM cells, at the same concentration, as a comparator.

**Decorin Experiments**

BTM cells were treated with decorin to investigate its influence on CLAN formation. Recombinant human decorin (95% purity; R&D Systems) was reconstituted in hydrochloric acid (HCl; 0.04 mM) and added to confluent BTM cells to provide a dose-response curve (100 ng/mL to 25 μg/mL). Based on this finding, recombinant decorin at a concentration of 25 μg/mL was added to confluent cultures of BTM cells with DMEM containing 1% FCS.

To assess the effect of the whole proteoglycan on TM cells, decorin from bovine articular cartilage (Sigma) reconstituted in sterile phosphate-buffered saline (PBS) was added to confluent cultures of BTM cells at concentrations ranging from 100 ng/mL to 25 μg/mL.

**Microscopy**

Actin was stained with Alexa-Fluor 488 phalloidin (Invitrogen, Carlsbad, CA) to allow for identification of CLANs; when required, nuclei were stained using propidium iodide (PI). Slides (Lab Tek; Thermo Fisher Scientific, Rochester, NY) were mounted (Fluoro-Mount; Dako, Carpinteria, CA) and were viewed by confocal microscopy (Bio-Rad Laboratories, Hercules, CA). Low-power images (>25 objective) and observations were made of the cultures followed by higher power observations (>60 oil immersion objective) needed for identification of CLANs. CLAN and nuclei quantification was obtained from counts in 20 fields of view per well on masked slides.

For quantification, we imposed a minimum structure of five or more identifiable hubs and at least three triangulated arrangements of actin spokes to constitute a CLAN; this made our counting consistent with that of previous reports. A CLAN territory has been further defined as the area of the cell containing characteristic actin hubs and spokes, the perimeter of which embodies the outermost spokes involved in a triangulation. Any hubs that cannot be linked in this manner are not considered part of the structure.

Statistical analysis of CLAN incidence under each of our treatment conditions was performed using ANOVA or the Student’s t-test. When analysis of variation between groups was required, ANOVA was used in conjunction with the Tukey test.

**Western Blot Analysis**

BTM cells either were treated with TGF-β2 (2 ng/mL) or were pretreated with SIS-3 (25 μM) before the addition of TGF-β2. After 2 hours, the cells were harvested in lysis buffer containing a broad-spectrum phosphatase inhibitor cocktail (Sigma) containing EDTA, and total protein was determined with a Bradford assay (BioRad). Total protein (25 μg) was loaded onto each well, and proteins were resolved by SDS-PAGE (12% polyacrylamide gel). After this, the proteins were transferred to a nitrocellulose membrane and were probed with anti-phosphoSmad-3 antibody (1:1500 dilution; Abcam) overnight. After three washes, the membranes were probed with goat anti-rabbit HRP antibody (Sigma), incubated with enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ), and exposed using an image capture system (ChemiDoc; BioRad).

**RESULTS**

**FCS Effects**

Our HTM primary cultures and the HTM5 cell line did not survive more than a few days in FCS-free DMEM. However,
when viable cells were still present and examined, invariably they were free of CLANs. Increasing FCS in the media up to 10% had a dramatic effect on the health of these cells, and CLANs were identified in 2% to 5% of cells in both our primary HTM and the HTM5 cultures (Fig. 1). Primary cultures of BTM cells showed a clear FCS dose-response effect because <4% of cells had CLANs in the presence of 0.5% FCS for 7 days, whereas approximately 12% of cells had CLANs in 10% FCS (Fig. 1). Primary BTM cells are robust and serve as effective test cells for our subsequent CLAN investigations, but clearly there are as yet unidentified CLAN inducers in FCS to which BTM cells are particularly responsive.

**TGF-β2 Experiments**

Increasing the concentrations of TGF-β2 from 1 ng/mL to 2 ng/mL increased CLAN incidence from 32% to 45% (Fig. 2); however, increasing the concentration to 5 ng/mL and above did not significantly affect CLAN incidence ($P > 0.05$) and could have a deleterious effect on cell health. Tim-response
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To further investigate the link between TGF-β2 and CLAN induction, we treated BTM cells with several agents that would block or inhibit the TGF-β2 action. Agents that blocked the TGF-β receptors RI and RII were at least partially effective at reducing TGF-β2 CLAN formation in BTM cells. The TGF-RII blocker LY-364997 reduced CLAN incidence to 31% after 7 days (a reduction of 32%), and the TGF-βRI/Alk5 blocker SB-431542 reduced CLAN incidence to 34% (a reduction of 25%). However, these values failed to reach statistical significance ($P > 0.05$) (Fig. 5a). In contrast, the specific Smad-3 inhibitor SIS-3 totally negated CLANs when incubated with TGF-β2 and reduced CLANs to 1% when added to TM cells without the growth factor ($P < 0.001$).

