Loss of Cell–Matrix Cohesiveness After Phagocytosis by Trabecular Meshwork Cells

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Purpose. To investigate the response of trabecular meshwork cells to phagocytic events.

Methods. Cultured bovine trabecular meshwork cells were established and exposed to latex microspheres for 40 to 44 hours. After phagocytosis, the cohesiveness of cells to their underlying matrix was measured by the susceptibility to trypsin, as indicated by the time needed to be liberated from culture plates. The amounts of two cell attachment proteins, fibronectin and laminin, in both the phagocytically challenged and the control cultures were measured at various postphagocytosis time points with an enzyme-linked immunosorbent assay. The fibronectin and laminin network was visualized with immunostaining. The mRNA levels were analyzed by Northern blot. Zymography using gelatin-containing gels was also performed to examine the gelatinase activities.

Results. Compared with controls, cells in phagocytically challenged cultures were more sensitive to trypsin. At the 4- and 8-hour postphagocytosis time points, the trypsinization time needed to suspend cells from tissue culture plates was significantly shorter for phagocytically challenged cells. Also, at these two time points, reduced amounts of fibronectin and laminin, as well as disruption of the fibronectin-laminin network, were observed in the phagocytically challenged trabecular meshwork cultures. The mRNA level for fibronectin was reduced, and a slightly increased gelatinase activity was noted. The fibronectin and laminin levels returned to normal by 24 hours.


The trabecular meshwork has been suggested to function in vivo as a self-cleaning filter, contributing thereby to the regulation of aqueous humor outflow and control of the intraocular pressure.1 This specialized tissue is composed of sheets of trabecular beams made up of a variety of connective tissue elements.2–5 Lining the beams are trabecular meshwork cells thought to play essential roles in the maintenance of the normal outflow system.

Phagocytosis is one of the major activities of trabecular meshwork cells.6–17 These cells have been shown to phagocytose avidly materials or debris that have been accumulated under normal or pathologic conditions. Previous studies using animals, including monkeys,6,7 rabbits,8 and cats,9,10 have demonstrated that after engulfing foreign materials, trabecular meshwork cells would round up, detach from trabecular beams, and subsequently migrate. A similar sequence of events has also been observed in the outflow system in humans.11 The mechanism of such in vivo events, however, has yet to be clarified. We hypothesize that the trabecular meshwork cells lose their cohesiveness to the underlying beams after phagocytosis, leading to their detachment and eventual departure from the trabecular lamellae. Using tissue-cultured trabecular meshwork cells, we tested our hypothesis by examining the response of these cells to phagocytosis. The susceptibility of trabecular meshwork cells to
trypsin treatment after phagocytosis was determined as an indicator of their cohesiveness to the matrix underneath. In addition, the levels of two cell attachment proteins, fibronectin and laminin, in the cultures were measured to evaluate the effects of phagocytosis on these proteins and to correlate the findings with the trypsinization results.

MATERIALS AND METHODS

Cell Culture

Fresh bovine eyes were obtained from a local slaughterhouse. Trabecular tissues were excised from the eyes and cultured on Falcon Primaria flasks (Falcon Laboratories, Becton-Dickinson, Oxnard, CA), as previously described. The medium included Eagle's minimum essential medium (EMEM), 10% fetal bovine serum, 5% calf serum, essential and nonessential amino acids, and antibiotics (all from JRH Biosciences, Lenexa, KS). When the cells reached confluence, they were trypsinized and subcultured.

