
Glucocorticoid-Induced Formation of Cross-Linked Actin Networks in Cultured Human Trabecular Meshwork Cells

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Purpose. To determine the effects of glucocorticoid treatment on the microfilament structure of cultured human trabecular meshwork cells. Topical or systemic administration of glucocorticoids can lead to the development of ocular hypertension and to the development of vision loss, which is clinically similar to primary open angle glaucoma. However, the mechanism(s) by which glucocorticoids cause ocular hypertension is not well defined. Alterations in the trabecular meshwork, the site of drainage of aqueous humor from the eye, have been linked to the development of ocular hypertension.

Methods. Human trabecular meshwork cells were cultured in the presence and absence of glucocorticoids for 0 to 21 days. The microfilament organization of the cultured trabecular meshwork cells was examined by epifluorescent and transmission electron microscopy.

Results. Glucocorticoids caused a progressive change in the organization of microfilaments in the trabecular meshwork cells, but not in other cultured ocular cells. By fluorescence microscopic analysis, the actin stress fibers found in control trabecular meshwork cells were reorganized on treatment with glucocorticoids into cross-linked actin networks that resembled geodesic-dome-like polygonal lattices. The cross-linked actin networks were reversible on withdrawal of the glucocorticoid treatment. Dose-response data for dexamethasone, relative ranking of activity with glucocorticoid potency, and partial inhibition with glucocorticoid antagonists all suggest the involvement of the trabecular meshwork glucocorticoid receptor in cross-linked actin network formation. The reorganization of the trabecular meshwork cytoskeleton alters cell function because glucocorticoid treatment of cultured trabecular meshwork cells also inhibited trabecular meshwork cell migration and proliferation.

Conclusion. The steroid-induced alteration in trabecular meshwork cytoskeleton may be an important factor in the development of steroid-induced ocular hypertension and may play a role in the ocular hypertension associated with primary open angle glaucoma. *Invest Ophthalmol Vis Sci.* 1994;35:281-294

Glucocorticoids appear to play an important role in the development of corticosteroid glaucoma. Both systemic¹ and topical ocular²⁻⁶ administration of glucocorticoids can lead to the development of ocular hypertension in a subset of the population. In addition, in patients with Cushing's syndrome, who have endogenously elevated levels of cortisol, ocular hypertension often develops,⁷ and prolonged steroid-induced ocu-

lar hypertension can lead to a disease remarkably similar to primary open angle glaucoma.^{2,3} Animal models have shown that eyes topically treated with glucocorticoids also develop ocular hypertension.⁸⁻¹¹ The elevated intraocular pressure associated with steroid treatment has been shown to be caused by increased aqueous humor outflow resistance^{1,4} and is associated with morphologic changes in the trabecular meshwork (TM).¹²⁻¹⁵ Recent experiments have also demonstrated dexamethasone (DEX)-induced ocular hypertension in isolated perfusion-cultured human eyes.¹⁶

A variety of studies have demonstrated glucocorticoid-induced changes in cultured trabecular meshwork cells. Human trabecular meshwork cells contain glucocorticoid receptors^{17,18} and therefore are likely

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A portion of this work was presented at the 1990 Annual Meeting of the Association for Research in Vision and Ophthalmology in Sarasota, Florida.

Submitted for publication: October 21, 1992; revised June 16, 1993; accepted June 21, 1993.

Proprietary interest category: N.

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targets for glucocorticoid action. The treatment of human trabecular meshwork cells with glucocorticoids has been shown to increase the expression of the extracellular matrix molecules collagen,¹⁹ glycosaminoglycans,²⁰ elastin,²¹ and fibronectin²² as well as induce the expression of a 55 kD sialoglycoprotein.^{23,24} Glucocorticoid treatment also decreased the expression of several extracellular proteinases including fibrolytic enzymes²⁵ and stromolysin.²⁶ TM phagocytic activity^{23,27} and hydraulic conductivity²⁸ are both decreased by steroid treatment. In addition, glucocorticoid treatment of cultured TM cells caused a marked enlargement of TM nuclei and DNA content.²⁹

A number of studies have shown that the aqueous humor outflow facility can be modified by agents that alter the cytoskeleton of the TM.³⁰⁻³⁶ There are three major classes of cytoskeletal elements, microfilaments, microtubules and intermediate filaments, all of which have been characterized in the TM.³⁰ The cytoskeleton is essential for controlling cell shape, adherence to the extracellular matrix, motility, cytokinesis, as well as phagocytic activity.³⁷⁻⁴⁰ In some cases, protein synthesis can be regulated by the cytoskeleton.³⁹ The purpose of the current study was to examine the effect of glucocorticoid treatment on the cytoskeletal microfilaments of cultured human TM cells.

MATERIALS AND METHODS

Cell Culture

Human donor eyes (age range, 18–83 years) were obtained from regional eye banks, and TM cells were generated as previously described.^{22,41} Briefly, the TM cells were grown in Ham's F10 Media (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine and 100 U each of penicillin/streptomycin (Gibco Laboratories, Grand Island, NY). The TM cells were incubated at 37°C in 7% CO₂/93% air, and the media was changed two times each week. TM cells were passaged from confluent monolayers using Cytodex 3 microcarrier beads (Sigma Chemical Co., St. Louis, MO) as described previously.²² The sterile Cytodex beads were added to the confluent TM monolayer approximately 1 week before passage of the cells, which allowed the beads to become coated with cells. The cell-coated beads were then aspirated with a Pasteur pipette into culture media and transferred to a new culture plate. The cells migrated off the beads onto the surface of the plate and grew to form a new monolayer. Nine different primary TM cell lines were obtained from different donors (Table 1). To compare TM cytoskeletal characteristics with other cell types, additional primary cell lines were also generated and characterized as previously described including: bo-

TABLE 1. Cell Strains Tested for Dexamethasone-induced CLAN Formation

<i>Cell Strain</i>	<i>Tissue</i>	<i>DEX-induced CLANs</i>
TM 1	Trabecular meshwork	+
TM 2	Trabecular meshwork	+
TM 4	Trabecular meshwork	+
TM 6	Trabecular meshwork	–
TM 10B	Trabecular meshwork	+
TM 13B	Trabecular meshwork	+
TM 16A	Trabecular meshwork	+
TM 26	Trabecular meshwork	+
TM 29	Trabecular meshwork	+
CM 16D	Ciliary muscle	–
RPE 1	Retinal pigment epithelium	–
RPE 2	Retinal pigment epithelium	–
LEPI	Lens epithelium	–
CornStr	Corneal stroma	–
NPE	Ciliary nonpigmented epithelium	–
BVE	Bovine coronary artery vascular endothelium	–

vine coronary artery vascular endothelial cells⁴² (provided by William Campbell, University of Texas Southwestern Medical Center, Dallas, TX), human corneal stroma fibroblasts,⁴¹ human lens epithelial cells,^{43,44} human retinal pigment epithelial cells,⁴⁵ human ciliary body nonpigmented epithelial cells⁴⁶ and human ciliary muscle cells.⁴⁷ All ocular cell lines were grown in Ham's F10 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. Bovine coronary artery vascular endothelial cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics.

