Bovine Trabecular Cells Produce TIMP-1 and MMP-2 in Response to Mechanical Stretching

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Abstract: Bovine trabecular cells in growth phase were exposed to cyclic mechanical stretching of the bottom of a culture dish at a cycle of 30 seconds for 72 hours. The stretched cells produced significantly larger amounts of metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1) after 72 hours, compared with cells in nonstretched control. In contrast, TIMP-2 and MMP-9 levels were not influenced by mechanical stretching. Trabecular cells would modify extracellular matrix in response to such mechanical stimuli as bending of trabecular meshwork or aqueous flow by the production of TIMP-1 and MMP-2. **Jpn J Ophthalmol 1998;42:90–94** © 1998 Japanese Ophthalmological Society

Key Words: Cyclic mechanical stretching, matrix metalloproteinase-2 and 9 (MMP-2 and MMP-9), tissue inhibitor of metalloproteinase-1 and 2 (TIMP-1 and TIMP-2), trabecular cells.

Introduction

The trabecular meshwork is a major site for aqueous outflow, and its resistance to the flow is considered responsible for the development of primary open-angle glaucoma.[1,2] Accumulation of extracellular matrix components such as collagens and proteoglycans in the trabecular meshwork has been found in eyes with primary open-angle glaucoma, and the abnormal balance between their production and degradation has been advocated as one of the underlying pathogenic factors.[1–3]

Matrix metalloproteinases (MMPs) are a group of enzymes involved in the degradation of collagens and proteoglycans. Tissue inhibitors of metalloproteinases (TIMPs) inhibit the activity of MMPs by binding to them.[4] These two kinds of proteins play a cooperative role in modulating degradation of the extracellular matrix. Trabecular cells have also been shown to produce TIMPs and MMPs.[5–8]

The trabecular meshwork in vivo is exposed to mechanical forces generated by aqueous flow and changing levels of intraocular pressure. We previously demonstrated that the elevation of hydraulic pressure to around 25 mm Hg induced a transient rise of intracellular calcium in human trabecular cells in a culture flask.[9] Furthermore, mechanical stretching applied to the bottom of a culture dish with bovine and porcine trabecular cells resulted in the increased production of prostaglandin $F_{2\alpha}$.[10,11] In this study, we measured the production of TIMP-1, TIMP-2, and MMPs by bovine trabecular cells in culture in response to mechanical stretching, to test whether or not mechanical force would influence the metabolism of extracellular matrix in the trabecular meshwork.

Materials and Methods

Culture of Bovine Trabecular Cells[10–12]

Bovine eyes were obtained from a local slaughterhouse within 3 hours after sacrifice and disinfected by soaking in 0.2% povidone iodine for 10 minutes and then in 70% alcohol for 30 seconds. The eyes were washed several times in phosphate-buffered saline (PBS) and cut circumferentially into halves. After the removal of the lens and iris from the anterior half of the globe, the trabeculum was trimmed from the cornea at the Schwalbe's line and then from the sclera under a dissecting microscope, as previously described.[10,11] Each fragment of the trabeculum was

placed in a six-well multidish (Nunc, Naperville, IL, USA) containing Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 15% fetal calf serum (FCS), 100 mg/L streptomycin, and 100 mg/L ampicillin, and incubated in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C. Cells grown out of the fragments were transferred to 6-cm petri dishes with air vents (Nunc) and treated with 0.25% trypsin and 1 mM EDTA in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (Gibco BRL, Grand Island, NY, USA).

Cyclic Mechanical Stretching[10,11]

Trabecular cells were seeded in 6-cm petri dishes at the density of 9.67×10^4 cells in 10 mL DMEM with 15% FCS per dish and incubated for 24 hours before the stretching, to let the cells attach to the bottom. Three dishes were placed securely on the apparatus for cyclic mechanical stretching, with a piston under the dish moving up and down to cause the deformation of the dish bottom, as reported previously.[10,11] The piston was moved at a cycle of 30 seconds to stretch the dish bottom at the maximum magnitude of a strain of 4.5 mm/m (4500 microstrains).