The phosphorylation and, hence, activation of Smad-3 was tested using a phosphospecific Smad-3 antibody. The phosphorylated state of Smad-3 was investigated after treatment with TGF-β2, TGF-β2 plus SIS-3, or SIS-3, and all were compared with the control. These data showed that TGF-β2 induced Smad-3 phosphorylation, whereas SIS-3 pretreatment inhibited this phosphorylation (Fig. 5b).

Addition of a TGF-β2-neutralizing antibody (1.6 μg/mL) resulted in a reduction of CLANs from 45% with 2 ng/mL TGF-β2 to within baseline levels (4.6%) at day 7, which represents a percentage decrease of 90%. However, the addition of an IgG (goat) control in the presence of TGF-β2 caused a reduction of CLAN incidence from 44% to 30% (Fig. 6) (a percentage decrease of 32%), indicating a level of nonspecific CLAN reduction. Therefore, of the 90% decrease observed in the addition of TGF-β2–neutralizing antibody, the percentage decrease associated with TGF-β2-specific neutralization was 59%.

Aqueous Humor Experiments

BTM cells treated with aqueous humor, diluted 1:1 with DMEM containing 1% FCS, expressed CLANs in 12% of cells at 3 days, but by 7 days the incidence was 37% (Fig. 6). Some CLANs were unusually large, taking up most of the cells’ cytoplasm (Fig. 7a). As with TGF-β2, CLANs appeared not to be uniformly distributed but were clustered in hotspots of neighboring cells. However, in structure and arrangement of hubs and spokes, aqueous humor–induced CLANs were identical with those induced by DEX and TGF-β2.

After the addition of TGF-β2-neutralizing antibody to our BTM cells in aqueous humor (diluted 1:1 with DMEM containing 1% FCS), the predominant actin arrangement was parallel rows of stress fibers in many of our cells (Fig. 7b). CLAN incidence after TGF-β2–neutralizing antibody in aqueous hu-
mor at the 7-day point was significantly reduced to 12% (Fig. 6) (P < 0.05), representing an overall reduction of 66% in CLAN incidence. Further, the TGF-β2–neutralizing antibody at optimum levels did not deplete CLAN numbers that developed when BTM cells were exposed to DMEM containing 1% FCS, showing a CLAN incidence of 13% (±14.1%) at the 7-day point. The addition of an IgG control was found to have no significant effect on CLAN incidence when added in combination with DMEM containing 1% FCS (7.8% ± 6.5%).

**Decorin**

Preliminary studies showed that decorin was a CLAN-producing agent when added to confluent cultures of BTM cells (Fig. 8a), producing CLANs similar in appearance and structure to those shown previously. Dose-response results indicated that recombinant human decorin levels of 100 ng/mL did not induce CLANs above baseline levels, whereas increasing the concentration to 250 μg/mL increased CLAN incidence to a level of 69% at 7 days (P < 0.001) (Fig. 8b). At higher concentrations, CLAN incidence reached a plateau, and concentrations were not taken above 100 μg/mL.

Cells treated with decorin isolated from bovine articular cartilage at levels higher than 10 μg/mL showed cytoplasmic vacuolation and some cell detachment after 5 days’ exposure. When examined at 7 days, many remaining cells were shrunk and showed nondescript actin staining, which was too inconsistent for quantification. Of the cells still attached, CLAN incidence was found to be within baseline levels.

**DISCUSSION**

Although the stress fiber pattern of actin predominates in TM cells, other cytoplasmic F-actin arrangements, such as CLANs, may be of importance. Recent observations made it entirely improbable that CLANs are an artifact of the in vitro tissue culture environment and also suggested that CLANs are not just a consequence of corticosteroid action.

A small number of CLANs are sometimes found in well-established human and murine (unpublished observations, 2009) cultures of TM cells exposed to nothing more than medium containing FCS. We have shown that BTM cells grown in DMEM containing varying levels of FCS also form CLANs. CLAN incidence in BTM cultures was dependent on FCS concentration (investigated up to 10% FCS) and time in culture. Interestingly, a similar pattern was not seen with primary HTM cells. The apparent decrease in CLANs in HTM cultures exposed to media containing 10% FCS may be an artifact associated with the close packing of the abundant postconfluent cells, making CLANs more difficult to identify. Given that there are virtually no detectable levels of corticosteroid in the FCS and media we use, TGF-β2 is present in FCS; however, the exact concentration is variable and is not stated by manufacturers. Total protein content ranges from 0.03 to 0.04 g/mL (taken from analytical data sheets and personal communications, May 2010), suggesting that the concentration of a specific growth factor would be very low but may contribute to a cocktail of unknown inducers present in FCS. Further, all attempts to reduce the baseline incidence of CLANs in BTM cells with TGF-β2–neutralizing antibody were unsuccessful. Perhaps with the relatively low basal incidence of CLANs in BTM cells grown in FCS (though higher than in other species we have studied), there was insufficient sensitivity to identify a neutralization effect. However, the more probable explanation is that there is a CLAN stimulant in our culture medium that remains to be identified.

