Proteins Secreted by Human Trabecular Cells

Glucocorticoid and Other Effects

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The capacity of cultured human trabecular meshwork (HTM) cells to secrete an extracellular matrix was studied by indirect immunofluorescence. Synthesis of nine extracellular matrix (ECM) proteins known to be present in the normal trabecular meshwork was assessed in three HTM cell lines. Fourteen primary antibodies were used and cultures were labeled two and four weeks after confluence. The HTM cell lines showed consistent labelling patterns for the normal extracellular connective tissue constituents including collagens (types I, III, IV, V and VI), glycoproteins (laminin and fibronectin) and a basement membrane-associated proteoglycan. These antigens were localized to the basal cell surface in an extracellular reticular pattern corresponding to cell margins. Dextran addition at confluence helped to intensify the staining of these components, but ascorbate had no apparent effect. Interestingly, elastin, another normal component of the trabecular meshwork, was not identified under standard conditions, or after addition of ascorbate or dextran. However, elastin could be detected intracellularly following dexamethasone treatment for three days, and extracellularly in punctate deposits when this treatment was used for 1 or 2 weeks. Our findings indicate that HTM cells may be responsible for the secretion and maintenance of all the major ECM constituents of the trabecular meshwork. The elastin results suggest a possible mechanism contributing to obstruction of outflow in steroid glaucoma if increased amounts of elastin are also produced in vivo. This approach can also serve as a useful baseline for comparison with HTM cell lines treated with glaucoma medications or obtained from patients with glaucoma. Invest Ophthalmol Vis Sci 30:2012–2022, 1989

The connective tissue of the trabecular framework is an important determinant of its mechanical properties. To function properly, the trabecular meshwork must be sufficiently compliant to alter its configuration with changes in intraocular pressure, accommodation or other forces. These alterations in turn modify other structures, such as the aqueous channels which may open or close to control aqueous outflow. The connective tissue must also be resilient enough to return promptly to its original status at baseline conditions. We have recently found that, concomitant with these roles, the connective tissues of the meshwork appear both complex and highly organized, containing a large number of collagen types as well as glycoproteins/proteoglycans. These constituents are distributed in an orderly fashion within defined subregions of the uveoscleral cords and corneoscleral sheets.

There are some similarities between the supporting matrix of the meshwork and the matrices of other organs and tissues such as lung, uterus and blood vessels. These other organs, like the trabecular meshwork, must have a compliant connective tissue framework in order to respond to changes in pressure and other forces. A common finding in all these tissues is that they possess both collagen types I and III and that these two collagen types are found closely associated with each other. In the lung, loss of compliance and resilience has been related to specific connective tissue changes. In the trabecular meshwork, sclerosis is also a prominent alteration which was noted in the earliest pathologic studies of glaucoma. The relevance of sclerosis to the trabecular dysfunction in primary open angle glaucoma is not so readily apparent. Moreover, such changes may be preceded by disorders affecting the trabecular cells. Thus, at the present time, the relationship between cellular and connective tissue alterations remains unclear.

The synthesis and maintenance of many connective tissues are carried out by fibroblasts which are found throughout the stroma. However, the cords

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and sheets of the trabecular meshwork have no fibroblasts within their stroma, and only lining trabecular cells can be found. Since it was important to know whether trabecular cells have the capacity to secrete the variety of extracellular proteins present in the meshwork, we examined the synthetic capabilities of our human trabecular meshwork (HTM) cell cultures. Here we report the results of an immunofluorescence study employing a battery of fourteen antibodies to analyze the synthesis by these cells of nine extracellular proteins known to exist in the trabecular meshwork. A preliminary report has been published elsewhere. The cultured trabecular cells appear to be capable to secrete the major constituents of the extracellular matrix (ECM) of the trabecular meshwork. This finding is discussed in view of the known cellular and connective tissue alterations of the trabecular meshwork in nonglaucomatous normals and in glaucoma.

