

TIMP-1 Production by Bovine Retinal Pigment Epithelial Cells Increases in Response to Cyclic Mechanical Stretch

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Purpose: The effect of mechanical stretch was examined on cultured retinal pigment epithelial (RPE) cells in order to observe changes in their production of tissue inhibitor of metalloproteinase-1 (TIMP-1) and vascular endothelial growth factor (VEGF) in response to physiological strain.

Methods: Bovine RPE cells in near-confluent culture were exposed to mechanical stretch of the bottom of a 6-cm petri dish at the maximum magnitude of 4500 microstrain and at a cycle of 30 seconds for 72 hours. TIMP-1 and VEGF levels in the medium following 24, 48, and 72 hours of cyclic stretch were measured by enzyme immunoassay.

Results: The growth of RPE cells during the 72-hour period of stretching did not show a significant difference from that of nonstretched control cells. RPE cells in the stretched group produced a significantly larger amount of TIMP-1 at 48 and 72 hours after stretch, compared with nonstretched control ($P = .044$ and $P = .027$, respectively, Student *t*-test). The levels of VEGF produced by RPE cells were not significantly different between the stretched group and nonstretched control group.

Conclusions: The secretion of TIMP-1 by bovine RPE cells was enhanced by cyclic mechanical stretch. Mechanical strain is one factor in regulating the secretion of TIMP-1 by RPE cells. **Jpn J Ophthalmol 2001;45:470-474** © 2001 Japanese Ophthalmological Society

Key Words: Retinal pigment epithelial cells, TIMP-1, VEGF, mechanical stretch.

Introduction

Retinal pigment epithelial (RPE) cells are crucial for the survival of retinal photoreceptor cells. RPE cells have been shown to secrete a wide range of growth factors and cytokines, including vascular endothelial growth factor (VEGF).¹ In addition, they are known to produce collagens, proteoglycans, matrix metalloproteinases (MMPs), and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs).² The balance between synthesis of extracellular matrix components and their degradation is crucial for the maintenance of extracellular matrices such as

Bruch's membrane and interphotoreceptor matrix. The degradation of extracellular matrix is regulated by a delicate balance between levels of active MMPs and TIMPs. The activation of MMPs is also an important step in regulating the degradative activity of MMPs.³

We previously demonstrated that trabecular cells of the eye in culture responded to cyclic mechanical stretch at a magnitude as small as 4500 microstrain (0.45% stretch) and produced prostaglandin F₂-alpha, TIMP-1, MMP-2, and a new gene (oculomedin).⁴⁻⁶ We also showed that TIMP production was regulated by cyclic mechanical stretch in scleral fibroblasts and retinal glial cells.^{7,8} In the present study, bovine RPE cells were exposed to this small magnitude of cyclic mechanical stretch and their production of TIMP-1 and VEGF was measured in order to test a hypothesis that RPE cells would respond to a physiological range of mechanical stretch.

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Materials and Methods

Culture of Bovine RPE Cells

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 and cut circumferentially into halves.^{4,5} After the removal of vitreous together with the anterior half, the retina was peeled off and cut at the optic disc. The posterior half was incubated with 0.25% trypsin and 1 mM EDTA in Ca^{2+} , Mg^{2+} -free Hanks' balanced salt solution (Gibco BRL, Gaithersburg, MD, USA) for several minutes.⁹ The RPE cells were dissociated by pipetting and transferred to wells of a six-well multidish containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 mg/L ampicillin, and 100 mg/L streptomycin. Half of the medium was changed twice a week.