In preparation for trypsinization experiments and for enzyme-linked immunosorbent assay (ELISA), first- or second-passaged cells were plated in triplicates onto Corning six-well plates (Corning Laboratory, Corning, NY) at 100,000 cells/well. For immunostaining experiments, cells were plated onto four-well glass chamber slides at 5,000 cells/well. Five days after plating, when confluency was reached, the cells were exposed to media containing latex beads (300 μg/ml, 0.99 μm diameter; Polysciences, Warrington, PA) for 40 to 44 hours. Those cultures that did not receive latex beads served as controls. After the exposure, the cells were washed extensively with PBS and were incubated in serum-free media for varying periods of time (2, 4, 8, 24, 48, 72, or 96 hours). Enzyme-linked immunosorbent assay plates (Costar, Cambridge, MA) were incubated at 37°C for 2.5 hours with 50 μl of either the fibronectin or laminin standards (Collaborative Research, Bedford, MA) or the samples (media or the dialyzed cell solution in 1:1, 1:2, or 1:4 dilutions). At the end of the coating period, excess solution was removed, and the wells were washed two times with phosphate-buffered saline (PBS) containing Tween 20. One hundred microliters each of 3% BSA in PBS, rabbit anti-human fibronectin (1:2000; DAKO, Santa Barbara, CA) or rabbit anti-mouse laminin (1:500, Collaborative Research), goat anti-rabbit IgG (1:500; Accurate Chemical, Westbury, NY), and rabbit peroxidase anti-peroxidase (1:1000; DAKO) were then sequentially added to each well. Each incubation was at 37°C for 30 minutes, with extensive washings in between. Finally, 100 μl of the peroxidase substrate O-phenylenediamine-2HCl (Abbott Laboratories, North Chicago, IL) was added to each well. The color developed was measured in a Micro-ELISA reader (Biotek, Burlington, VT) at 490 nm.

The condition that we selected for the assay gave a linear standard curve with fibronectin and laminin between 0.75 and 12 ng. This curve was used to determine the fibronectin or laminin content in our samples. The fibronectin and laminin levels were normalized to the cell protein in each sample, and the two-tailed Student's t-test was used to analyze the statistical significance of the data. ELISA was repeated at least three times.

Western Blot Analysis

Fibronectin and laminin standards, as well as aliquots of the culture media equivalent to 5 μg of cell protein, were electrophoresed on 6% sodium dodecyl sulfate (SDS) gels under reducing conditions. The proteins were electroblotted overnight onto a nitrocellulose membrane (0.2 μm; Schleicher and Schuell, Keine, NH). After blocking with 5% nonfat dry milk (Carnation, Los Angeles, CA), the membrane was allowed to react with either rabbit anti-human fibronectin (1:10,000; Collaborative Research), rabbit anti-mouse laminin (1:10,000; Collaborative Research), or normal

ELISA

After phagocytosis, the cells were washed extensively (six times) with serum-free EMEM. After a further incubation for 4, 8, 24, 48, 72, and 96 hours in serum-free medium, the medium in each well of the six-well plates was collected, and the cells were harvested in 1.5 ml of 0.05N NaOH. Both fractions were analyzed for the fibronectin and laminin content. Protein content in the cell fraction was measured by Lowry's method using bovine serum albumin (BSA) as the standard.

The wells of the flat-bottom 96-well enzyme immunoassay plates (Costar, Cambridge, MA) were incubated at 37°C for 2.5 hours with 50 μl of either the fibronectin or laminin standards (Collaborative Research, Bedford, MA) or the samples (media or the dialyzed cell solution in 1:1, 1:2, or 1:4 dilutions). At the end of the coating period, excess solution was removed, and the wells were washed two times with phosphate-buffered saline (PBS) containing Tween 20. One hundred microliters each of 3% BSA in PBS, rabbit anti-human fibronectin (1:2000; DAKO, Santa Barbara, CA) or rabbit anti-mouse laminin (1:500, Collaborative Research), goat anti-rabbit IgG (1:500; Accurate Chemical, Westbury, NY), and rabbit peroxidase anti-peroxidase (1:1000; DAKO) were then sequentially added to each well. Each incubation was at 37°C for 30 minutes, with extensive washings in between. Finally, 100 μl of the peroxidase substrate O-phenylenediamine-2HCl (Abbott Laboratories, North Chicago, IL) was added to each well. The color developed was measured in a Micro-ELISA reader (Biotek, Burlington, VT) at 490 nm.

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rabbit IgG (1:10,000; Cappel, Durham, NC), followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000; Cappel). Immunoreactive bands were visualized with use of the luminol-based enhanced chemiluminescence system (Kirkgaard and Perry Laboratories, Gaithersburg, MD) and recorded on Reflection films (DuPont NEN, Boston, MA). Prestained molecular weight markers (Bio-Rad, Richmond, CA) were run in parallel.