Materials and Methods

Cell Culture

Three different cultured HTM cell lines from 14-, 30-, and 57-year-old nonglaucomatous donor eyes were used. The 14- and 30-year-old lines were previously characterized as showing the features of HTM cells in situ. As in the earlier studies, the line from the 57-year-old donor eye was examined by electron microscopy to verify its distinctive HTM cell ultrastructure. Informed consent was obtained from patients or relatives according to standard procedures. Approval from the University of California, San Francisco Committee on Human Research was also obtained. Culture media and procedures followed to grow the HTM cells were similar to those previously described. Briefly, third or fourth passage HTM cells from frozen stocks were defrosted at room temperature, grown in 10 cm culture dishes and split at a ratio of 1:20. The tissue was then transferred directly into 35 mm dishes at a ratio of 1:10 onto 35 mm dishes or were plated directly into 35 mm dishes at a ratio of 1:20. The tissue culture media consisted of Dulbecco’s modified Eagle’s medium (DME) supplemented with 15% fetal bovine serum, 2 mM glutamine, 50 µg/ml gentamycin, and 2.5 µg/ml fungizone. No growth factors were added. Media was changed every 2 days. When cells reached confluence the serum concentration was reduced to 10%. All of the HTM cultures were assayed for extracellular matrix (ECM) synthesis by immunofluorescence methods after they had formed stable monolayers at both 2 or 4 weeks after confluence.

Indirect Immunofluorescence

The antibodies primarily employed for these studies were obtained from David A. Newsome, MD (New Orleans, LA). Preparation techniques were as previously described. Monospecific, polyclonal antibodies against human collagen types I, III and IV were elicited in sheep and those against human collagen types V and VI, fibronectin (FN), and elastin (EL) were prepared in rabbits. Mouse antigens were used to elicit antibodies against laminin (LN) in sheep and against basement membrane proteoglycan (BMPG) in rabbits.

Rabbit anti-sheep or goat anti-rabbit antibodies conjugated to FITC were used as the secondary antibodies (Cooper Biomedical, Inc., Malvern, PA). As controls, anti-human IgG antibodies made in rabbit or goat were used to block nonspecific binding of the secondary antibodies. Other controls included incubation with sheep or rabbit serum, or incubation in PBS alone rather than with primary antibody, or in PBS rather than secondary antibody.

Fluids were removed from the culture dishes by suction and the cells were fixed for 15 min in 4% formalin in PBS, (0.02 M phosphate buffer, pH 7.4 with 0.15 M NaCl), followed by rinsing in PBS three times over 20 min. Thirty microliters of primary antibody diluted in PBS were applied to a designated portion of the culture dish, and incubated for 1 hr at room temperature in a moistened environment. Cells were then rinsed with PBS four times over 1 hr and treated in the dark for 1 hr with 30 µl of FITC-conjugated secondary antibody, diluted 1:100 in PBS and centrifuged at 12,000 RPM for 5 min prior to use. Following four PBS rinses over 1 hr in the dark, dishes were stored in a moistened, dark environment until examined with the fluorescence microscope.

A coverslip was applied to each dish with a drop of anti-oxidant medium (2% N-propyl-gallate and 90% glycerol in Alcon balanced salt solution, pH 8.0) to preserve fluorescence. The dishes were viewed in a Zeiss photomicroscope under blue light epi-illumination (Zeiss filters, blue glass BG12, FT50 chromatic splitter, and barrier filter 50). Photographs were taken with AGFA 1000 film according to the following manually timed exposures: ×40 microscope magnification, 1 min; ×100, 30 sec; ×160, 15 sec; ×250, 10 sec. Timing of exposures allowed comparison of relative fluorescence between cultures treated under various conditions. Photography was done either the day of antibody treatment or the next day and there was no observable difference between the cultures photographed at those times.

Effect of Antibody Source on ECM Immunofluorescence

A variety of antibody types other than the monospecific polyclonal antibodies described above is becoming available. We made a particular effort to

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compare the nine polyclonal antibodies listed above to five other monoclonal and polyclonal antibodies acquired from various sources. These antibodies included goat anti-mouse laminin, (kindly provided by Hynda Kleinman, Bethesda, MD), rabbit anti-mouse laminin, (Collaborative Research, Inc., Bedford, MA) and a monoclonal mouse anti-human laminin (developed by Eva Engvall, La Jolla, CA and obtained from the Developmental Studies Hybridoma Bank, Baltimore, MD). A monoclonal antibody to human type VI collagen was also obtained by Dr. Newsome (from Dr. E. P. Kay, Dallas, TX). A further supply of anti-mouse BMPG prepared in rabbits was the generous gift of John R. Hassell (Bethesda, MD). Appropriate FITC-conjugated secondary antibodies, blocking steps and serum controls were used for this additional series of antibodies. Altogether 14 different antibodies raised against nine types of ECM proteins were used as primary antibodies in the labeling experiments.

