Permissive Effect of Fibronectin on Collagen Gel Contraction Mediated by Bovine Trabecular Meshwork Cells

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Purpose. The effect of fibronectin on the contractility of trabecular meshwork (TM) cells was investigated.

Methods. The contractility of bovine TM cells was evaluated by culture of the cells in a collagen gel and measurement of the change in the diameter of the gel under various conditions. The formation of stress fibers and the localization of integrin α5 and β1 chains (which together form a fibronectin receptor) in bovine TM cells were investigated by laser confocal microscopy of cells stained with phalloidin and antibodies to the integrin subunits.

Results. The addition of fibronectin to collagen gels containing bovine TM cells induced marked gel contraction in a time- and concentration-dependent manner. Cytochalasin D (an inhibitor of microfilament formation) and the peptide GRGDS (Gly-Arg-Gly-Ser-Pro), a fibronectin receptor antagonist, each inhibited this effect of fibronectin, whereas nocodazole (an inhibitor of microtubule polymerization) and the control peptide GREGSP (Gly-Arg-Gly-Glu-Ser-Pro) did not. Furthermore, fibronectin induced the spreading of cells, the formation of actin stress fibers, and the expression of integrin α5 in the collagen gel–embedded TM cells.

Conclusions. Fibronectin promotes collagen gel contraction mediated by bovine TM cells. Moreover, the formation of actin stress fibers and upregulation of integrin α5 appear to contribute to this permissive effect of fibronectin. The interaction of fibronectin with TM cells may thus be a determinant of the contractility of TM tissue. (Invest Ophthamol Vis Sci. 2003;44:4331–4336) DOI:10.1167/iovs.03-0068

Intraocular pressure (IOP) is determined by the balance between the production and drainage of aqueous humor. In human eyes, the outflow of aqueous humor occurs predominantly through the trabecular meshwork (TM), which is located at the angle of the eye. In addition to TM cells, TM tissue consists of various extracellular matrix (ECM) proteins, including collagen (types I, III, IV, and V), proteoglycans, laminin, fibronectin, and elastin. Many studies have examined regulation of ECM protein metabolism in TM cells. For example, in porcine TM cells, the synthesis and secretion of fibronectin are stimulated by transforming growth factor (TGF)-β, and the levels of gelatinase B and of tissue inhibitor of matrix metalloproteinases (TIMP) are modulated by various growth factors and cytokines. Glucocorticoids also increase the expression of fibronectin in human TM cells. The abnormal deposition of ECM proteins in TM tissue may result in an increase in resistance to aqueous humor outflow and a consequent increase in IOP. The amount of fibronectin has thus been shown to be increased in the TM tissue of human glaucomatous eyes.

The flow of aqueous humor from the anterior chamber of the eye, through the TM tissue, to the episcleral vein occurs by a pressure-dependent filtration process that does not affect the fluid’s composition. Contraction of TM tissue is thought to increase the resistance to aqueous humor outflow. For example, agents that induce TM tissue contraction also increase IOP by reducing the rate of fluid outflow. We have studied TM tissue contractility in a model that allows evaluation of collagen gel contraction mediated by bovine TM cells in culture. Collagen gel contraction in such a system is thought to depend on the interaction between cells and ECM proteins. Fibronectin is thus required for collagen gel contraction mediated by corneal fibroblasts. Cells express various types of integrin molecules on their surfaces that are responsible for adhesion to ECM proteins. One of the principal receptors for fibronectin is composed of integrin α5 and β1 chains.

We have now investigated the effects of ECM proteins, especially those of fibronectin, on the contraction of TM cells. In these studies, we monitored the contraction of collagen gels mediated by bovine TM cells. We have shown that TGF-β1–induced collagen gel contraction mediated by bovine TM cells results in part from the formation of actin stress fibers. In the present study we investigated the effects of fibronectin on the formation of such stress fibers as well as on the localization of integrin α5β1 in TM cells, with the use of fluorescence-based laser confocal microscopy. Furthermore, we examined the effects of an inhibitor of actin polymerization and of an RGD peptide that blocks the binding of integrin α5β1 to fibronectin on collagen gel contraction mediated by bovine TM cells.

