Intracellular calcium response to hydraulic pressure in human trabecular cells

Toshihiko Matsuo, Nobuhiko Matsuo

Abstract

Aims—To understand the mechanism for regulation of intraocular pressure, human trabecular cells were examined to determine whether they could respond to the change in hydraulic pressure.

Methods—Human trabecular cells were cultured from trabeculum tissue fragments excised during trabeculectomy in four eyes of three patients with primary open angle glaucoma and exposed to the change of hydraulic pressure in a tissue culture flask connected to a glass syringe. The pressure was exerted by automatic infusion of the piston of the syringe and monitored by a pressure gauge. The intracellular calcium concentration was measured in real time with a calcium binding fluorescent dye, fluo-3.

Results—A small number (about 10%) of cells appearing morphologically to be trabecular cells showed transient elevations or oscillations of the intracellular calcium concentration in response to the elevation of hydraulic pressure to 20–30 mm Hg, indicating that a part of the human trabecular cells could sense the change in hydraulic pressure.

Conclusion—Some cells in the human trabecular tissue seem to sense the change in intraocular pressure and might play a role in its regulation.

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Trabecular meshwork is a major site for aqueous outflow and its abnormalities lead to the elevation of intraocular pressure.^{1, 2} Cells covering beams of the trabecular meshwork and in the juxtacanalicular tissue near the Schlemm's canal are referred to as trabecular cells which naturally consist of heterogeneous populations.^{3–5} The trabecular cells produce various kinds of biologically active substances as growth factors⁶ and prostaglandins,^{7, 8} and also synthesise extracellular matrix components,^{9–11} indicating that they play a role in physiological homeostasis of the trabecular meshwork as well as in maintaining its anatomical architecture.

Intraocular pressure (IOP) is maintained by the balance between the production of aqueous humour by the ciliary epithelium and its outflow, mainly at the trabecular meshwork but also by the uveoscleral route. However, it is not known how the IOP is maintained in a normal range and whether there is a sensing mechanism for IOP. In addition to the previous observations of many nerve endings in the trabecular meshwork,¹² Tamm *et al*¹³ recently

showed by electron microscopy the presence of nerve endings characteristic of mechanoreceptors in the scleral spur, supporting the old hypothesis that the central nervous system is involved in the regulation of IOP.^{14–20} We previously demonstrated that cyclic mechanical stretching of cultured bovine and porcine trabecular cells resulted in their increased production of prostaglandin F_{2α},²¹ and suggesting that they sensed the pressure. In this study, we developed an in vitro model system to exert controlled hydraulic pressure on cultured cells and demonstrated that intracellular calcium concentration in human trabecular cells could change in response to the elevation of hydraulic pressure.

Materials and methods

CULTURE OF HUMAN TRABECULAR CELLS^{22–25}

Trabeculum tissue fragments excised during trabeculectomy in patients with primary open angle glaucoma were placed in wells of a 24 well multidish (Nunc, Naterville, IL, USA) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 100 µg/ml streptomycin, and 100 µg/ml ampicillin and incubated under a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C. Cells grew out of the tissue fragments obtained from three eyes of four patients (Table 1) usually 2 weeks after the start of culture. Cells were then dislodged by 5 minute treatment with 0.25% trypsin and 1 mM EDTA in Hanks' balanced salt solution (Gibco BRL, Grand Island, NY, USA), and transferred to a six well multidish (Nunc) and then to a 10 cm petri dish (Nunc). The second to fourth passage of cells were used for the following experiments.

ELECTRON MICROSCOPIC OBSERVATION OF CULTURED CELLS²⁶

Cells dispersed by treatment with 0.25% trypsin and 1 mM EDTA for 5 minutes were transferred to a 24 well multiplate for suspension culture (Sumilon, Tokyo, Japan) and incubated for 1 month in DMEM supplemented with 15% fetal calf serum. Media were changed twice a week. Cells formed floating

Table 1 Rates of response to hydraulic pressure in trabecular cells derived from patients with primary open angle glaucoma

Patient No/age (years)/sex/laterality	No of responding cells/No of cells observed
1/70/female/right	5/52 (9.6%)
1/70/female/left	8/78 (10.3%)
2/70/male/left	6/49 (12.2%)
3/60/male/right	0/45 (0%)

Department of
Ophthalmology,
Okayama University
Medical School,
Okayama City, Japan
T Matsuo
N Matsuo

Correspondence to:
Dr Toshihiko Matsuo,
Department of
Ophthalmology, Okayama
University Medical School,
2-5-1 Shikata-cho, Okayama
City, Okayama 700, Japan.