Microfilament Staining

Primary cell lines were grown to confluency on glass coverslips in 24-well Costar (Cambridge, MA) culture plates. To evaluate the effects of steroids on the cytoskeleton of cultured cells, steroids were added to the media by diluting 1 μ l of steroid stock solution per milliliter of media. Steroid stock solutions were prepared by dissolving steroids in absolute ethanol at 10³ higher concentrations than were needed in the media. Control cells received an equivalent volume of ethanol. All the steroids (DEX, prednisolone, cortisol, progesterone, cortexolone and methyltestosterone) were from Sigma. The cells were treated for 0 to 21 days, then their microfilament structure was examined. For rhodamine-phalloidin staining, the cells on the coverslip were rinsed in serum-free Ham's F10 media and fixed for 60 minutes in 1% glutaraldehyde (Sigma), 0.5% Triton X-100 (Sigma) in 50 mM phosphate buffer (pH 7.2). The cells were rinsed in phosphate-buffered saline (PBS), stained with rhodamine-

phalloidin (Molecular Probes Inc., Eugene, OR) for 20 minutes, rinsed in PBS and examined by epifluorescent microscopy. For smooth muscle α -actin staining, the TM cells on coverslips were rinsed in PBS then fixed in methanol at -20°C for 10 minutes. The cells were rinsed with PBS and incubated for 1 hour with a monoclonal antismooth muscle α -actin antibody (Sigma) diluted 1/50 in PBS containing 1% bovine serum albumin. The cells were rinsed three times in PBS and stained for 1 hour with fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim Corp., Indianapolis, IN) diluted 1/20 in PBS containing 1% bovine serum albumin. The cells were rinsed two times in PBS and two times in distilled water before examination by epifluorescence microscopy. The percentage of cells that developed cross-linked actin networks (CLANs) was determined by examining a minimum of 200 cells selected from random fields ($300\times$). All assays were performed at least two times and the average variability between assays was approximately 5–10%. Photomicrographs were taken with a Nikon (Garden City, NY) Optiphot Microscope using Kodak (Rochester, NY) Ektochrome 64 ASA or 800 ASA film.

Electron Microscopy

TM cells were grown on Formvar-coated (Formvar, Electron Microscopy Sciences, Fort Washington, PA) nickel grids so the microfilament arrangement could be examined ultrastructurally. Control and DEX-treated (10^{-7} M DEX) cells were grown for 14 days and processed according to the method described by Schliwa et al⁴⁸ for the preparation of whole mount cells. Briefly, the grids were first rinsed in serum-free Ham's F10 media, rinsed in PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl_2)

and then extracted for 1.5 minutes in PHEM buffer containing 0.15% Triton X-100. The cells were subsequently fixed for 30 minutes in 1% glutaraldehyde in PHEM buffer, rinsed, osmicated for 2 minutes, dehydrated, critical point dried, and examined in a Zeiss (Thornwood, NY) CEM-902 transmission electron microscope.

Cell Migration, Proliferation, and Size

The effects of DEX treatment on TM cell migration and proliferation was studied using Cytodex 3 microcarrier beads. A sparse layer of sterile Cytodex beads was added to a confluent monolayer of TM1 cells. The beads were left on the surface of the monolayer for 2 weeks until all beads were saturated with cells. The beads were then transferred by aspiration with a Pasteur pipette to a new 6-well plate containing media with and without 10^{-7} DEX (2 wells of each). The TM cells were grown for 10 days, fixed in phosphate-buffered formalin and stained with Geimsa (VWR, Sugarland, TX). The area of cell proliferation and migration around each bead was determined with a Bioquant (R & M Biometrics, Nashville, TN) image analyzer. The effect of DEX treatment on TM cell size was measured by image analysis of phase contrast photomicrographs of confluent TM16A cells incubated in the presence or absence of 10^{-7} M DEX for 14 days. The cells were fixed in 2.5% glutaraldehyde and stained with toluidine blue. The average area of more than 400 individual control and DEX treated cells was calculated. Statistically significant differences were determined using Student's *t* test.

RESULTS

The cultured human TM cells used in this study had the same characteristics as TM cells reported previ-

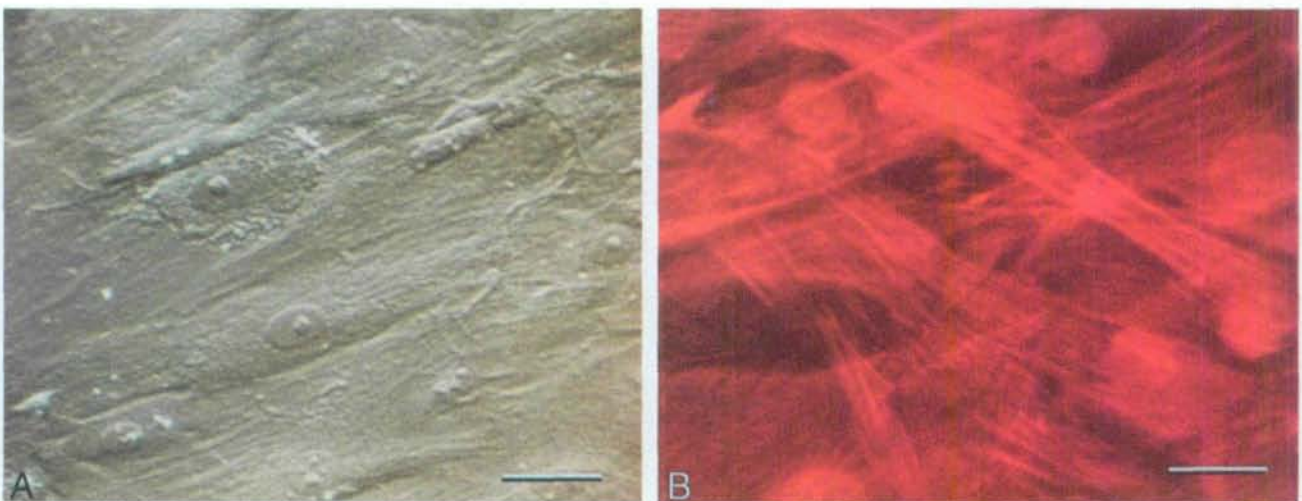


FIGURE 1. Hoffman (A) and rhodamine-phalloidin epifluorescent (B) photomicrographs of confluent human trabecular meshwork cells grown in the absence of dexamethasone. Bar = 50 μm .

ously.^{21,22,41,49,50} The TM cells (Fig. 1A) are broad, flat, and partially elongated, and they are packed closely together with multiple cell processes contacting other cells. When the cells are confluent, they form a single monolayer with overlapping cell boundaries. They have a relatively smooth surface and a centrally located oval nucleus. These cultured TM cells also synthesize fibronectin, laminin, and types I, III, and IV collagen (data not shown) consistent with the results of others.^{21,50}

The outline of microfilament bundles (stress fibers) is evident along the longitudinal axis of normal TM cells using Hoffman contrast microscopy (Fig. 1A). There is a change in the organization of the cytoskeleton, especially around the nucleus, in TM cells exposed to DEX for 7–14 days (Fig. 2A). When untreated TM cells stained with rhodamine-conjugated phalloidin were examined by epifluorescence microscopy, they showed the normal linear arrays of stress fiber microfilament bundles (Fig. 1B). TM cells treated with glucocorticoids showed a dramatic change in microfilament organization with the generation of cross-linked actin networks (CLANs) (Fig. 2B–F). The CLAN structure consisted of a three-dimensional geodesic-dome-like organization of microfilaments. The DEX-induced CLANs may incorporate only a portion of the cell's cytoskeleton (Fig. 2B) or change the entire microfilament structure of the cell (Fig. 2, C to E). The extent of glucocorticoid-induced cytoskeletal reorganization varied between the cell lines and time of exposure to the steroids (Fig. 3). It generally required 3–4 days before DEX-induced CLANs were evident, and there was a progressive accumulation of CLANs during subsequent weeks. Not all cells of a specific TM cell population were responsive to the glucocorticoid-induced alteration in cytoskeleton. Usually only 30% to 70% of the cells developed CLAN structures after exposure to DEX for 14 days (Fig. 3). One TM cell line (TM6) did not form CLANs when exposed to DEX for up to 3 weeks.