Methods

Materials

Eagle’s minimum essential medium (MEM), fetal bovine serum, and trypsin-EDTA (0.5% and 5.3 mM [tetrasodium salt], respectively) were obtained from Invitrogen-Gibco (Rockville, MD); acid-solubilized type I collagen from porcine tendon (_Cellmatrix type I-A; 3 mg/mL; native form) and type IV collagen from bovine anterior lens capsule (Cellmatrix type IV; 3 mg/mL; prepared by pepsin treatment), as well as reconstitution buffer (50 mM NaOH, 260 mM NaHCO3, 200 mM HEPES [pH 7.5]) were from Nitta Gelatin (Yao, Osaka, Japan). Chondroitin sulfates A (sodium salt; whale cartilage), chondroitin sulfate C (sodium salt; shark cartilage), dermatan sulfate (sodium salt; hog skin), heparan sulfate (sodium salt; bovine kidney), keratan sulfate (sodium salt; bovine cornea), and hyaluronic acid (sodium salt; hog skin) were from...
Seikagaku (Tokyo, Japan). Dulbecco's phosphate-buffered saline (PBS) was from Nissui Pharmaceutical (Tokyo, Japan), bovine serum albumin (BSA; fraction V) was from Nacalai Tesque (Kyoto, Japan), fibronectin (human plasma) was from Roche Molecular Biochemicals (Mannheim, Germany), and laminin (1 mg/mL; purified from Engelbreth-Holm-Swarm [EHS] mouse tumor basement membrane, classic laminin) was from Upstate Biotechnology (Lake Placid, NY). Rabbit antiserum to integrin α5 and mouse monoclonal antibodies to integrin β1 were from Chemicon (Temecula, CA), Cytochalasin D, nocodazole, paraformaldehyde, Triton X-100, and Tween 20 were from Sigma-Aldrich (St. Louis, MO). The peptides GRGDSP and GRGESP were from American Peptide (Sunnyvale, CA). Cell culture clusters (24-well) and 162-cm² cell culture flasks were from Corning (Corning, NY), and glass-bottomed dishes were from Matsunami Glass (Kishiwada, Osaka, Japan). Fluorescein-labeled phalloidin (Alexa Fluor 568) and goat antibodies to mouse or rabbit IgG (Alexa Fluor 488) were from Molecular Probes (Eugene, OR). Normal mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA), and normal rabbit serum was from Cappel (Durham, NC).

**Cell Culture**

Fresh bovine eyes were obtained from a local abattoir. Bovine TM cells were prepared as described[3,14] and cultured under 5% CO₂ in plastic culture flasks containing MEM supplemented with 10% fetal bovine serum. Experiments were performed with cells subjected to five passages.

**Collagen Gel Contraction Assay**

Cell culture clusters (24-well) were coated with 1 mL of 1% BSA per well at 37°C for 1 hour. Cultured bovine TM cells were collected after exposure to trypsin-EDTA for 5 minutes, washed twice with unsupplemented MEM, and resuspended in MEM at a density of 1.1 × 10⁶/mL. Type I collagen, 10× MEM, reconstitution buffer, bovine TM cell suspension, and soluble fibronectin or other ECM proteins were mixed on ice in a volume ratio of 6.6:1:1:0.2:2.2 (final concentration of type I collagen, 1.8 mg/mL; final cell density, 2 × 10⁶/mL). A portion (0.5 mL) of the mixture was added to each BSA-coated well of the cell culture clusters and was induced to form a collagen gel by incubation at 37°C in 5% CO₂ for 90 minutes. Unsupplemented MEM (0.5 mL) was then added on top of each collagen gel and, after 1 hour, the gel was freed from the side of the well with a microspatula. The diameter of each collagen gel was measured with a ruler every 24 hours during 5 days of culture, and the extent of gel contraction was calculated by subtracting the diameter of the gel at each time point from the initial diameter. All experiments were performed at least in triplicate and were repeated at least twice with different bovine TM cell preparations.