**Stimulation of ECM Production by Various Agents**

Preliminary results indicated to us that synthesis of the ECM proteins could be enhanced to yield a greater production of the antigens studied. Further, in the case of elastin, a glycoprotein we had localized in the meshwork interstitium, but which appeared to be undetectable in vitro, we decided to determine whether its synthesis could be stimulated by various agents. Thus the cultured cells were exposed to three different substances known to affect the synthesis of an extracellular matrix (ECM) and the results were compared to those obtained in the absence of such stimulation.

Dextran was used because it is thought to enhance the secretion of most, if not all, of the ECM proteins produced by such cells as cultured bovine corneal endothelium. In two separate experiments, we evaluated the effects of dextran addition at two different stages. In the first experiment, cultures about to become confluent received 4% dextran every other day for 1 week. Following this treatment, dextran was no longer used and the ECM production was evaluated either 1 or 3 weeks later. In the second experiment, the HTM cells did not receive dextran treatment until they had been confluent for 3 weeks. Dextran was given for 1 week and the labeling experiments were carried out 1 week later. Control dishes received new media (without dextran) every other day.

Ascorbate has been reported to increase collagen production in fibroblasts and corneal endothelium. To evaluate the effect of this substance on our cultured human trabecular cells, we conducted short and longer term experiments. To one set of dishes we added 50 μg/ml of ascorbate just before confluence every other day with each media change for 1 week. Other sets received either 25 or 10 μg/ml of ascorbate every other day for 2 weeks.

Dexamethasone has been shown to have an enhancing effect on elastin synthesis in other cultured cells, and we have previously reported protein induction following dexamethasone treatment. In one set of experiments, the HTM cells received 10⁻⁷ M dexamethasone with each media change for 3 consecutive days just prior to the immunofluorescent labeling. In another set of experiments, 5 × 10⁻⁷ M dexamethasone was added every other day with each media change for 1 or 2 weeks.

**ECM Pattern following Increased Exposure of Antigenic Sites**

Other experiments were carried out to assess the effect of increasing the exposure of the ECM to the antibodies. In one set of such tests, we treated the cultured cells with saponin to make the monolayer of trabecular cells more permeable and thereby promote the exposure of our antibodies to additional antigenic sites, intracellular as well as extracellular. Here all PBS rinses were replaced with PBS-saponin rinses, and antibodies were also diluted with PBS-saponin. In a second study, we widened the intercellular space by treating the confluent cultures with 10⁻⁵ M cytochalasin B for at least 60 min. The cells were observed with the phase contrast microscope to determine that they had become rounded up and that the intercellular space had expanded prior to fixation and antibody treatment. Finally, we removed the cell monolayer with 0.05% Triton X-100, to examine the underlying ECM.

**Results**

At 2 and 4 weeks postconfluence, HTM cell cultures formed a typical tightly packed monolayer of wide, flat cells which had extensive branching processes. Ultrastructural characteristics in all three lines were as previously reported. All controls were negative and no discernible differences in staining pattern were found when the blocking step was eliminated.

**Effect of Antibody Source on ECM Immunofluorescence**

An important factor in these immunofluorescent studies is the specificity of the antibodies used. While ours are “monospecific” antibodies, we thought it important to compare them with other polyclonal...
antibodies as well as with certain monoclonal antibodies which became available to us. A series of preliminary experiments were conducted to determine the antibody dilution which would yield satisfactory results. We found that the nine monospecific polyclonal antibodies could be used at a dilution of 1:20.

When the presence of laminin in the ECM of cultured HTM cells was assayed using four different anti-LN antibodies, we found that the most intense labeling was obtained with the polyclonal sheep anti-human antibody, where there was a marked reaction at a 1:20 dilution. The two other polyclonal antibodies also were effective but only at 1:10 dilutions. The monoclonal antibody was positive when used at 1:10 or higher concentrations, although the fluorescence intensity was less than for the polyclonal antibodies used at 1:20 dilution. Thus the cultured HTM cells tested positively for laminin production regardless of antibody source.