Most of the human TM cells express several different actin isoforms including smooth muscle α -actin. To determine which isoform of actin is involved in CLAN formation, we stained the glucocorticoid-treated TM cells with antibodies against smooth muscle α -actin. Polygonal lattices of smooth muscle actin (ie, CLANs) were seen in the DEX-treated TM cells (Fig. 2F). It is likely that several different isoforms of actin are involved in formation of these networks because the CLANs are more common and extensive when visualized with the nonisoform-specific phalloidin–rhodamine reagent compared to the visualization with the smooth muscle actin antibody.

Ultrastructural studies of the control TM cells showed cytoskeletal bundles arranged in normal linear arrays that ran the length of the cell (Fig. 4A). These

bundles varied in thickness from 90 to 120 nm and were composed of individual filaments that were likely microfilaments. In contrast, DEX-treated TM cells had the geodesic-dome-like structures like those seen by epifluorescence microscopy. These CLANs averaged 6 to 8 microfilament bundles radiating from electron dense vertices (Fig. 4B). The individual vertices were separated by approximately 3 to 5 μ m, which correlates with distances shown by light microscopy.

The steroid-induced formation of CLANs in cultured TM cells was specific for glucocorticoids (Fig. 5). The glucocorticoids DEX, prednisolone, and cortisol were able to induce CLANs in TM cells whereas other steroids (progesterone, methyltestosterone, and cortexolone) were incapable of CLAN generation. The ability of glucocorticoids to generate CLANs in the TM cells closely matched their glucocorticoid potency (DEX > prednisolone > cortisol). Various glucocorticoid antagonists (progesterone, methyltestosterone, and cortexolone) were able to partially block the ability of DEX to induce CLANs. Administered alone these antagonists also decreased the baseline level of CLANs in the control cell cultures. Baseline levels of CLANs appeared to be caused by the presence of cortisol in the fetal bovine serum used in the media. Extensive dialysis or activated charcoal/dextran treatment of the fetal bovine serum caused a dramatic reduction in baseline CLANs to less than 1% of the population (data not shown). The ability of various concentrations of DEX to induce CLAN formation is shown in Figure 6. The concentration of DEX that caused half the maximal response (EC_{50}) was approximately 2 nM.

The glucocorticoid-induced formation of CLANs appeared to be relatively specific for TM cells. Eight of nine of the TM cell lines responded to DEX by altering their microfilament organization (Table 1). Ocular cell lines derived from other regions of the eye (corneal scleral fibroblasts, ciliary body epithelial cells, retinal pigment epithelial cells, ciliary body smooth muscle cells) did not form glucocorticoid-induced CLANs. Likewise, cultured bovine coronary artery vascular endothelial cells were unresponsive to DEX-induced cytoskeletal changes.

The DEX-induced CLAN formation in cultured TM cells was time dependent (Fig. 3). There was a lag period of 2–4 days after initiation of DEX treatment and before the onset of CLAN formation. CLAN development was progressive and linear during a 2- to 3-week period in three TM cell lines and appeared to plateau after 10 days of DEX treatment in another three TM cell lines. The various TM cell lines had different sensitivities to DEX-induced CLAN formation and not all the treated cells in a culture population developed CLANS. In some cell lines (TM1 and TM16A) only 30% of the cells formed CLANS after 2

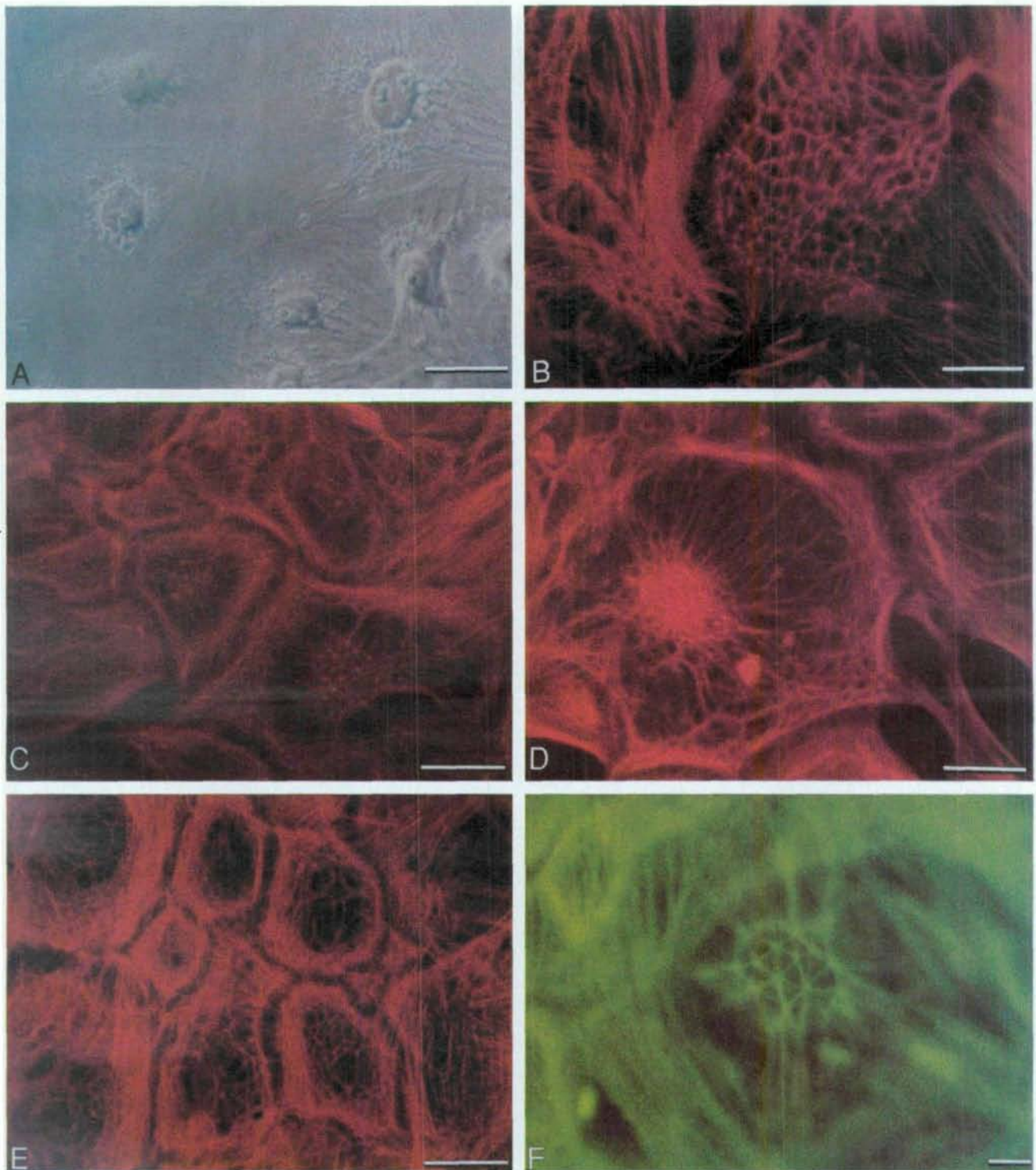


FIGURE 2. Hoffman (A), rhodamine-phalloidin epifluorescent (B, C, D, and E) and smooth muscle alpha-actin immunofluorescent (F) photomicrographs of confluent human trabecular meshwork cells grown in the presence of 10^{-7} M dexamethasone for 8 to 14 days. Bar = $50\ \mu\text{m}$.