Immunocytochemical Staining

At the 4- and 24-hour time points after phagocytosis, cells on chamber slides were fixed in paraformaldehyde–lysine–periodate fixative (pH 6.2),23 rinsed in PBS, incubated for 4 minutes in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% BSA and 0.2% Triton X-100, and rinsed again in PBS. After incubation for 45 minutes at room temperature in 10% heat-inactivated normal goat serum, the cells were allowed to react for 90 minutes with rabbit anti-human fibronectin (1:150; Collaborative Research) or rabbit antimouse laminin (1:50; Collaborative Research),24 and rinsed with PBS. Those serving as negative controls received normal rabbit IgG. The slides were then incubated with fluorescein-conjugated goat anti-rabbit IgG (1:20; Cappel) for 45 minutes in the dark, mounted in mounting fluid (Vector Laboratories, Burlingame, CA), and photographed.

Northern Blot Analysis

Total RNA was extracted from bovine trabecular meshwork cells in phagocytically challenged and control cultures by the acid guanidinium thiocyanate–phenol–chloroform method.25 Approximately 10 to 20 μg of RNA was collected from each culture flask. The isolated RNA samples were electrophoresed (5 μg per lane) on 1.5% formaldehyde-agarose gel and transferred to the positively charged nylon membrane (Amersham, Arlington Heights, IL).26 The membrane was exposed to ultraviolet light for 3 minutes to immobilize the RNA. 32P-labeled fibronectin (clone FN771,27 1.3 kb, from American Type Culture Collection, Rockville, MD) and β-actin (Clontech, Palo Alto, CA) cDNA probes were prepared using the oligo-labeling kit (Amersham). The nylon membrane was prehybridized and then hybridized with the 32P-labeled cDNA. After washing in 2× standard saline-citrate buffer, 0.1% SDS at room temperature, and 0.1× saline-citrate buffer, 0.1% SDS at 60°C, the membrane was allowed to dry, and autoradiography was performed using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) to detect the 32P-labeled cDNA–mRNA hybrids. The relative fibronectin to β-actin mRNA level in the samples was measured with a computerized densitometer (Bio-Rad).

Zymography

Zymography was performed as previously described28 in SDS-polyacrylamide gel electrophoresis with the use of gels containing gelatin to analyze the content of specific gelatinase. Briefly, 11% SDS-polyacrylamide gels were prepared, and 1.5 mg/ml of bovine skin gelatin (type A; Sigma) was copolymerized in the gel. Aliquots of the culture media that were equivalent to 5 μg of cell protein were loaded onto the gel without boiling and electrophoresed at 4°C at 70 V under non-reducing conditions. The gel was then shaken for 10 minutes in a 2.5% solution of Triton X-100 (Sigma) and for 15 minutes in 50 mM Tris buffer, pH 8.0, containing 0.15M NaCl, 1 mM CaCl2, 0.03% NaN3, and 0.1% Triton X-100 at room temperature to remove the SDS and incubated at 37°C in a reaction buffer (50 mM Tris, pH 7.5, 10 mM CaCl2, 0.03% NaN3) overnight to allow proteinase digestion of the substrate. After staining with Coomassie brilliant blue R-250 (Bio-Rad), the positions of enzymatic activities were identified as clear bands in the stained background. Molecular weight standards (Bio-Rad) were run in parallel.

Some of the described experiments were also performed using trabecular meshwork cells established from donor tissues obtained from the Illinois Eye Bank, Chicago. The donors, 5 and 35 years of age, did not have any ocular diseases. Methods used to secure human tissue were humane. Proper consent and institutional approval were obtained, and the procedure complied with the Declaration of Helsinki.