**Immunofluorescence Staining**

Collagen solutions containing bovine TM cells in the absence or presence of fibronectin were prepared as just described, and 50 µL of each solution was spread on glass-bottomed dishes that had been coated with 0.2 mL of 1% BSA at 37°C for 1 hour. Collagen gels were formed and then overlaid with 2 mL of MEM with or without fibronectin. After culture for 2 days, the gels were washed with PBS, and the cells were fixed for 30 minutes at room temperature with 1% paraformaldehyde in PBS. Fixed specimens were washed with PBS, allowed to dry in air, and then incubated for 30 minutes at room temperature with PBS containing 1% BSA to block nonspecific binding, washed again with PBS, and incubated for 1 hour at room temperature with rabbit antiserum to integrin α5 (1/500 dilution) or mouse monoclonal antibodies to integrin β1 (10 µg/mL), both diluted in PBS containing 1% BSA. The specimens were washed with PBS containing 0.1% Tween 20 (T-PBS) and then incubated for 1 hour at room temperature with fluorescence-conjugated (Alexa Fluor 488; Molecular Probes) goat antibodies to mouse or rabbit IgG (10 µg/mL; diluted with PBS containing 1% BSA). After the specimens were washed with T-PBS, they were incubated for 30 minutes at room temperature with fluorescence-conjugated phalloidin (5 U/mL Alexa Fluor 568, Molecular Probes; diluted with PBS containing 1% BSA) to stain F-actin. Finally, the specimens were washed with T-PBS and observed with a laser confocal microscope (Fluoview; Olympus, Tokyo, Japan).

**RESULTS**

**Effect of Fibronectin on TM Cell Contraction**

We first examined the effects of various ECM proteins and glycosaminoglycans on the contraction of collagen gels mediated by bovine TM cells. The inclusion of fibronectin or dermatan sulfate in the collagen gel increased the extent of contraction by 46% and 25%, respectively (Table 1). Other molecules examined, including type IV collagen, laminin, chondroitin sulfate A, chondroitin sulfate C, keratan sulfate, heparan sulfate, and hyaluronic acid, did not affect gel contraction. The extent of the fibronectin-induced collagen gel contraction was dependent both on the time of incubation (Fig. 1) and on the concentration of fibronectin (Fig. 2). To confirm that the observed changes in collagen gel diameter induced by fibronectin were not attributable to an increase in collagen degradation, we measured the amount of hydroxyproline in the incubation medium after acid-heat hydrolysis; fibronectin had no effect on the amount of this collagen degradation product (data not shown).

**Effect of an RGD Peptide on Fibronectin-Induced TM Cell Contraction**

We next investigated whether the fibronectin-induced contraction of bovine TM cells is mediated by the interaction of fibronectin with its integrin α5β1 receptor. Inclusion of GRGDSP (Gly-Arg-Gly-Ser-Pro), a peptide mimic of the cell-binding domain of fibronectin, in the collagen gel resulted in a concentration-dependent inhibition of fibronectin-induced TM cell contraction that was significant at a concentration of 25 µg/mL (Fig. 3). In contrast, the peptide GRGESP (Gly-Arg-Gly-Glu-Ser-Pro), an inactive analogue of GRGDSP, did not affect fibronectin-induced TM cell contraction at concentrations up to 250 µg/mL.
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Effects of Cytochalasin D and Nocodazole on Fibronectin-Induced TM Cell Contraction

To evaluate the role of the intracellular cytoskeletal system in fibronectin-induced TM cell contraction, we examined the effects of cytochalasin D and nocodazole, inhibitors of microfilament and microtubule assembly, respectively. The addition of cytochalasin D to the overlay medium resulted in a concentration-dependent inhibition of fibronectin-induced collagen gel contraction mediated by bovine TM cells (Fig. 4). In contrast, similar addition of nocodazole did not result in a marked inhibition of fibronectin-induced TM cell contraction. These results suggest that the formation of microfilaments, but not that of microtubules, contributes to fibronectin-induced TM cell contraction.