Cyclic Mechanical Stretch

RPE cells near confluency in 6-cm petri dishes (Figure 1) received 10 mL of fresh medium just prior to beginning the stretching procedure. Three dishes at a time were placed securely on the apparatus for cyclic mechanical stretch (Figure 2), with a piston under each dish moving up and down to cause the deformation of the dish bottom, as reported previously.^{4,5} The piston was moved at a cycle of 30 seconds to stretch the dish bottom at a maximum strain magnitude of 4.5 mm/m (4500 microstrain, 0.45%

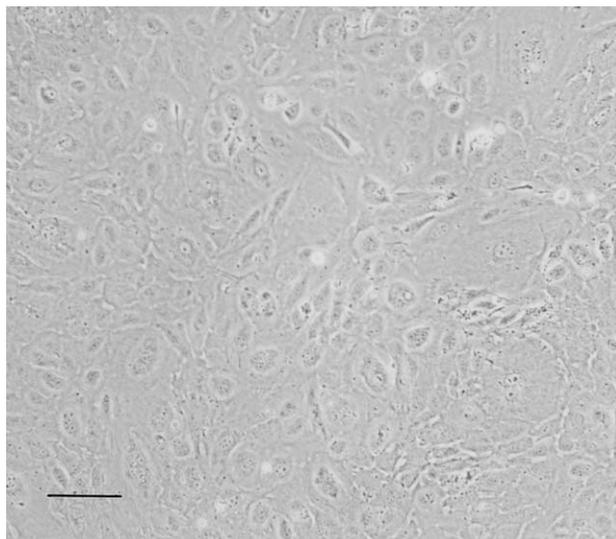


Figure 1. Phase-contrast micrograph of bovine RPE cells in culture exposed to cyclic mechanical stretch. Bar = 100 μm .

stretch). The number of cells was counted with a blood cell counting plate 0, 24, 48, and 72 hours after the beginning of stretching by dislodging cells with 0.25% trypsin and 1mM EDTA.

Of the three petri dishes secured on the stretching apparatus at one time, one dish was used for cell counting and measurements of TIMP-1 and VEGF in the medium at 24 hours. The second and third dishes served in the cell counting and the measurements in the medium at 48 hours and at 72 hours, respectively. This series of experiments was repeated four or five times to obtain four or five sets of data. Cell counting and the measurements in the medium were also done for dishes before stretching. Control petri dishes, not subjected to stretching, were placed for the same time period in the same incubator and used the same as described for stretched dishes.

Measurements of TIMP-1 and VEGF

Measurements of TIMP-1 and VEGF were done by enzyme immunoassay using a human TIMP-1 kit (Dai-ichi Kagaku Yakuhin, Tokyo), and Quantikine VEGF immunoassay kit (R&D Systems, Minneapolis, MN, USA). Both kits were tested to confirm reaction with bovine TIMP-1 and VEGF. For TIMP-1, 10 μL of fivefold-diluted samples or standards, mixed initially with 150 μL of horseradish peroxidase-conjugated anti-human TIMP-1 monoclonal antibody, were transferred to microplate wells pre-coated with another anti-human TIMP-1 monoclonal antibody, and incubated for 30 minutes at room temperature. After washing three times with 10 mM phosphate buffer (pH 7.5) containing 50 mM sodium chloride and 0.05% Tween-20, each well was incubated with 0.5% o-phenylenediamine and 0.02% hydrogen peroxide for 15 minutes. Color develop-

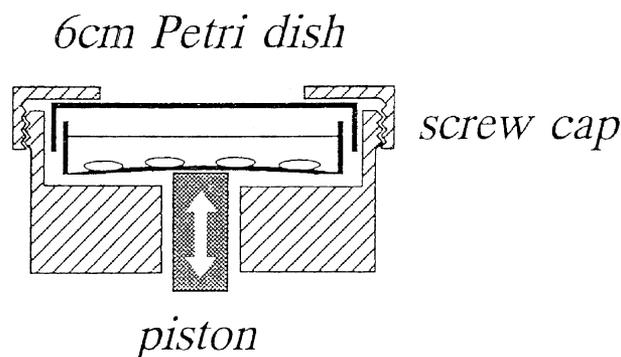


Figure 2. Schematic drawing of apparatus for cyclic mechanical stretch. Piston under 6-cm petri dish secured on apparatus moves up and down to cause deformation of dish bottom.^{4,5}

ment was stopped by the addition of 1 M sulfuric acid, and optical density was read at 492 nm with a microplate reader (EIA Reader, Model 550; Bio-Rad Laboratories Japan, Tokyo). This TIMP-1 kit could measure free TIMP-1 as well as TIMP-1 bound to MMPs.