weeks of DEX treatment. Approximately half of the cell population in the other cell lines developed the glucocorticoid-induced cytoskeletal rearrangement. The DEX modification in microfilament structure was reversible on withdrawal of DEX from the media (Fig.

7). The disappearance of CLANs on removal of DEX was time dependent requiring approximately 5 to 6 days to return to control levels.

We evaluated the functional consequences of glucocorticoid treatment of cultured TM cell migration

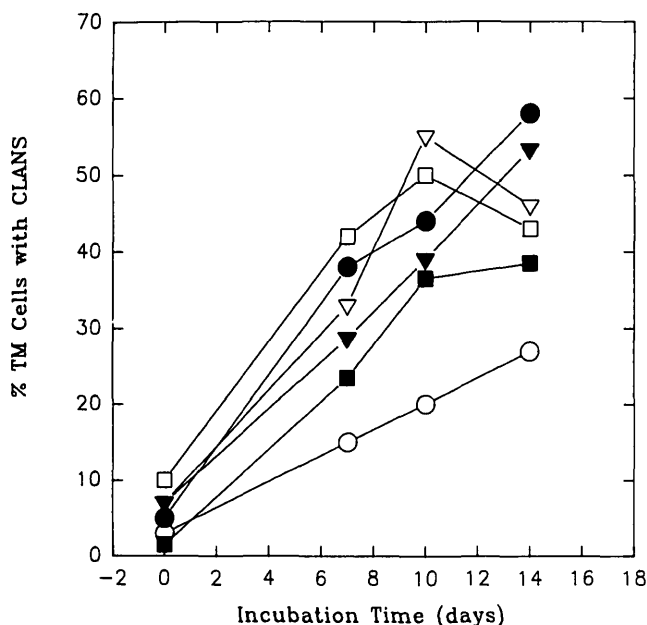


FIGURE 3. Time-dependent generation of cross-linked actin networks in cultured human trabecular meshwork cells. Confluent monolayers of trabecular meshwork (TM) cells were treated with 10^{-7} M dexamethasone for 0 to 14 days. The cells were fixed and stained with rhodamine-conjugated phalloidin to examine the microfilament structures. Each experiment was done in duplicate and the results are the average of two to five experiments. The average percentage of cells containing cross-linked actin networks were determined by the examination of >200 cells per coverslip. TM1 (○), TM2 (●), TM4 (▽), TM10B (▼), TM13B (□), and TM16A (■).

and size. In a microcarrier bead migration assay, Cytodex beads coated with TM cells were plated in the absence (Fig. 8A) or presence (Fig. 8B) of 10^{-7} M DEX and grown for 10 days in culture. In the control wells, the cells migrated and proliferated radially from the Cytodex beads. In contrast, there were many fewer cells and the area of migration was significantly decreased in the DEX-treated cells. The migration area for untreated control cells and DEX-treated cells was determined by image analysis. Treatment with DEX for 10 days caused a 70% to 80% decrease in TM cell migration (Fig. 9A). We also examined the effect of DEX treatment on TM cell size. Cell size was calculated by image analysis of >400 cells grown in the absence or presence of 10^{-7} M DEX for 14 days. The average size of control TM cells ($n = 464$) was $1.62 \pm 0.045 \times 10^3 \mu\text{m}^2$ (mean \pm SEM) compared to $2.46 \pm 0.086 \times 10^3 \mu\text{m}^2$ for the DEX-treated cells ($n = 474$). There was a large variation in the range of cell sizes in the control (230 to $7403 \mu\text{m}^2$) and the DEX-treated (280 to $9554 \mu\text{m}^2$) TM cells. The DEX-treated cells were significantly ($P < 0.001$) larger by an average of 55% than the untreated control cells (Fig. 9B).

DISCUSSION

The microfilament structure of a variety of cultured cells has been well documented. Bundles of microfilaments, known as stress fibers, form submembranous cables of actin filaments on the basal side of the cell membrane attached to the substratum. In some cells, stress fibers also occur above the nucleus and beneath the apical membrane surface.⁵¹ Stress fibers are not unique to cultured cells; they have also been identified in vivo in vascular endothelial cells⁵² and in cells of the human TM.⁵³⁻⁵⁵ Actin microfilaments are also found at the periphery of cultured cells in membrane ruffles, microspikes, and blebs.⁵¹ Several earlier studies have reported stress fibers in cultured human TM cells.^{36,56} Data from the current study have also shown that cultured human TM cells contain prominent actin stress fibers. In addition, a small percentage of untreated TM cells had a rather unique microfilament structure in which actin was arranged into polygonal networks or geodesic-dome-like structures. These polygonal networks consist of CLANs that are present in varying degrees among the nine TM cell lines examined. The addition of glucocorticoids to the culture media of TM cells led to a progressive and time-dependent significant increase in the proportion of TM cells that developed CLANs. This reorganization of microfilaments was reversible on removal of the steroid from the media. This steroid-induced reorganization of the microfilaments was specific for glucocorticoids; other steroids were not capable of inducing CLANs. The ability of glucocorticoids to generate CLANs in the TM cells was related to glucocorticoid potency and was partially inhibited by concomitant treatment with glucocorticoid antagonists. In the dose-response studies, the EC_{50} for DEX-induced CLAN generation closely matched the affinity of DEX for the human TM glucocorticoid receptor.¹⁷ Finally, the glucocorticoid induction of CLANs appeared to be unique to the cultured TM cells. A variety of other cell lines tested did not generate CLANs on DEX exposure.