Similar studies were performed for type VI collagen, for which we had available a polyclonal and monoclonal antibody. Both antibodies gave similar results, diluted to 1:5. Thus, we conclude that in the case of laminin and collagen type VI, the monospecific antibodies used in these studies appear to provide very similar results to those obtained with monoclonal and other polyclonal antibodies.

Production of Extracellular Matrix Proteins In Vitro

As in the study of the extracellular matrix in situ, we have divided the assortment of nine types of antibodies into two groups. The first group contains five antibodies which bind to antigens usually present in basement membranes and which are also found in the subendothelial region of the trabecular meshwork beams. The second group contains four types which bind constituents usually present in the stroma or substantia propria of tissue and which we have localized in the core or central portion of the trabecular beams.

Basement membrane-associated antigens: The five types of basement membrane-associated antigens are laminin, fibronectin, basement membrane proteoglycan and collagen types IV and V. All five of these antigens were also present in the ECM of cultured HTM cells, as shown in Figure 1. We found certain reproducible differences among the various antigens when all of the antibodies were used at a 1:20 dilution. The most intense staining occurred for the glycoproteins LN and FN (Fig. 1A-D), an intermediate intensity for BMPG and collagen type IV (Fig. 1E-H), and the weakest for type V collagen (Fig. 1I-J). This variability was related to the intensity of the labeling reaction as well as to the extent of deposition of the antigen, that is, the area covered by the fluorescent material.

The pattern of fluorescence was similar for all the proteins in this group, in that irregular deposits of extracellular fluorescent material were found along the cell margins. These deposits appeared as uneven strands which formed a reticular network along the surface of the culture dish (or the basal surface of the cells). The thickness of each strand varied considerably along its length, giving a rather irregular appearance to the network. The fluorescence most often corresponded to zones of intercellular contact, although not every cell was completely outlined with reactive material, so that a group of two or more cells sometimes was circumscribed. Very occasionally, fine, labeled strands occurred just inside the cell margins, but no label was visible under nuclei. At multiple points of contact between cells, there was often an additional accumulation of ECM where several labeled strands intersected. This pattern was especially apparent for the glycoproteins LN and FN. The deposition of all of the labeled ECM was on the surface of the culture dish, which corresponds to the basal or subendothelial surface of the HTM cells in situ. The cultured HTM cells, then, maintain their polarity under in vitro conditions.

Interstitial collagen types and elastin: The four antigens included among the interstitial proteins are collagen types I, III, VI and elastin. Type VI collagen, a recently identified interstitial antigen, is localized within the beam cores in situ when either the polyclonal or monoclonal antibodies available to us was used (unpublished observations). Under standard conditions in vitro the HTM cells were labeled with strong extracellular fluorescence for three of the four interstitial proteins, as shown in Figure 2. These are collagen types I, III and VI. The pattern of deposition differed somewhat from that of the basement membrane associated antigens, in that the collagen networks appeared to be composed of smoother and more regular filaments. Type I collagen (Fig. 2A, B) was present as smooth, curved strands of uniform diameter extending between the cells; type III had a similar pattern as well as some fine short fibers intersected along the strands (Fig. 2C, D). Type VI collagen (Fig. 2E, F) was distinct from types I and III in that it appeared as solid strands with diffuse thickened regions. These long threads often seemed to be unconnected to others so that there was an incomplete reticular pattern. As was the case for the basement membrane group of proteins, all of the ECM appeared to be on the basal surface, and no intracellular reactivity was detectable for these three antigens. Despite the synthesis of the three interstitial collagen types, no elastin could be detected in these
Fig. 1. Basement membrane-associated proteins produced by HTM cells in vitro. Fluorescent staining is in a reticular pattern corresponding to borders of the polygonal HTM cells. Two- or four-week post-confluent cultures, polyclonal antibodies. (a) and (b), Fibronectin; (c) and (d), Laminin; (e) and (f), Basement membrane proteoglycan; (g) and (h), Type IV collagen; (i) and (j), Type V collagen. Left-hand column (×240); right-hand column (×380).
Fig. 2. Interstitial proteins produced by HTM cells in vitro. Two- or four-week postconfluent cultures, polyclonal antibodies. (a) and (b), Type I collagen; (c) and (d), Type III collagen; (e) and (f), Type VI collagen; (g) and (h), Elastin, 2-week dexamethasone treatment. Punctate staining is at the basal surface. (i) Rabbit serum control (Rabbit Serum + FITC), (j) PBS control (PBS + FITC). Controls using primary antibody and no FITC were identical to (i) or (j) where; only weak cellular autofluorescence is visible. (a), (c), (e) and (g), ×240; (b), (d), (f), (h), (i) and (j), ×380.
HTM cell cultures. Thus, extracellular staining for eight of nine of the ECM antibodies used was observed, and this was true for both 2- and 4-week post-confluent cultures of HTM cells.