RESULTS

By phase-contrast microscopy, bovine trabecular meshwork cells, after incubation with latex beads for approximately 40 hours, retained their normal appearance. Almost all the cells were particle laden in the cytoplasm. The cells in the phagocytically challenged cultures and those in the controls were subjected to trypsinization in parallel experiments. It was found that the time needed for cells to begin to contract and shrink was significantly (P < 0.0082) shorter in phagocytically challenged cultures (Table 1) than in control cultures at both the 4- and 8-hour post-phagocytosis time points. Phase-contrast micrographs (Fig. 1) further demonstrated that at these time points, cells in phagocytically challenged cultures (Figs. 1A to 1D) were more sensitive to trypsin than those in controls (Figs. 1E to 1H). The phagocytically challenged cultures (Figs. 1B to 1D) had a greater number of cells rounding up and fewer cells remaining even after a shorter period of trypsin treatment than the controls (Figs. 1F to 1H). At the 0-, 2-, and 24-hour time points, no discernible difference was observed.
TABLE 1. Effects of Phagocytosis on the Susceptibility of Cultured Bovine Trabecular Meshwork Cells to Trypsin Treatment

<table>
<thead>
<tr>
<th>Postphagocytosis Time Points (hours)</th>
<th>Culture</th>
<th>Time Needed for Cells to Start Contraction and Shrink (seconds)</th>
<th>Time Needed for 95% of the Cells to Become Refractile (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Control</td>
<td>14.5 ± 2.1</td>
<td>149.5 ± 23.3</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>15.5 ± 0.7</td>
<td>165.5 ± 25.5</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>14.5 ± 0.7</td>
<td>165.5 ± 30.4</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>16.0 ± 1.4</td>
<td>164.5 ± 9.2</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>19.2 ± 3.3</td>
<td>257.0 ± 32.9</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>13.5 ± 2.1*</td>
<td>136.8 ± 47.9*</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>19.3 ± 4.2†</td>
<td>479.7 ± 189.0</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>14.0 ± 2.0†</td>
<td>356.3 ± 130.9</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>18.5 ± 0.7</td>
<td>361.5 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>18.5 ± 2.1</td>
<td>270.0 ± 39.6</td>
</tr>
</tbody>
</table>

Data are mean ± SD averaged from values obtained from three to six independent cultures. Experimental cultures indicate phagocytically challenged cultures. When exposed to trypsin, almost all the cells in a culture dish would round up, become refractile, and break away with time. There is, however, always a residual number (approximately 5%) of cells that remain spread and attached, seemingly resistant to trypsinization. The time needed to reach this stage is referred to as "the time needed for 95% of cells to become refractile."

* $P = 0.0078$ compared with control cultures; † $P = 0.014$ compared with control cultures; ‡ $P = 0.0081$ compared with control cultures.

The amounts of fibronectin and laminin in control and phagocytically challenged bovine trabecular meshwork cultures were below the detection limits at the 0-hour time point. The level of fibronectin increased with time (Fig. 2), whereas the amount of laminin produced (data not shown) leveled off after 24 hours. Both protein products were found mostly in the culture medium. The amount of fibronectin in phagocytically challenged cultures was significantly ($P < 0.028$) lower than that in control cultures (Table 2) 4 and 8 hours after phagocytosis. The fibronectin level returned to its normal value by 24 hours, and the normal level was maintained through the 48-, 72-, and 96-hour time points. A similar decrease at the
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FIGURE 2. Accumulation of fibronectin in control bovine trabecular meshwork cultures as a function of time after media change. The amount of fibronectin (ng/µg cell protein) associated with the cell layer and secreted to the media was determined by ELISA. The total was the sum of the cell layer and the media amounts. The mean values of fibronectin found in the cell layer were 0.54, 1.34, 1.06, 1.00, 1.30, and 0.94 ng/µg of cell protein, respectively, for the 4-, 8-, 24-, 48-, 72, and 96-hour time points. The corresponding values in the media fractions were 6.5, 18.4, 25.2, 259.1, 530.2, and 849.3 ng/µg of cell protein.

4- and 8-hour time points was also noted with laminin levels (Table 2).

By Western blot analyses, one major protein band immunoreactive to an anti-fibronectin antibody was observed at a molecular weight of approximately 250 kd (Fig. 3A). Using an antibody to laminin, two bands with molecular weights in the 220-kd range and one minor band in the 400-kd range, presumably representing laminin β1, γ1, and α1 chains, were found (Fig. 3B). Confirming the ELISA data, the amounts of fibronectin and laminin in the media of phagocytically challenged cultures were lower than those found in samples of control cultures (lanes 2 and 4). No difference was seen at the 24-hour time point.