Polygonal actin networks have been previously reported in other cultured cells, although these structures were not shown to be induced with glucocorticoids. Lazarides^{57,58} has shown that when first plated, primary cultures of rat embryo cells transiently form actin polygonal nets. Primary cultures of chick cardiac myocytes have been shown to transiently form similar actin polygonal networks,⁵⁹ and these networks have also been reported in up to 15% of the cells in the epidermal cell line NB-11^{51,60} as well as in a 3T3 mouse fibroblast line.⁶¹ The functional role of CLANs in cultured cells, however, is not known. Lazarides has shown that CLANs occur transiently in some cultured cells when the cells are initially placed in culture and

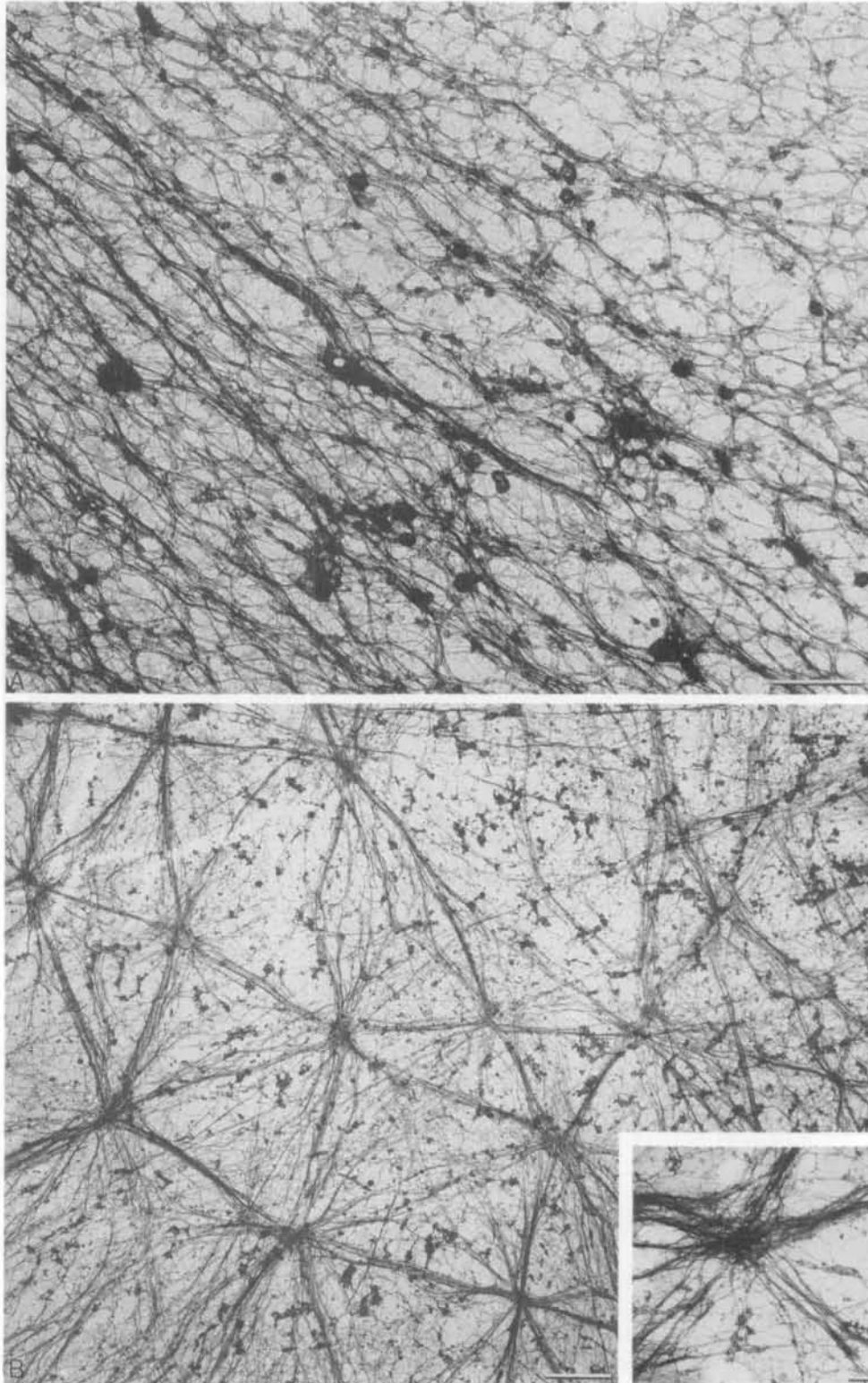


FIGURE 4. Whole mount transmission electron micrographs of the cytoskeleton of control trabecular meshwork cells (**A**) and trabecular meshwork cells exposed to 10^{-7} M DEX for 14 days (**B**). The stress fibers in the control cells are arranged in normal linear arrays while DEX-treated microfilaments are grouped into 90–120 nm bundles radiating from electron dense vertices. Bar (**A**) = 0.5 μ m; Bar (**B**) = 2 μ m; Bar (**B, inset**) = 0.5 μ m.

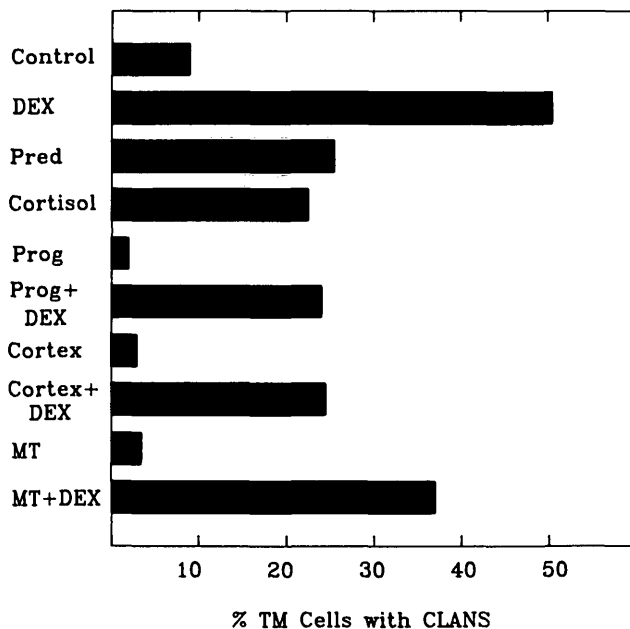


FIGURE 5. Effect of steroids on cross-linked actin network (CLANs) formation in cultured human trabecular meshwork cells. Confluent cultures of TM2 were treated with steroids [10^{-7} M dexamethasone (DEX), 10^{-6} M prednisolone (Pred), 10^{-6} M cortisol, 10^{-6} M progesterone (Prog), 10^{-6} M corticosterone (Cortex) and/or 10^{-6} M methyltestosterone (MT)] for 14 days. The trabecular meshwork cells were fixed and stained with rhodamine-phalloidin and cross-linked actin networks were determined by the examination of >200 cells per treatment group. The results shown are the average values of two experiments with each treatment done in duplicate.

has proposed that the CLANs are an intermediate structure in the reorganization of the cytoskeleton to allow the cells to adhere to and spread on the surface of the plate.^{57,58} Osborn and colleagues⁵¹ have speculated that these polygonal networks may play some sort of role in the structure and function of the cell. Okabe and Hirokawa⁶¹ determined the turnover rates of actin microfilaments in cultured fibroblasts and have shown the slowest rate of actin turnover to be in the polygonal networks compared to actin incorporated in stress fibers or membrane ruffles. Support for glucocorticoid-mediated stabilization of microfilaments comes from a report on cultured cells treated with glucocorticoids that were more resistant to cytochalasin-induced disruption of the actin microfilaments.⁶² It therefore appears that the CLANs are the most stable form of microfilaments in the cell and this stability may play an important functional role.