Effect of Various Agents on ECM Secretion

When dextran was added for a period of 1 week to cultures near confluence, we found that staining for all of these proteins was slightly intensified, compared to the experiment where dextran was added after the cells had been confluent for 3 weeks. The enhancement of ECM production with dextran is consistent with the results reported by Gospodarowicz, who began its use in vitro. However, its mechanism of action is apparently unknown. We found that dextran addition did not alter the types of proteins produced, since when it was not added to the HTM cultures, we still found that the same eight ECM constituents were labeled and formed similar patterns. As noted, elastin secretion was not detected when standard conditions were used, nor could we observe extracellular staining for elastin when the cultured cells were stimulated with dextran.

When ascorbate was used, either for 1 or 2 weeks as described in Methods, we found no qualitative or quantitative differences in the extracellular proteins secreted by the HTM cells, nor was there any notable intensification of the staining pattern. It is possible that longer treatment or different dosage of ascorbate could be effective in promoting collagen secretion, as reported for other cell types. or that HTM cells

did not respond to the stimulus. The fact that ascorbate also apparently failed to induce synthesis of elastin is consistent with results in other cell types, where elastin production is depressed by this treatment, or is unaffected.

When the cells were stimulated by the addition of 10^{-7} M dexamethasone for only 3 days, we found some elastin-labeled material intracellularly. When stimulation was carried out for 1 or 2 weeks at a slightly higher dose (5 \times 10^{-7} M), we found a punctate distribution containing granules of various sizes, either in the culture medium, loosely attached to the cells, or on the basal cell surface (Fig. 2G, H). Thus, dexamethasone treatment appeared to have a specific effect characterized by the induction of synthesis of elastin as well as deposition of this protein in a distinct manner. That is, all of the other ECM constituents are secreted onto the basal surface in a well-defined pattern such that a reticular network was formed at the cell borders. Elastin synthesis under glucocorticoid stimulation resulted in secretion of this protein into the extracellular medium, as well as onto the basal surface where the material appeared to be bound to the dish/cell surface. For elastin, we were able to detect an absolute difference (ie, presence vs. absence) following dexamethasone treatment. Examples of serum and PBS controls for experiments reported in this paper are given in Figure 21, J.

For the remaining antigens, which were detected regardless of dexamethasone, certain other effects were observed with this treatment. Among these differences was a reduction in the amount of staining for type I collagen in cultures given dexamethasone. Here the heavily stained, thick strands of ECM normally seen for type I collagen were replaced with smaller patches of stain composed of thinner threads. Collagen type III may also have been reduced, but only to a minor extent. Suppression of collagen biosynthesis by glucocorticoids has been reported for other cell types. We also detected a reduction in staining for FN. Others have observed that as cells become more tightly packed in the confluent monolayer, FN fluorescence is reduced as it becomes covered by cells. It is possible that a similar effect may have lead to the apparent alteration in FN staining described here.

In contrast to the observed reductions in collagen type I and FN, there was an apparent increase in staining intensity for collagen type VI. Cells treated with dexamethasone had a reticular network composed of thicker fibrils of type VI than did the untreated controls. For BMPG and LN, there was a much more diffuse network of fine fibers than in controls. In the case of LN these fibers were not only confined to the cell borders but extended under the nuclei as well. These alterations could represent either an increased antigen production, a change in its pattern of secretion or some other unspecified effect.

Collagen types IV and V were not visibly affected by glucocorticoid treatment. Whether any of these alterations (other than that seen for elastin) represent quantitative changes resulting from glucocorticosterone treatment or simply reflect configurational changes in cell shape or packing could not be ascertained in the present experiments.