The number of cells was counted with a blood cell counting plate 24, 48, and 72 hours after the beginning of stretching by dislodging cells with 0.25% trypsin and 1 mM EDTA.

TIMP-1 and TIMP-2 Measurement

The measurements were done by enzyme immunoassay using the Human TIMP-1 Kit (Dai-ichi Kagaku Yakuhin, Tokyo, Japan) and Biotrak TIMP-2, Human ELISA System (Amersham, Buckinghamshire, England). For TIMP-1, 10 μL of 41-fold–diluted samples or standards, mixed initially with 150 μL of horseradish peroxidase-conjugated antihuman-TIMP-1 monoclonal antibody, were transferred to wells of a 96-well microplate coated with another antihuman-TIMP-1 monoclonal antibody, and incubated for 30 minutes at room temperature. Each well was washed three times with 0.4 mM phosphate buffer (pH 7.5) containing 1% Tween-20, and then incubated with 0.5% o-phenylenediamine and 0.02% hydrogen peroxide for 15 minutes. Color development was stopped by the addition of 2 N sulfuric acid, and read at 492 nm with a microplate reader (EIA Reader, Model 2550, Bio-Rad Laboratories Japan, Tokyo). For TIMP-2, 50 μL of 5-fold–diluted samples and 50 μL of peroxidase-conjugated antibody to TIMP-2 were incubated in wells of a microplate coated with an-

other antibody to TIMP-2 for 2 hours at room temperature. After washing, wells were incubated with 3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide in 20% dimethylformamide for 30 minutes. The color was read at 450 nm after the addition of 1 N sulfuric acid.

Gelatin Zymography[13–15]

Seven microliters of culture medium samples, mixed with a loading buffer without reducing agents (×4 buffer: 36% sucrose, 8% sodium dodecyl sulfate [SDS], 40 mM Tris-HCl pH 6.8, 10% Bromphenol-blue), were applied to a 2.5% polyacrylamide gel (5 cm vertical × 10 cm horizontal in size) containing 1% SDS and 2% gelatin (type B: from bovine skin, Sigma Chemical Co., St. Louis, MO, USA), and electrophoresed for 2 hours at 20 mA. The gel was washed with 2.5% Triton X-100 for 30 minutes to remove SDS, and then incubated with Tris buffer (50 mM Tris, 10 mM $CaCl_2$, pH 7.6) for 15 hours at 37°C. The gel was stained with 2% Coomassie Brilliant Blue in 10% acetic acid and 40% methanol for 10 minutes, and destained with 8% acetic acid and 10% methanol. The gels were sandwiched between cellophane sheets, dried, and then submitted to densitometric analysis with Scanning Imager 300SX and ImageQuant Software Version 3.3 (Molecular Dynamics, Sunny Vale, CA, USA). The inverted optical density integrated for the whole area of each band was used as enzymatic activity.
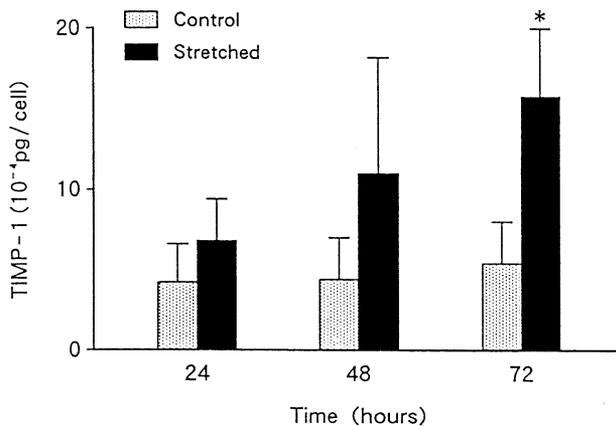
Results