Effects of Fibronectin on Actin Stress Fiber Formation and the Localization of Integrin α5 and β1 Chains in TM Cells

Finally, we examined the effects of fibronectin on the formation of stress fibers and on the expression and localization of integrin α5 and β1 chains in TM cells cultured in collagen gels for 2 days. Cells cultured without fibronectin were polygonal and small, with no marked protrusions or cellular processes (Figs. 5A, 6A). Fluorescence specific for F-actin was mostly punctate. Although short stress fibers were apparent in some cells, the localization of F-actin seemed unrelated to the few small cellular processes. The inclusion of fibronectin in both the collagen gel and overlay medium resulted in extensive cell spreading and the formation of both substantial cellular processes and prominent stress fibers (Figs. 5E, 6E).

TM cells cultured in collagen gels without fibronectin exhibited few fluorescence signals specific for integrin α5 (Fig. 5B). In contrast, in the presence of fibronectin, a punctate pattern of integrin α5 fluorescence was prominent in TM cells (Fig. 5F). Merging of the F-actin and integrin α5 fluorescence images suggested that the expression of integrin α5 was associated with the formation of stress fibers in cells cultured in the presence of fibronectin (Fig. 5G), but not in those cultured in its absence (Fig. 5C).

TM cells cultured in collagen gels in the absence of fibronectin exhibited a punctate pattern of integrin β1 fluorescence (Figs. 6B, 6C). In cells cultured in collagen gels in the presence of fibronectin, the integrin β1 fluorescence signals were also observed in association with the cellular processes (Figs. 6F, 6G).

Staining of cells with normal rabbit serum (Figs. 5D, 5H) or normal mouse IgG (Figs. 6D, 6H) as negative controls con-
firmed the specificity of staining with the antibodies to integrins α5 or β1, respectively. These results thus demonstrate that the fibronectin-induced spreading of bovine TM cells cultured in collagen gels was accompanied by expression of integrin α5.

DISCUSSION

In the present study, fibronectin induced collagen gel contraction mediated by bovine TM cells. This effect of fibronectin was inhibited by cytochalasin D and by the peptide GRGDSP, but not by nocodazole or the peptide GRGESP. Fibronectin also induced the spreading of bovine TM cells, and this effect was accompanied by the formation of actin stress fibers and by expression of integrin α5.

Although various ECM proteins and glycosaminoglycans have been detected in TM tissue, among the molecules tested in the present study only fibronectin and, to a lesser extent, dermatan sulfate significantly promoted collagen gel contraction mediated by bovine TM cells. The type IV collagen used for our experiments was not native, but rather comprised a mixture of degraded fragments. Furthermore, the laminin used may not be identical with that present in living tissue. It is thus possible that the failure of type IV collagen or laminin to affect collagen gel contraction mediated by TM cells may be due to a low biological activity of the preparations studied.

ECM proteins are implicated as a determinant of IOP. The abundance of fibronectin and other ECM proteins is increased in human glaucomatous eyes, for example, and matrix metalloproteinases, which degrade ECM proteins, have been shown to increase outflow facility in perfused human eyes in organ culture. These observations suggest that deposition of ECM proteins in TM tissue impairs aqueous humor outflow. Fibronectin is also present in aqueous humor and is a potent chemoattractant for bovine TM cells in vitro. Furthermore, aqueous humor from human glaucomatous eyes induces the chemotaxis of human TM cells, implicating fibronectin in the pathogenesis of glaucoma.

Although our present in vitro findings cannot be extrapolated directly to the pathobiology of aqueous humor dynamics, they suggest that the interaction between TM cells and extracellular collagen can result in cell-mediated collagen contraction and that fibronectin promotes this effect. We have shown that inhibitors of protein kinase C (PKC), of the small guanosine triphosphatase Rho, of myosin light chain kinase (MLCK), and of actin polymerization inhibit TGF-β1-induced collagen gel contraction mediated by bovine TM cells. Other researchers have shown that inhibition of PKC, of Rho-associated coiled-coil-forming protein kinase (ROCK), 20 which is a target of Rho; or of actin polymerization results in relaxation of TM tissue in vitro. Inhibitors of PKC, ROCK, MLCK, and actin polymerization also increase trabecular outflow facility in vitro. Moreover, IOP is reduced by administration of ROCK or MLCK inhibitors in vivo. Together, these observations suggest that agents that inhibit collagen gel contraction mediated by TM cells also induce the relaxation of TM tissue, increase aqueous humor outflow facility, and lower IOP.