ECM Pattern following Increased Exposure of Antigenic Sites

It is possible that the labeling patterns that we observed reflect limited accessibility to antibodies and not true antigenic distribution. As described in Methods, the cultured cells were permeabilized by saponin treatment. These studies showed that the HTM cells preserved their staining patterns regardless of exposure to saponin; neither intracellular nor a greater degree of basal labeling was discernible for the extracellular proteins. We also examined the possible
Fig. 3. Effect of cytochalasin B on antigenic sites in cultured HTM cells. Note increased fluorescence in treated cultures. Two-week postconfluent cultures, polyclonal antibodies. (a) Laminin, no cytochalasin B. (b) Laminin, cytochalasin B treatment. (c) Fibronectin, no cytochalasin B. (d) Fibronectin, cytochalasin B treatment. ×380.

effect on the labeling pattern produced by a visible widening of the intercellular space. To do this we exposed the cultured cells to cytochalasin B (CB) prior to fixation. This substance disrupts the cytoskeleton, so that cultured cells contract, disrupt cell junctions and become separated from their neighbors. In this situation, the basic staining pattern remained unaltered. However, the intensity as well as the area of the fluorescent label with CB treatment appeared to be greater than in untreated controls for the two antibodies tested, fibronectin and laminin (Fig. 3). Both of these experiments suggest that the reticular network pattern probably represents the chief deposition sites of the ECM constituents by the cultured HTM cells at the stages examined in these studies. This pattern appears to persist even when the cells are removed by Triton X-100. We observed that it is difficult to remove the cells without damage to the underlying ECM when the cells have been confluent for 2 to 4 weeks. However, the results with CB also point out that the access of the antibodies to the ECM constituents is somewhat limited, and patterns of staining may not reflect the true distribution of the ECM components. In any case, it appears that the early secretion of eight of the extracellular proteins is polarized, in that it occurs along the basal surfaces and preferably towards the intercellular spaces.

Discussion

Our cultured HTM cells secrete an array of extracellular matrix constituents which is almost identical to that identified in situ. Under these culture conditions, nine antigens which occur in situ in the subendothelial and interstitial portions of the meshwork beams are deposited extracellularly. One of these constituents, elastin, appeared to be secreted only when the cells were stimulated with dexamethasone. These results are indicative of maintenance of a differentiated state by these cultured cells. Not only do the HTM cells retain their in situ repertoire of synthesis, but we have also demonstrated that this pattern appears remarkably constant if cells are grown under appropriate conditions. A few other investigators have reported synthesis of some of these constituents by cell lines isolated from meshwork tissues, but ours is the first study to encompass a wide variety of antibodies, different cell lines, culture times and treatment with dexamethasone and other agents. The types and amounts of ECM produced in confluent cultures appear to be the same for three separate sources of cells and at 2 or 4 weeks after reaching confluence. It is interesting to note that Hernandez et al reported that preconfluent cells from the trabecular meshwork do not secrete laminin or type IV collagen but that these constituents appeared in the ECM after confluence. Our studies were only of postconfluent cells and the ECM appeared to be the same whether studied at 2 weeks, 4 weeks or 3 months postconfluence. Thus, the capacity for secretion of these components of the ECM does not seem to depend, within certain limits, upon donor age, nor does it require long incubation periods. The addition of dextran, ascorbate or other such stimulants to the
culture media was not required for production of eight out of nine constituents of the ECM, although addition of dextran at the confluent stage appeared to enhance the amount of labeled material. The ability to study ECM components of the HTM in vitro will help set the stage for future comparative studies involving cell lines from patients with glaucoma and cells exposed to various glaucoma medications and hormones.