For VEGF, a series of standard and undiluted samples were incubated for 2 hours at room temperature in wells of a microtiter plate coated with monoclonal antibody against human VEGF165. After washing the wells with the buffer provided, horseradish peroxidase-conjugated polyclonal antibody against VEGF was added to the wells to sandwich the VEGF immobilized during the first incubation. Following a wash, a substrate solution (tetramethylbenzidine) was added to the wells and color was developed for the measurement at 450 nm.

Results

The growth of bovine RPE cells in culture during a 72-hour period of stretching did not show a significant difference from that of nonstretched control RPE cells (Student *t*-test, Figure 3). Cyclic mechanical stretch at this magnitude did not induce morphological changes of the RPE cells (Figure 1).

RPE cells in the stretched group at 48 hours and 72 hours after stretching produced a significantly larger amount of TIMP-1 ($16.47 \pm 5.27 \times 10^{-4}$ ng/cell and $18.71 \pm 5.12 \times 10^{-4}$ ng/cell, respectively, mean and standard deviation, $n = 4$), compared with the nonstretched control ($9.14 \pm 2.39 \times 10^{-4}$ ng/cell

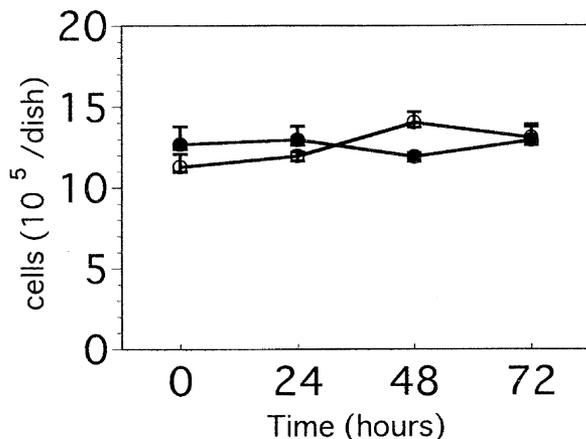


Figure 3. Increase in number of bovine retinal pigment epithelial cells during cyclic mechanical stretch. Growth does not show significant difference between stretched group and nonstretched control group during 72-hour period (Student *t*-test). T-bars indicate standard error. ○: control, ●: stretched.

and $10.46 \pm 2.54 \times 10^{-4}$ ng/cell, respectively, $n = 4$, $P = .044$ and $P = .027$, Student *t*-test, Figure 4). The level of TIMP-1 secretion per cell in a culture dish both in the stretched group and in the nonstretched control group increased significantly during the lapse of time (one-way analysis of variance [ANOVA], $P = .0016$ and $P = .0007$, respectively), indicating that the RPE cells continued to secrete TIMP-1 in culture media over the entire experimental period.

In contrast, the production of VEGF by bovine RPE cells did not show a significant difference between the stretched group and the nonstretched control group (Student *t*-test, Figure 5). The level of VEGF secretion per cell both in the stretched group and in the nonstretched control group remained unchanged for the period of 72 hours ($P = .1804$ and $P = .1127$, respectively, one-way ANOVA), indicating that the RPE cells did not continue to secrete VEGF.

Discussion

This study clearly demonstrated that bovine RPE cells in culture produced a significantly larger amount of TIMP-1 in response to cyclic mechanical stretch. It should be noted that the significant increase of TIMP-1 secretion occurred only after 48 hours of stretching. In contrast, levels of VEGF were not changed significantly by mechanical stretch. The magnitude of cyclic mechanical stretching used in this study was as small as 4500 microstrain,⁴ equivalent to 0.45% of stretching, and would be within a