Bovine trabecular cells grew exponentially during 72 hours of observation. Mechanical stretching did not influence the growth curve of cells compared with the nonstretched control (Figure 1). The trabecular cells showed the tendency to produce higher levels of TIMP-1 as a lapse of time during 72 hours both in the stretched group and in the nonstretched control (Figure 2). The secretion of TIMP-1 increased significantly 72 hours after the beginning of mechanical stretching in the stretched group (N = 6, $15.74 \pm 4.18 \times 10^{-4}$ pg/cell as mean ± standard deviation) compared with the nonstretched control (N = 6, $5.44 \pm 2.46 \times 10^{-4}$ pg/cell; Student's t-test, P = 0.0008). In contrast, mechanical stretching did not influence the level of TIMP-2 produced by trabecular cells (Figure 3). The TIMP-2 levels after 72 hours decreased significantly, compared with those after 24 hours, both in the stretched group and in the nonstretched control group (Student's t-test, P = 0.0331 and P = 0.0296, respectively).
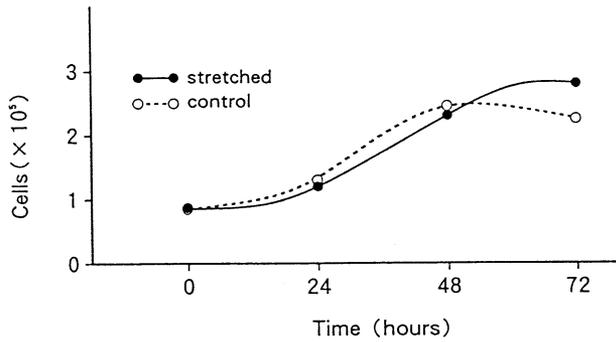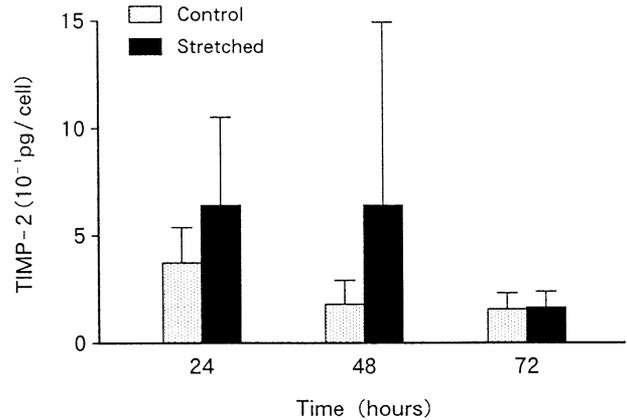
Figure 1. Growth curves of bovine trabecular cells during mechanical stretching, compared with the nonstretched control. Mechanical stretching does not affect the growth of cells.

The levels of TIMP-2 produced per cell were at three orders of magnitude as high as those of TIMP-1 (Figures 2 and 3). The level of TIMP-1 in DMEM and 15% FCS without cells was 5.07 ng/mL, by two orders of magnitude as low as that in the culture medium after 24 hours in the nonstretched control group (mean = 164.15 ng/mL). In contrast, the level of TIMP-2 in DMEM and 15% FCS without cells was 56.62 ng/mL, about the same as that in the culture medium after 24 hours in the nonstretched control group (mean = 55.00 ng/mL).