Microfilaments are essential to a variety of cell functions. Cell shape, cortical flow, mobility, phagocytosis and cytokinesis all depend on microfilament dynamics.³⁷⁻⁴⁰ The development of CLANs may dramatically change microfilament cycling and lead to more stable microfilament structures, which in turn may dra-

matically affect cellular function. Incubation of human TM cells with DEX (10^{-7} M) for 10 days led to dramatic microfilament CLAN generation and concomitantly caused a significant decrease in TM migration and proliferation. TM cell size also significantly increased after 14 days of DEX treatment. A glucocorticoid-mediated increase in cell size was previously reported in cultured human TM cells exposed to high concentrations of cortisol for 3 weeks.²⁹ In addition, TM cells are actively phagocytic,⁶³⁻⁶⁶ and cultured TM cell phagocytic activity is significantly decreased when treated with DEX.^{23,27} Furthermore, cultured TM cells exposed to cortisol for 3-4 weeks in culture have been reported to have larger nuclei and higher nuclear DNA content than control cells²⁹ suggesting that although DNA synthesis continues, the steroid-treated cells may not be able to divide. This suggestion is supported by the fact that cytokinesis requires the reorganization of actin microfilaments to form a circumferential "cleavage furrow" in the middle of the cell.³⁷ Cells that have their microfilaments "locked" into CLANs would be less likely to readily form cleavage furrows and therefore be less likely to divide.

The glucocorticoid-induced reorganization of the

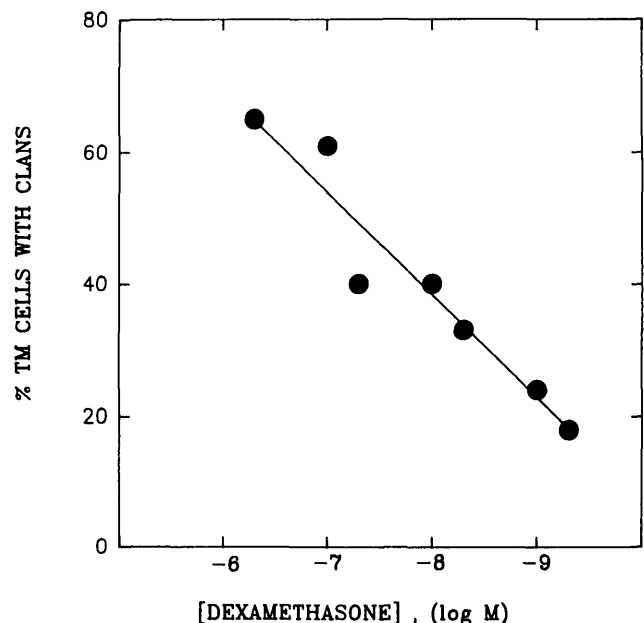


FIGURE 6. Effect of dexamethasone (DEX) concentration on cross-linked actin network (CLANs) formation in cultured human trabecular meshwork cells. Trabecular meshwork cells were incubated with various concentrations of DEX for 14 days. The cells were fixed and stained with rhodamine-phalloidin and cross-linked actin networks were determined by the examination of >200 cells per treatment group. The results shown are the average of two experiments with each DEX dose done in duplicate. The EC_{50} for cross-linked actin network formation was determined to be 2 nM. Correlation coefficient = 0.96.

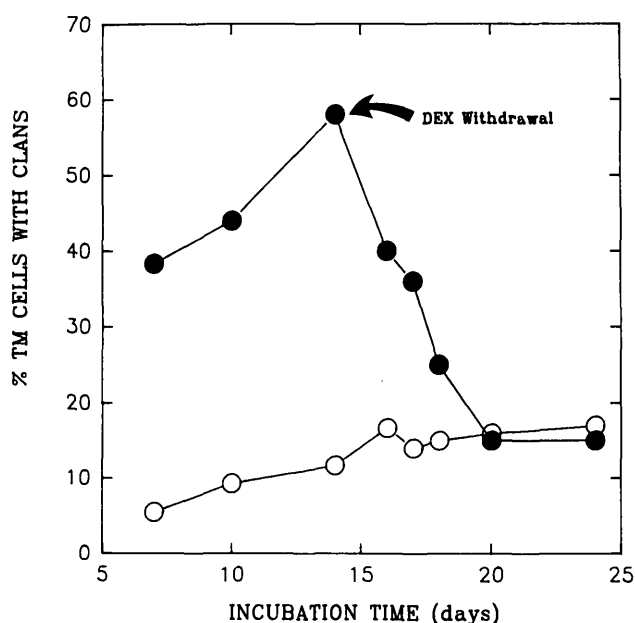


FIGURE 7. Effect of withdrawal of dexamethasone on cross-linked actin network formation in cultured human trabecular meshwork cells. Confluent cultures of TM2 were treated with 10^{-7} M dexamethasone for 14 days before removal of dexamethasone from the media. Cells were fixed and stained with rhodamine-phalloidin on days 7, 10, 14, 17, 18, 19, 20, and 24. The percentage of cells with cross-linked actin networks was determined by examination of >200 cells. The results shown are the average values of two experiments with each experiment done in duplicate. DEX-treated cells (●), control cells (○).

TM cell cytoskeleton may have important consequences in the function of the TM, which acts as a biologic filter for the aqueous humor leaving the eye. The actively phagocytic TM cells clean debris from the aqueous humor, and the TM cells have the capacity to generate and turnover the extracellular matrix of the aqueous humor outflow pathway.^{21,50,67} The TM also provides a modest resistance to aqueous humor outflow, which generates the intraocular pressure necessary for maintaining the shape of the cornea and globe. There are a number of potential explanations for how DEX-induced alterations in the TM cytoskeleton could directly lead to the development of ocular hypertension. (1) It has been suggested that glucocorticoid-induced ocular hypertension may be caused by inhibition of TM cell phagocytosis.⁶⁸ The DEX-induced inhibition of TM phagocytosis^{23,27} may therefore be the result of the DEX-induced reorganization of the TM cytoskeleton because a dynamic microfilament organization is necessary and essential for phagocytic activity. (2) There is a very close relationship between the cytoskeleton and the extracellular matrix. Changes in the expression and deposition of the cytoskeleton can dramatically affect the extracellular ma-

trix and the converse is also true.^{38,39} There is a strong association between actin microfilaments and the extracellular matrix glycoprotein fibronectin.⁶⁹ A previous study demonstrated that DEX treatment of cultured human TM cells caused a significant time-dependent accumulation of fibronectin.²² The time dependence for this DEX-induced effect on fibronectin was similar to the DEX-induced effects on the TM cytoskeleton. This may suggest a link between the effects of DEX on the cytoskeleton and the extracellular matrix. Fibronectin can regulate a number of cell functions including adhesiveness to the substrata and cell shape.^{38,39} Several studies have indicated an increased deposition of fibronectin in the outflow pathway of eyes from patients with open angle glaucoma,^{70,71} although it is currently not known whether this increased fibronectin deposition is the cause of elevated intraocular pressure in these glaucoma patients. (3) The cytoskeleton plays a major role in the regulation of cell shape and size. Microfilaments play a major part in the cytokinetic process during cell division when a microfilament cuff forms in the center of the cell to contract and divide the cell in half. It is possible that the DEX-induced reorganization of TM microfilaments prevents cytokinesis. Several pieces of evidence suggest that the DEX-treated TM cells cannot divide. The DEX-treated cells in this study did not proliferate. Cortisol-treated human TM cells have been shown to undergo endoreplication, with little cell division, which led to many TM cells containing greater than a diploid content of DNA.²⁹ The current study and the work of Tripathi and colleagues²⁹ have shown that glucocorticoid treatment of human TM cells caused a significant increase in TM cell size. This enlargement of TM cell size could have important consequences on the outflow facility function leading to decreased transcellular flow and to decreased porosity. Alvarado and colleagues²⁸ have shown that DEX exposure of human TM cells grown on filters leads to a significant reduction in the hydraulic conductivity (ie, increased resistance to fluid flow). DEX treatment of isolated perfusion cultured human eyes has also been shown to lead to the development of progressive increased intraocular pressure.¹⁶ (4) Recent evidence indicates that the TM contains contractile elements^{72,73} and that TM contractility may help regulate aqueous humor outflow.⁷⁴ We have shown that smooth muscle α -actin is one of the actin isoforms present in cultured human TM cells and that smooth muscle α -actin is involved in glucocorticoid-induced CLAN formation. The glucocorticoid-induced reorganization of actin (especially smooth muscle α -actin) may inhibit the normal contractility of the TM and lead to an inhibition of aqueous humor outflow. (5) The glucocorticoid-induced reorganization of microfilaments could dramatically alter the tensile integrity of TM cells. Briefly, cel-