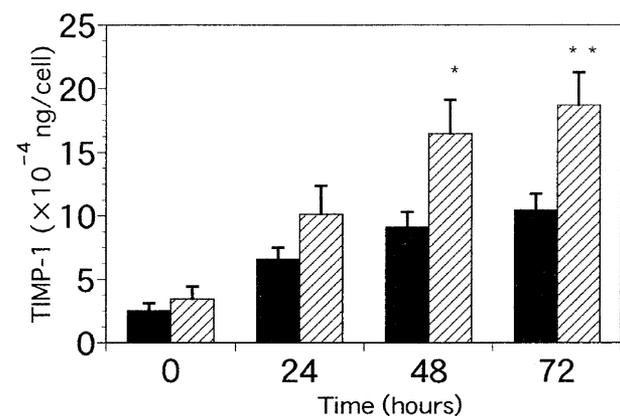


Figure 4. Tissue inhibitor of metalloproteinase-1 (TIMP-1) produced by bovine retinal pigment epithelial (RPE) cells during cyclic mechanical stretch. At 48 hours and 72 hours after beginning of cyclic mechanical stretch, RPE cells in stretched group produced significantly larger amount of TIMP-1, compared with nonstretched control group (* $P = .044$ and ** $P = .027$, Student *t*-test, $n = 4$). T-bars indicate standard error. ■: control, ▨: stretched.

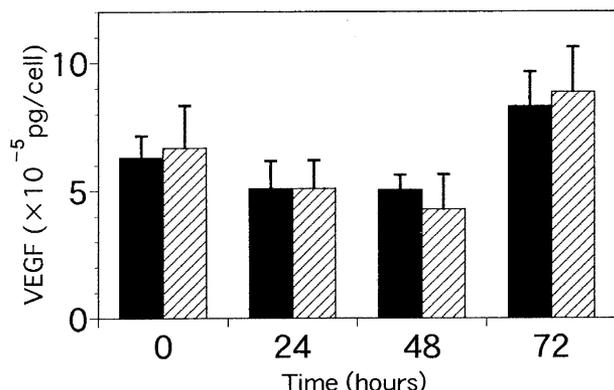


Figure 5. Vascular endothelial growth factor produced by bovine retinal pigment epithelial cells during cyclic mechanical stretch. No difference is noted between stretched group and nonstretched control group (Student *t*-test, *n* = 5). T-bars indicate standard error. ■: control, ▨: stretched.

physiological stimulus range. The stretching, indeed, did not influence the growth or morphology of bovine RPE cells in this study.

The levels of TIMP-1 and VEGF used for statistical analysis were expressed as those measured in the medium divided by the number of RPE cells in a culture dish to obtain the secreted amounts per cell. The TIMP-1 level was about 10,000× higher than the VEGF level in this study. The low level of VEGF secretion by RPE cells, compared with TIMP-1, may be one reason for no significant change in VEGF secretion by cyclic mechanical stretch.

In a previous study, rabbit RPE cells cultured on silicone rubber membrane was stretched by 10% and shown to secrete a lysosomal enzyme, N-acetyl-β-glucosaminidase, in response to this single stretch.¹⁰ In parallel with our study, rat RPE cells were exposed to pulsatile 10% or 15% stretch by a commercially available apparatus (Flexercell Strain Unit), and shown to express and secrete VEGF in 1 hour.¹¹ The secretion of VEGF in that study was not normalized by the number of RPE cells, despite the fact that cell growth was expected. In addition, such a magnitude of stretch up to 10%, as used in the two previous studies, is considered not within the physiological range.

TIMPs are present in interphotoreceptor matrix¹² and also detected in the subretinal fluid in patients with retinal detachment.¹³ TIMPs in interphotoreceptor matrix are produced by RPE cells.¹⁴ TIMPs, at least TIMP-3, are also detected in Bruch's membrane.^{15,16} TIMP-1 produced by RPE cells in response to mechanical stretch would suppress degradation of

interphotoreceptor matrix and, thus, protect retinal photoreceptor cells from damage caused by mechanical stretch. In the *in vivo* eye, mechanical stretch of the retina evoked by movement of vitreous gel and fluid would be transmitted to RPE cells because outer segments of photoreceptor cells are firmly surrounded by apical microvilli of RPE cells. Under these circumstances, the presence or the absence of posterior vitreous detachment would be a critical factor for the response of RPE cells because mechanical stress exerted by the vitreous on the retina and then transmitted to RPE cells would be different at the magnitude between these conditions.

In conclusion, mechanical stretch was shown as one kind of stimuli to increase the secretion of TIMP-1 by RPE cells.

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