The trabecular cells also had the tendency to produce higher levels of MMP-2 (gelatinase A, 72 kDa gelatinase) as a lapse of time both in the stretched
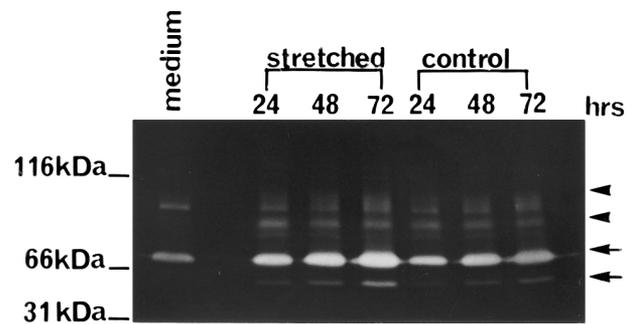

Figure 3. Production of TIMP-2 by bovine trabecular cells during mechanical stretching, compared with the nonstretched control. The level of TIMP-2 produced per cell does not change significantly between the stretched group and nonstretched control group. The TIMP-2 levels after 72 hours decreased significantly, compared with those after 24 hours, both in the stretched group and nonstretched control group (Student's t-test, $P = 0.0331$ and $P = 0.0296$, respectively). Each column and bar represents a mean and standard deviation of 5 measurements.

group and in the nonstretched control (Figures 4 and 5). In contrast, the secretion of MMP-9 (gelatinase B, 92 kDa gelatinase) remained stationary during the same period. Most of MMP-2 produced by trabecular cells was its latent form with a larger molecular weight, while both the active and the latent form of MMP-9 were equally secreted in the media (Figure
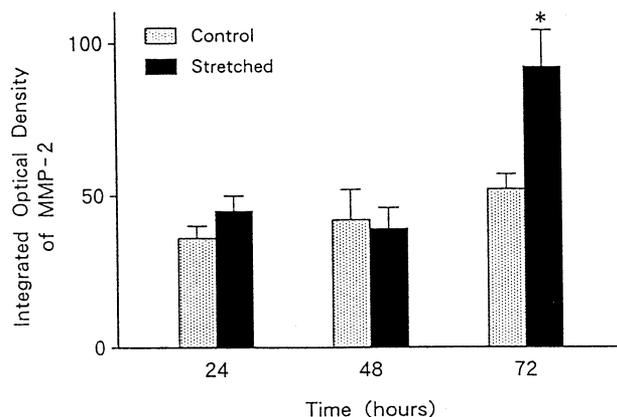

Figure 2. Production of TIMP-1 by bovine trabecular cells during mechanical stretching, compared with the nonstretched control. The level of TIMP-1 produced per cell increases significantly 72 hours after mechanical stretching, compared with the nonstretched control (*Student's t-test, $P = 0.0008$). Each column and bar represents a mean and standard deviation of 6 measurements.

Figure 4. Gelatin zymogram. Bovine trabecular cells produce a higher level of MMP-2 (gelatinase A, arrows; upper arrow, latent form; lower arrow, active form), but not of MMP-9 (gelatinase B, arrowheads; upper arrowhead, latent form; lower arrowhead, active form) as a lapse of time in the mechanically stretched group compared with the nonstretched control group. Increased production of the latent form of MMP-2 (upper arrow) is most evident. The leftmost column gives molecular markers in kilodaltons. "Medium" indicates DMEM + 15% FCS without cells.

Figure 5. Production of the latent form of MMP-2 (gelatinase A, upper arrow in Figure 4) by bovine trabecular cells quantified by densitometric analysis of zymograms. Trabecular cells produce a significantly higher level of the latent form of MMP-2 72 hours after the beginning of stretching, compared with the nonstretched control (*Student's *t*-test, $P = 0.014$). Each column and bar represents a mean and standard deviation of triplicate measurements. Integrated optical density is given as arbitrary units.

4). Mechanical stretching induced the significantly increased production of the latent form of MMP-2, but not of MMP-9, 72 hours after the beginning of stretching as compared with the nonstretched control (N = 3, Student's *t*-test, $P = 0.014$ for MMP-2, Figure 5), The active form of MMP-2, although neither quantified nor tested statistically, also appeared to be secreted at a higher level in the stretched group than in the nonstretched control group (Figure 4).