Immunostaining with an antibody to fibronectin revealed an extensive fibronectin network in control cultures at both the 4-hour (Fig. 4A) and 24-hour (Fig. 4C) postphagocytosis time points. In phagocytically challenged cultures, the network was visually disrupted at the 4-hour time point (Fig. 4B), but it was recovered by 24 hours (Fig. 4D). A disruption of the laminin network was similarly demonstrated (photograph not shown).

The Western blot and immunostaining experiments were also performed with human trabecular meshwork cells. The results found with bovine trabecular meshwork cells were reproduced with the human cells, albeit to a lesser degree. After phagocytosis, less fibronectin and laminin were found in the phagocyt-
FIGURE 4. Immunostaining of bovine (A to D) and human (E,F) trabecular meshwork cells with anti-fibronectin 4 hours (A,B,E,F) and 24 hours (C,D) after phagocytosis. Cells were from either control (A,C,E) or phagocytically challenged (B,D,F) cultures. The fibronectin network seen in control cultures (A,E) was disrupted in phagocytically challenged cultures (B,F) at the 4-hour postphagocytosis time point. No difference in the fibronectin network was seen at the 24-hour time point (C,D). Bar = 100 μm.

FIGURE 5. Northern blot analysis of RNA isolated from control (lanes 1, 3, 5) and phagocytically challenged (lanes 2, 4, 6) bovine trabecular meshwork cultures 4 hours (lanes 1, 2), 8 hours (lanes 3, 4), and 24 hours (lanes 5, 6) after phagocytosis. The RNA (10 μg) was electrophoresed on a 1% agarose–formaldehyde gel, transferred to nylon membrane, and hybridized to either 32P-labeled FN711 (arrowhead) or β-actin (arrow) probe.

DISCUSSION

Phagocytosis is one of the most important activities of trabecular meshwork cells. Failure or inadequacy to phagocytose is thought, at least in part, to be the basis of pathologic conditions, such as exfoliation glaucoma and pigmentary glaucoma. The phagocytosis by trabecular meshwork cells has been shown to involve a complex sequence of cellular events, including engulfing of foreign materials, cell detachment, and migration.6–11 We examined the response of trabecular meshwork cells to phagocytosis using a well-defined in vitro system. This system, unlike that in vivo, eliminates the possible involvement and influence of inflammatory reactions and is suitable for the unraveling of the basic mechanisms of phagocytic events.

Our findings are in agreement with those of previous studies12–19 that, in culture, trabecular meshwork cells have the ability to avidly phagocytose. After phagocytic challenge, our results showed that the cells remained attached to culture plates. They were, however, more easily trypsinized and liberated than control cells, substantiating the hypothesis that the cohesiveness of cells to the underlying connective tissue was approximately 20% to 30% higher in phagocytically challenged cultures 4 and 8 hours after phagocytosis.

Northern blot analysis using a fibronectin probe detected an mRNA of approximately 7.8 kb in trabecular meshwork cultures (Fig. 5). The amount of fibronectin mRNA in phagocytically challenged cells, when normalized to the β-actin mRNA level, was approximately 30% to 50% lower than that in control cells for the 4- and 8-hour postphagocytosis time points. The mRNA level at the 24-hour time point also was lower in phagocytically challenged cultures. However, the protein level, as measured by ELISA (Table 2), had already rebounded by 24 hours. This apparent conflict may be related to the scenario that translational or secretory control may override transcription regulation or the half-life of the transcript.