We have shown previously and in our present study that aqueous humor also has CLAN-inducing properties. Aqueous humor contains a number of bioactive agents, including further studies.
the multifunctional cytokine TGF-β. A wide range of cellular responses are associated with activated TGF-β, and many of these, such as altered extracellular matrix synthesis, proteolytic activity, and altered signaling pathways, are evoked in TM cells. This investigation has shown TGF-β2 to be a highly effective CLAN-inducing agent in BTM cells at the 2-ng/ml level, corresponding to known levels within aqueous humor.

Use of a TGF-β2-neutralizing antibody effectively reduced CLAN incidence to baseline levels in our BTM target cells. Three main classes of TGF-β cell membrane receptors have been identified to date: receptor types RI, RII, and RIII. The RI
and RII types are transmembrane serine/threonine kinase receptors. Confluent and postconfluent cultured TM cells express TGF-β receptors, and these receptors are also expressed by TM tissues in vivo. It was, therefore, of interest that our RI and RII receptor blocking agents partly suppressed TGF-β2-induced CLAN production when added separately.

We also demonstrated that specific inhibition of Smad-3, a downstream target of canonical TGF-β2 signaling, totally negated TGF-β2-induced CLAN formation. TGF-β2-Smad2/3 signaling has been linked to the expression of α-smooth muscle actin.

We have identified a well-established pathway involved in CLAN formation that can be further investigated to find agents that inhibit the development of CLANs within the TM cell cytoplasm. Enhanced α3-integrin signaling has been reported to influence CLAN formation by what appears to be an inside and outside signaling pathway in TM cells, representing another potential direction for the perturbation of CLANs.

Research directed toward the identification of CLAN inhibitors is being conducted in our laboratory. Clark et al. identified tetrahydrocortisol as a potent inhibitor of CLANs produced by DEX. We have shown that CLAN induction by TGF-β2 can be at least partially inhibited by TGF-β RI and RII receptor blocking agents, Smad disrupting agents (SIS-3), and a TGF-β2-neutralizing antibody. The identification of CLAN inhibitors is in its infancy but may prove invaluable in understanding the pathways involved in CLAN formation.

Decorin has been reported to bind to TGF-β, thus modulating its cellular action; as such, the proteoglycan has been used to modify gliosis and has even been investigated as a potential adjunct to promote the success of glaucoma surgery. Intuitively we had expected the inhibition of TGF-β2 CLAN induction, but instead the recombinant human decorin was found to be an effective CLAN inducer. In contrast, decorin extracted from bovine cartilage was not supportive of BTM cell survival at levels associated with CLAN stimulation. This difference may be explained by the molecular differences in these two decorin preparations. Recombinant decorin is not glycosylated and has a much lower molecular weight than does extracted decorin, which may alter its ability to interact with receptors on the TM cells. It would seem more likely that the core protein is responsible for CLAN induction, but the mechanism of action was not explored in this study. Possible CLAN modulation by the decorin glycosaminoglycan chains still has to be investigated, as does the possibility that other closely related members of the small leucine-rich proteoglycan family, such as biglycan and lumican, have CLAN inducing actions.
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We were convinced based on appearance and organization that the CLANs associated with TGF-β2, aqueous humor, decorin, and DEX were identical, but it will be valuable in the future to examine the CLANs for known corticosteroid CLAN-associated proteins such as α-actinin, 24-25 phosphatidylinositol 4,5-bisphosphate, and syndecan-4. 55 DEX induction of CLANs has been shown in cultured human 26 and bovine 27 TM cells and in the intact TM tissue ex vivo. 28 Future studies must look at the ex vivo or even the in vivo effects of inducers such as TGF-β2 and decorin on CLAN formation in the cells of the outflow system and at whether CLANs contribute to increased outflow resistance and elevated intraocular pressure. DEX 29-35 and other corticosteroids have cell shape and extracellular matrix effects, and that is also the case for TGF-β2. 41 Decorin has a cell shape–altering and migration-inhibiting action 42 and is also an effective antiscarring agent 54 and even aqueous humor has a TM cell shape-changing action. 59 Filla et al. 26 implicate β3 integrin activation and αvβ3 signaling as CLAN promoters; their suggestion of inside-out signaling mechanisms as crucial to CLAN formation has heightened their significance in light of the characteristics of the CLAN inducers identified in the present study.

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References