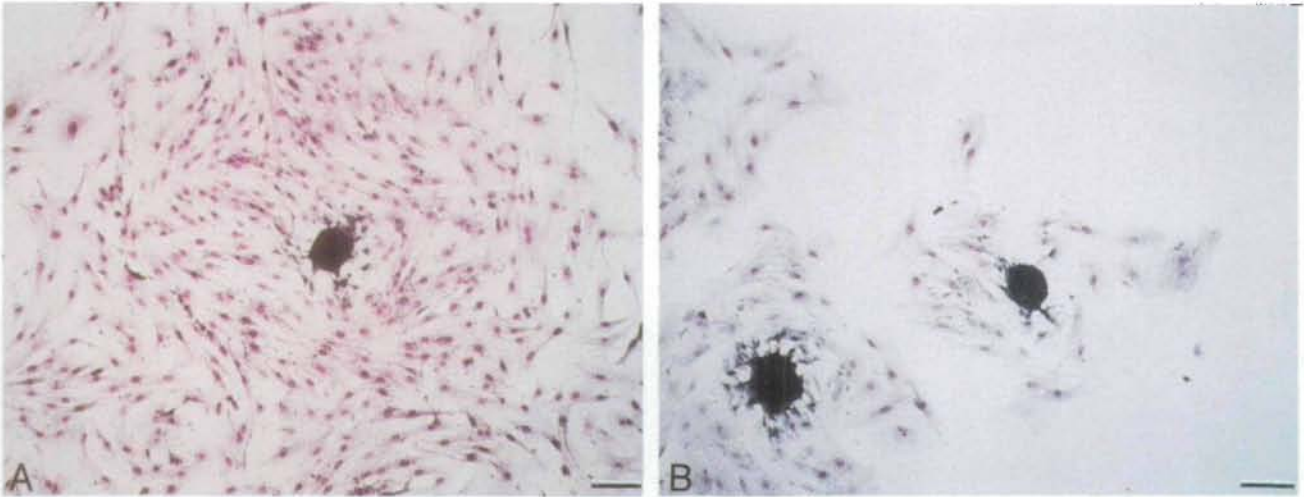


FIGURE 8. Effect of dexamethasone treatment on trabecular meshwork cell migration. Micro-carrier Cytodex beads coated with trabecular meshwork cells were transferred to new culture dishes and grown in the absence (A) or presence (B) of 10^{-7} M DEX for 10 days. The cells were fixed and stained, and the area of migration determined by image analysis. Bar = $100 \mu\text{m}$

lular tensile integrity refers to the global connectivity of cytoskeletal elements, integrins, and extracellular matrix.⁷⁵ Tensile integrity can account for the cytomechanical stress on cells, which is a dynamic interplay between the forces involved in cell spreading (microfilaments and adherens junctions) and the forces respon-

sible for cell rounding (microtubule compression). Cellular tensile integrity can determine cell shape, the mechanical responsiveness of cells, and nuclear response to cell shape change as well as regulate cytoskeletal assembly and signal transduction.^{75,76} The major reorganization of the TM cytoskeleton caused by glu-

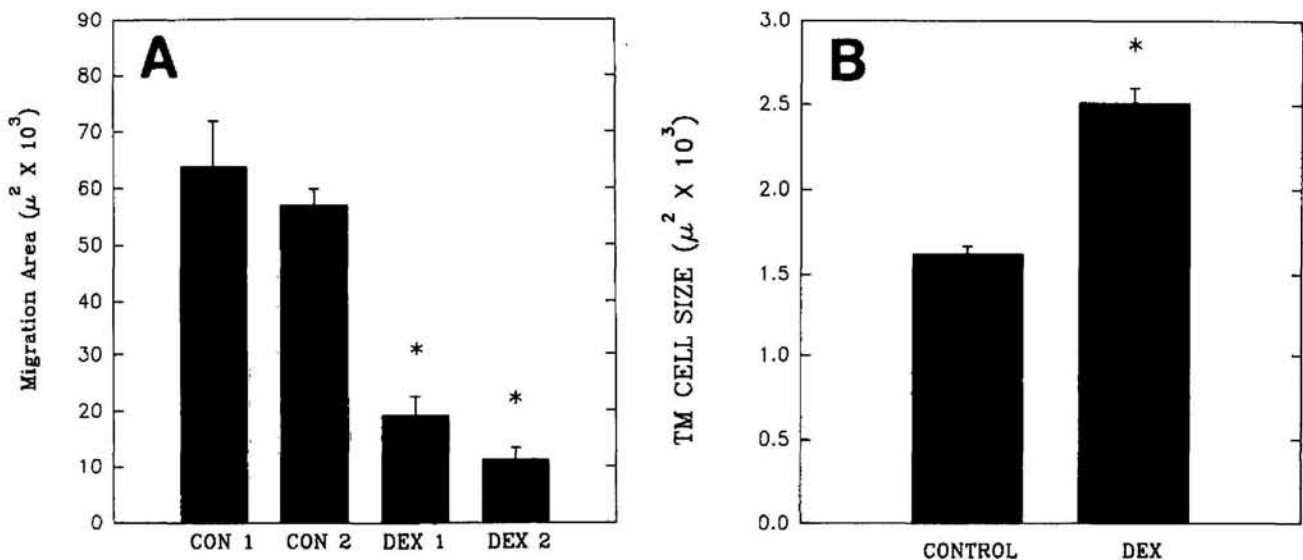


FIGURE 9. Effect of dexamethasone treatment on trabecular meshwork cell migration and cell size. (A) The average migration area \pm standard error for 6 to 10 beads per well was determined for trabecular meshwork cells grown in the absence (CON = control) or presence of 10^{-7} M dexamethasone (DEX) for 10 days (see Fig. 8). Each bar represents experimental results from individual wells. DEX treatment significantly ($P < 0.005$) inhibited trabecular meshwork cell migration by 70 to 80%. (B) Confluent trabecular meshwork cells were grown in the presence or absence of 10^{-7} M DEX for 14 days and the average cell size \pm standard error was determined by image analysis of >400 individual cells for each group. DEX treatment significantly ($P < 0.001$) increased trabecular meshwork cell size by an average of 55%.

glucocorticoid treatment may therefore alter many of the cell functions controlled by tensile integrity. It is also possible that the DEX-induced changes in the TM cytoskeleton could be synergistically mediated by any combination of these effects.