Elastin is the only antigen we identified in meshwork tissues in situ which was apparently not manufactured (or was synthesized in such minute amounts that we could not detect its presence) by HTM cells in vitro cultured under our standard conditions. Production of this matrix component was not induced by ascorbate or dextran treatment. However, intracellular fluorescence was observed when the cells were stimulated by dexamethasone for only 3 days; and, the addition of dexamethasone for 1 or 2 weeks resulted in secretion of elastin into the extracellular medium. There has been evidence that glucocorticoids and other factors could influence the production of elastin in vitro by other cell types.26,27,40–43 Previously, Polansky et al have shown induction of protein synthesis by dexamethasone in cultured HTM cells in studies using biochemical methods.18,28 These proteins appear as discrete bands on SDS polyacrylamide gels, and one of these is in the same KD range as elastin. The present study shows that the HTM cells can be induced to synthesize and secrete elastin or an elastin-like protein that is released into the culture medium as well as onto the basal surface. The secretion of a protein such as elastin into the trabecular cell medium could suggest a possible mechanism for the obstruction of aqueous outflow in steroid glaucoma and perhaps even in POAG. If this important new observation is confirmed to occur in vivo, it may point towards unexplored avenues of investigation regarding glucocorticoid effects. Recently, elastin was shown to be present within the aqueous channels as well as in the interstitial tissues of the meshwork beams.44 It is also pertinent that our results indicate an effect of glucocorticoids on synthesis of other important ECM components such as type VI collagen and BMPG. While these proteins may be secreted in a greater quantities, others such as collagen type I may be reduced. These observations need to be confirmed by quantitative methods in both in vitro and in vivo experiments.

Overall, our finding that these nine antigens are produced in vitro by HTM cells strongly suggests that these cells are responsible for the secretion and maintenance of most, if not all, of the major connective tissue constituents of the trabecular framework in situ. Therefore, other cell types are not required for the production of the meshwork connective tissue components.

In view of the crucial role of HTM cells in ECM synthesis implied by our results, it is appropriate to recall our previous studies in which the relationship between cell loss and connective tissue alterations was investigated. We found that in the “core” of the trabecular beams and cords there is a transformation of the connective tissues to an abnormal basophilic material.45 Similar alterations had previously been noted by others.9–11 However, we were the first to establish a relationship between these connective tissue alterations and changes in the density of trabecular cells.12 There is a regional, gradient pattern in both the transformation or “accumulation” of the basophilic material and in alteration of the cell density. Thus, the inner meshwork has the highest content of abnormal material and the greatest loss of cells, whereas the outer portions have the lowest concentration of the basophilic material as well as the least loss of cells.12,45 In view of the present findings which show that the trabecular cells are potentially capable to synthesize the ECM of the trabecular beams and cords, it is not surprising that loss of such cells is accompanied by alterations of the associated connective tissues.

The temporal sequence of events was examined by comparing cell loss and accumulation of basophilic material in both nonglaucomatous normals and POAG specimens. Here we found that initially there is a considerable loss of the trabecular meshwork cells before there are measurable changes in basophilic material. It is only when the meshwork has lost a large number of cells that there is a notable accumulation of such material. Again, this sequence of events can be readily understood in view of the role of the trabecular cells in the synthesis and maintenance of the trabecular connective tissues. Cell loss and/or dysfunction may lead to accretion of the abnormal connective tissue as a phenomenon secondary to the cell dropout.

Alteration of the meshwork ECM would certainly be expected to affect compliance and resilience and thereby the ability to control intraocular pressure. Since we have shown that the beam cores contain collagen types I, III and VI as well as elastin,2 all of which are important in determining tissue compliance and resilience, it is possible that the altered ECM is a modification of one or more of these proteins. Indeed, elevation in the proportion of collagen type I compared with type III has been associated with increased tissue rigidity.7 In the one biochemical study of normal meshwork tissue, an age-related increase in total type I collagen was found, along with a modified peptide profile, in meshworks from individuals over...
the age of 40, indicative of age-related modifications in that collagen molecule.\textsuperscript{46} In contrast, no changes in the elastin content with age were reported, and no type III collagen was detected by these methods. There has been only a single biochemical study which compares the ECM in meshworks of nonglaucomatous normals and glaucomatous patients.\textsuperscript{47} These authors reported no differences in the trabecular meshwork content of collagen-specific amino acids; however, specific collagen types were not identified. More detailed antibody and/or biochemical studies are required to discover the precise nature of the connective tissue changes and to learn whether the same alterations occur in normal aging and in glaucoma. Now, our in situ and in vitro studies of the localization and production of a large battery of specific antigens in the normal trabecular meshwork should provide the requisite baseline information for investigation of such questions.

**Key words**: human trabecular meshwork, cell culture, extracellular matrix, immunofluorescence, dexamethasone

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