Discussion

The present study demonstrated that mechanical stretching increased the production of TIMP-1 and MMP-2 (gelatinase A) by bovine trabecular cells in their exponential growth phase. In contrast, the production of MMP-9 (gelatinase B) and TIMP-2 was not affected by mechanical stretching. The unchanged levels of MMP-9, measured in this study, could not be attributable to the concurrent presence of TIMP-1, inhibitor for MMP-9, because these two proteins were separated electrophoretically in the gel before zymography. Furthermore, the whole activity of gelatinases, including not only active forms but also latent forms with a larger molecular weight, could be measured by zymography, since their proenzymes were activated by pretreatment with SDS in electrophoresis before zymography.[13]

Both free TIMP-1 and TIMP-1 bound to MMPs could be measured by the kit used in this study (manufacturer's instruction). In contrast, TIMP-2 bound to the proenzyme of MMP-2 could not be measured by the kit, while free TIMP-2 and TIMP-2 bound to active MMPs could be measured (manufacturer's instruction). The significant decrease of TIMP-2 after 72 hours, both in the stretched group and in the nonstretched control group, would be, therefore, attributable to the increased level of the proenzyme of MMP-2 secreted by trabecular cells.

Trabecular tissue contained type I and type IV collagens as the main constituents of its extracellular matrices.[3] Trabecular cells have been shown to produce a high level of MMP-2 (gelatinase A) and a low level of MMP-9 (gelatinase B).[6] MMP-2 degrades type IV collagen, while MMP-9 degrades types I and IV collagens.[4] The increased production of MMP-2, but not of MMP-9, in response to mechanical stretching, might have a specific role in balanced degradation of extracellular matrices in the trabecular tissue. The increased secretion of TIMP-1 also suppresses the activity of MMP-9 under the circumstances.

Trabecular cells in growth phase, in the presence of 15% FCS, were exposed to mechanical stretching in this study. Trabecular cells at confluency would better mimic an in vivo environment of the trabecular tissue than cells in exponential growth. However, trabecular cells at confluency did not survive in the absence of FCS for as long as 24 hours, whereas cells at confluency still showed some growth in the presence of FCS.

In this study, mechanical stretching of trabecular cells in vitro would correspond to such an in vivo situation as bending of the trabecular beams by aqueous flow and by changing levels of the intraocular pressure. The selective increases in the production of TIMP-1 and MMP-2, but not of MMP-9 and TIMP-2, suggest that cells would modulate extracellular matrices in the trabecular tissue in response to the mechanical force that they perceive. Abnormal response of trabecular cells to such mechanical forces might lead to the disturbed balance of extracellular matrices in the trabecular tissue.

Histopathologically, the decreased number of cells[16] and accumulation of such extracellular matrix components as collagens and proteoglycans[1,2] in the trabecular tissue have been observed in the eyes of the elderly, as well as in eyes with primary open-angle glaucoma. Under these circumstances, trabecular tissue as a whole might lose elasticity and show reduced sensitivity to mechanical forces, which would, in turn, result in abnormal regulation of matrix metalloproteinases and their inhibitors.

Gonzalez-Avila et al[17] showed that aqueous humor in eyes with primary open-angle glaucoma contained significantly higher levels of TIMP-1 and significantly lower collagenase activity than those in normal eyes and in eyes with other types of glaucoma. They suggested that a decrease of collagen degradation, as a result, might contribute to excessive deposition of collagen with loss of the trabecular cells during the development of primary open-angle glaucoma.[17] Other studies also demonstrated the presence of metalloproteinases in human aqueous humor.[18,19] The present results suggest that TIMP-1 and metalloproteinases in aqueous humor are produced, in part, by trabecular cells as a consequence of mechanical forces applied to the trabecular meshwork.

Mechanical forces have been shown to influence production of extracellular matrix components and metalloproteinases in other types of cells, such as fibroblasts,[20,21] vascular smooth muscle cells,[22] and vascular endothelial cells.[23] This study demonstrated that mechanical forces also played a part in regulating the production of TIMP-1 and MMP-2 in trabecular cells.

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