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933434/)

**Figure 4.** Effects of cytochalasin D (A) and nocodazole (B) on fibronectin-induced collagen gel contraction mediated by bovine TM cells. Cells were cultured for 5 days in collagen gels containing fibronectin (0.1 mg/mL) and with overlay medium containing the indicated concentrations of cytochalasin D or nocodazole, after which the extent of collagen gel contraction was determined. Data are the mean ± SD of results from three independent experiments. *P < 0.05, **P < 0.01 versus fibronectin alone (Dunnett test).

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933434/)

**Figure 5.** Effects of fibronectin on the distribution of F-actin and integrin α5 in bovine TM cells cultured in collagen gels. Cells were cultured for 2 days in collagen gels in the absence (A–D) or presence (E–H) of fibronectin (0.1 mg/mL in both the gel and overlay medium). They were then stained with fluorescence-labeled phalloidin (A, E) as well as with rabbit antiserum to integrin α5 and fluorescence-labeled secondary antibodies (B, F). Merged images are shown in (C) and (G), respectively. Normal rabbit serum was used as a negative control for staining with the antiserum to integrin α5, and the merged images with F-actin staining are shown in (D) and (H). Scale bar, 50 μm.
Interactions between cells and ECM proteins are mediated by integrins. Thus, antibodies to integrin α5β1 inhibit the binding of fibroblasts to fibronectin.27 In the present study, fibronectin induced the upregulation of integrin α5 in association with fibronectin-induced spreading in bovine TM cells. Both integrin α5 and β1 chains may thus be responsible for the collagen gel contraction mediated by TM cells in the presence of fibronectin. Integrin β1 is also a component of receptors for other ECM proteins in addition to that for fibronectin. For example, integrins α1β1 and α2β1 function as receptors for collagen and laminin.58,25 Moreover, the contraction of collagen gels mediated by other cell types30–31 has been shown to depend on integrins α1β1 or α2β1. It is therefore possible that integrin β1 was constitutively expressed at the surface of bovine TM cells in the present study as a result of its interaction with the type I collagen gel.

We have shown that the formation of actin stress fibers in bovine TM cells contributes to the collagen gel contraction mediated by these cells in response to TGF-β1.7 In the present study, the inhibition of microfilament polymerization by cytochalasin D blocked the collagen gel contraction mediated by bovine TM cells in response to fibronectin. Microfilament formation may thus be important for TM cell contraction, regardless of the stimulus. In contrast, inhibition of microtubule formation by nocodazole did not markedly affect fibronectin-induced bovine TM cell contraction, even though these cells contain a network of microtubules (data not shown), suggesting that these structures do not play an important role in this effect of fibronectin. The disruption of microtubules in human TM cells52 and other cell types33–34 has been shown to result in an increase in contractility. It is possible that a stimulatory effect of nocodazole on bovine TM cell contraction in the present study was masked by the large effect of fibronectin.

Fibronectin is also implicated in the increase in IOP associated with steroid-induced glaucoma. Glucocorticoids thus increase the synthesis of fibronectin in human TM cells.4,5 Induce ocular hypertension in association with increased fibronectin expression at the anterior angle in perfused human eyes in culture,35 and increase the expression of TM-inducible glucocorticoid response (TIGR) protein,36 which is also known as myocilin, binds to fibronectin, and is thought to function in the regulation of aqueous humor outflow. The interaction of fibronectin with TM cells may thus contribute to the regulation of aqueous humor outflow and therefore provide a new target for therapeutic intervention in glaucoma.

References