There is evidence that manipulation of the TM cytoskeleton can alter intraocular pressure. A variety of agents that perturb the cytoskeleton of the TM cells have been shown to affect intraocular pressure. Intracamerally injected cytochalasin B into monkey eyes caused disassembly of TM microfilaments and a concomitant increase in the aqueous humor outflow facility (a decrease in intraocular pressure) because of changes in TM cell shape.^{31,32} Pretreatment with the microfilament stabilizing agent phalloidin inhibited this cytochalasin increase in outflow facility.³³ Cultured TM cells incubated with cytochalasin B undergo significant cell shape changes,³⁰ and the treatment of human TM cells grown on filters with cytochalasin has been shown to increase hydraulic conductivity.³⁴ Ethacrynic acid has also been shown to quickly (within minutes) increase the outflow facilities of monkey and enucleated calf eyes when injected intracamerally³⁵ and has been reported to cause dramatic changes in microtubule structure and cell shape in cultured cells.³⁶ In contrast, glucocorticoids cause a slowly progressive increase in intraocular pressure after topical ocular administration in humans,^{2,6} rabbits,^{8,9} cats,¹⁰ and monkeys.¹¹ This ocular hypertension often takes several weeks to develop and is generally, although not always, reversible on discontinuation of therapy.^{4,6,13} The *in vitro* effects of glucocorticoids on the cytoskeleton of cultured TM cells closely matches the time course for both development and reversibility of glucocorticoid-induced ocular hypertension.

Glucocorticoid treatment of cultured human TM cells does not uniformly lead to a cytoskeletal reorganization in all of the cells. Cell line TM6 has all the characteristics of a cultured TM cell, but does not respond to DEX-treatment. There are differing degrees of responsiveness in all of the other TM cell lines tested. Cell line TM1 was slowly responsive to DEX treatment with only 25% of the cells developing CLANs by 14 days of treatment. In cell lines TM2 and TM10B, approximately 60% of the cells developed DEX-induced CLANs. DEX treatment did not cause CLAN formation in a number of other ocular cultured cells that are distinct from TM cells. There are several possible explanations for this heterogeneity in DEX responsiveness. (1) The cells of the TM are themselves heterogeneous. It is quite possible that uveal meshwork cells, corneoscleral meshwork cells, juxtacanalicular cells, and Schlemm's canal inner wall cells are different and therefore respond differently to glucocorticoids. Each of our TM cell lines may have different subpopulations of these TM cells and only one of these subpopulations

may respond to DEX-induced cytoskeletal changes. (2) Every TM cell in culture may not contain receptors for glucocorticoids; therefore only those cells with receptors would develop DEX-induced CLANs. (3) The glucocorticoid-induction of CLANs in cultured TM cells appears to be dependent on the glucocorticoid receptor. This response is: dose-dependent with an EC_{50} that matches the affinity of DEX for the receptor; partially blocked by glucocorticoid antagonists; and specific for glucocorticoids showing higher activity for the more potent glucocorticoids. Binding of the glucocorticoid to the glucocorticoid receptor generates an active receptor that acts as a ligand-responsive transcriptional factor that regulates gene expression.⁷⁷⁻⁷⁹ Most physiological and pharmacologic responses to glucocorticoids are mediated by regulation of gene expression. The intracellular glucocorticoid-activated receptor complex binds to specific regions on the DNA known as glucocorticoid response elements. Although almost all cells in the body contain glucocorticoid receptors and the same complement of DNA, glucocorticoid-induced gene expression is cell specific. The reason for this specificity is that each cell type has a unique combination of glucocorticoid response elements accessible to the glucocorticoid receptor complex. In other words, there is a cell-specific masking of many of the glucocorticoid response elements. It is possible that only a subset of TM cells contain an accessible glucocorticoid response element(s) that regulates the genes required for DEX-induced CLAN formation and therefore only a portion of the cultured TM cells would respond. This same mechanism would account for the inability of glucocorticoids to cause cytoskeletal changes in the other cell types tested. Support for the lack of CLANs in other cell types also comes from studies that have shown TM cells to have cytoskeletal elements distinct from those of surrounding cells.^{55,72,73} Whether glucocorticoids directly or indirectly regulate specific cytoskeletal components responsible for CLAN formation is not clear. (4) There is heterogeneity in ocular hypertension responsiveness to glucocorticoid administration. Not all persons treated with glucocorticoids develop ocular hypertension, and there are distinct degrees of responsiveness.^{1,4-6} It may therefore be anticipated that there is considerable variation in DEX-induced changes in cultured TM cytoskeletal elements based on this heterogeneous *in vivo* responsiveness.

There is a population of patients that, when treated for prolonged periods with glucocorticoids, develops a disease that is clinically very similar to primary open angle glaucoma.^{2,3,6,13} In approximately 5% of the normal population significant elevations in intraocular pressure due to steroid treatment will develop, a third of the population are classified as moderate responders, and the rest are nonresponders.⁴⁻⁶ The ste-

roid "high responders" have a much higher risk of open angle glaucoma eventually developing.^{80,81} Elevated levels of serum^{82,83} and aqueous humor⁸² cortisol as well as abnormal ocular cortisol metabolism⁸⁴ have been reported in glaucoma patients. Because glucocorticoids have a variety of diverse biologic and pharmacologic effects, it is difficult to know if they are directly or indirectly involved in the generation of ocular hypertension. We have recently shown that isolated, perfused human organ cultured eyes treated with DEX develop a time-dependent elevation in intraocular pressure.¹⁶ Furthermore, these DEX-induced ocular hypertensive eyes had glaucoma-like morphologic changes in their TM.¹⁶ It therefore seems likely that glucocorticoids have a direct effect on the TM, and that this can directly lead to ocular hypertension. Previously, a defect in the microfilaments of TM cells obtained from patients with glaucoma was reported.⁸⁵ The current study has shown that glucocorticoids can dramatically affect the organization of the TM cell cytoskeleton. Accordingly, the steroid-induced reorganization in TM cell cytoskeleton may have important implications in the pathogenesis of glaucoma.

Our results suggest that glucocorticoids play a direct role in the pathogenesis of corticosteroid glaucoma. The dramatic glucocorticoid-induced reorganization of the cytoskeleton affects TM cell size, cell function, and may be involved in the alteration in composition of the TM extracellular matrix. This model system should prove useful for the molecular dissection of this unusual microfilament structure and may aid in the understanding of the pathogenic mechanisms involved in glucocorticoid-induced glaucoma and primary open angle glaucoma.

Key Words

trabecular meshwork, glucocorticoids, cytoskeleton, actin, glaucoma

Acknowledgments

The authors thank Cheryl Collins for help in preparing this manuscript and J. Chloe Bulinski and George Bloom for their encouragement and advice. The human ocular cell lines were obtained from human donor eyes acquired from the Central Florida Lion's Eye Bank (Tampa), the Medical Eye Bank of Western Pennsylvania (Pittsburgh) and the Rochester Eye and Human Parts Bank (Rochester, NY).

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