The zymographic pattern was similar to that reported by Alexander et al.27 A major gelatinase band at a molecular weight of 66 kd, identified previously27 as gelatinase A, was seen (Fig. 6). Minor bands at higher molecular weights were also noted. Compared with that in controls, the activity of the 66-kd gela-
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The reduction in cohesiveness was most pronouncedly seen at the 4-hour post-phagocytosis time point, which subsided at the 8-hour time point and became undetectable at the 24-hour time point. Along with these findings, ELISA and Western blot analysis results revealed that the amounts of fibronectin and laminin in phagocytically challenged cultures were reduced at the 4- and 8-hour time points but recovered by 24 hours. Fibronectin and laminin are two large extracellular matrix glycoproteins that have diverse functions. In view of their major roles in fundamental biologic processes, such as cell adhesion and spreading, and the close correlation of the trypsinization and ELISA data, we surmise that the diminished levels of fibronectin and laminin may be a contributing factor to the loss of cell-matrix cohesiveness in phagocytically challenged trabecular meshwork cultures. The reduction in the fibronectin and laminin levels was only moderate (Table 2), but the disruption of the extracellular fiber network was striking (Fig. 4), suggesting that even a relatively subtle change of protein levels may lead to a major impact in matrix structure and perhaps cellular behavior as well.

Our results also imply that the in vivo postphagocytosis detachment and migration of trabecular meshwork cells from beams may be a consequence of the reduced cohesiveness related to suppressed levels of fibronectin and laminin and the disruption of their network. It should be noted that the in vitro data demonstrated merely the loosening of the cells, which, though necessary, may not be sufficient for the detachment and subsequent migration seen in vivo. Perhaps for the chain of events to occur, further triggering or influence of factors other than phagocytic challenges is required. Such factors may encompass physical forces generated by the aqueous flow, as well as chemical mediators, including cytokines and chemotactic molecules present in the microenvironment or produced by inflammatory cells. This conjecture is consistent with a previous finding that only limited trabecular cell migration was observed after phagocytic challenges in a perfusion organ culture system.

Shirato et al., in examining the kinetics of phagocytosis in trabecular meshwork cells, has shown that by 40 hours, a majority (85% to 90%) of the cells have been recruited for phagocytosis. We therefore followed a similar protocol, exposing trabecular meshwork cells with latex beads for 40 to 44 hours. After phagocytosis, the latex beads were removed. The effects of phagocytosis, including the loss of cohesiveness and the reduction in fibronectin and laminin, were found to be elicited rapidly, mostly at the 4- and 8-hour time points, and faded within 24 hours. This relatively short time course for the changes is in line with results reported by Sherwood et al., who demonstrated that after injections of zymosan particles into monkey and cat eyes, some trabecular meshwork cells may complete the entire sequence of phagocytic events within 1 hour. The time course found in the in vivo system is thus even shorter than that in the in vitro cultures. The difference may simply reflect the difference of the two systems. The in vivo microenvironment is conceivably more complicated than that in vitro. Influences from other cell types and inflammatory reactions might have helped to accelerate the events in the former system.

The normal turnover rate for fibronectin in chick embryo fibroblast cultures has been shown to be approximately 30 to 36 hours. In view of the slow turnover rate, our Northern blot data suggest that the expression or synthesis of fibronectin by trabecular meshwork cells may be reduced by phagocytic challenges. An increased gelatinase activity also was ob-
served. This change may be contributory to the diminishment of fibronectin in phagocytically challenged trabecular meshwork cultures. The increase in enzyme activity was in line with our previous results demonstrating elevated lysosomal enzyme activities in trabecular meshwork cultures after phagocytosis.  

It is now recognized that cell–matrix interactions are mediated through a group of cellular receptors, mainly of the integrin superfamily, which are transmembrane heterodimers composed of two subunits, $\alpha$ and $\beta$. At least 16 distinct $\alpha$ and eight $\beta$ subunits have been identified and the combination of one $\alpha$ and one $\beta$ chain determines the ligand specificity. In addition to their roles in cellular adhesion and spreading, the integrins are also known to be involved in migration through their association with the actin-cytoskeleton within the cell. Studies are under way to determine whether the downregulation of fibronectin and laminin by phagocytosis that we observed is related to an altered expression of integrin receptors, reorganization of cytoskeletal structures, or modified migratory activities of trabecular meshwork cells.

**Key Words**

phagocytosis, trabecular meshwork, cell culture, fibronectin, laminin

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**References**

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