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Figure 1 Schematic diagram showing a system to exert controlled hydraulic pressure on cultured cells and to measure simultaneously their intracellular calcium concentration. The pressure in a flask filled with medium can be elevated by automatic movement of the piston of a syringe and monitored by an electric pressure gauge.

aggregates (multicellular spheroids) within a day under this non-adherent environment of culture.²⁷⁻³² Multicellular spheroids after 1 month were fixed with 2.5% glutaraldehyde in

0.1 M phosphate buffer (pH 7.4), then with 1% osmium tetroxide, dehydrated with a graded alcohol series, and embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. We used multicellular spheroids for morphological observation of the cells because cells, in general, showed more tendency to differentiate in multicellular spheroids than in monolayer growth.^{28-30, 32}

APPLICATION OF HYDRAULIC PRESSURE AND MEASUREMENT OF INTRACELLULAR CALCIUM CONCENTRATION

Human trabecular cells were transferred to tissue culture polystyrene flasks with an area of 25 cm² (Iwaki, Funabashi, Japan) and cultured for 4 days. The cells before reaching confluency were loaded with 10 μM of fluo-3 acetoxymethyl ester (fluo-3/AM; Molecular Probe, Eugene, OR, USA) for 30 minutes at 37°C in DMEM and incubated further in DMEM without fluo-3/AM.^{33, 34} The flask was then filled with HEPES buffered saline (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM glucose, pH 7.4).³⁵

The flask was placed on the stage of an inverted microscope (Olympus IMT-2; Olympus, Tokyo, Japan) attached to argon ion laser (laser scanning microspectrofluorometer ACAS570, Meridian Instruments, Okemos, MI, USA), and hydraulic pressure was exerted on the cells as shown schematically in Figure 1. The pressure inside the flask was elevated by controlled movement of the piston of a 10 ml glass syringe connected to the flask, by means of an automatic syringe injector (Syringe Infusion Pump, Model 980324, Harvard Apparatus, South Natick, MA, USA), and monitored continuously by a pressure gauge (LifeKit, Disposable Pressure Monitoring Kit; Nihon Kohden, Tokyo) connected through an amplifier (RMP-6004M; Nihon Kohden) to a printer (Mini Recorder SJ-3462; Atto, Tokyo, Japan).

The changes in intracellular calcium concentration of trabecular cells were observed as the changing intensity of fluorescence every 20 seconds. The fluorescent images of cells visualised by scanning argon laser at 488 nm as an excitation wavelength were captured through a ×20 objective lens by a photomultiplier tube and stored on a computer. One frame of the image usually contained 3-7 cells and the fluorescent intensity of each cellular area was measured by an image analysis program in Single Detector Kinetics Program Version 3.22 (Meridian).

Results

MORPHOLOGICAL CHARACTERISTICS OF CELLS

Cells could be grown out of about a half of trabecular explants tested and consisted apparently of two types of cells. The dominant type had a star-like or triangular shape with multiple cell processes and showed monolayer growth in confluency (Fig 2A), indicating the features of trabecular cells.²²⁻²⁵ The other type, which was observed less frequently, were elon-

Figure 2 Phase contrast micrographs of cells used in this study (derived from the right eye in patient 1). The predominant type of cells (A) are star-shaped, have multiple cellular processes, and grow in a monolayer, characteristic of human trabecular cells.²²⁻²⁵ The other type of cells observed occasionally as small foci in culture are spindle-shaped, and grow in superimposed layers, characteristic of scleral fibroblasts or keratocytes.²²⁻²⁵ Magnification, ×50.

Figure 3 Electron micrograph of cells used in this study (derived from the right eye in patient 1). Cells grown as a multicellular spheroid in a non-adherent environment for 1 month²⁶ have nuclei with the dark spiked band of peripheral chromatin, osmiophilic cytoplasm with various organelles, together with many villous projections and coated vesicles (arrowheads) along the cell surface, characteristic of human trabecular cells.²³⁻²⁵ An apoptotic cell (large arrow) is phagocytosed by a neighbouring trabecular cell and an intercellular junction is noted between cellular processes (small arrow). Uranyl acetate and lead citrate staining. Bar=1 μ m.

Figure 4 Transient elevations (oscillations) of the intracellular calcium concentration in a trabecular cell (arrows) in response to the elevation of hydraulic pressure. The responding cell shows the increased intensity of fluorescence represented as pseudocolours in a time course. Downward trends of fluorescence intensity in each cell as a lapse of time are due to defocusing of the image caused by expansion of the flask. Bar=50 μ m.

gated, spindle-shaped cells that grew in super-imposed layers as small foci (Fig 2B), characteristic of scleral fibroblasts or keratocytes.²²⁻²⁵

Electron microscopically, cells in multicellular spheroids 1 month after the beginning of culture showed many villous projections and coated vesicles along the cell surface, together with frequent intercellular junctions (Fig 3).

The osmiophilic cytoplasm contained various organelles such as ribosomes, mitochondria, Golgi apparatus, and different levels of phagosomes, while the nuclei showed the prominent dark spiked band of peripheral chromatin. All these features were consistent with those described for human trabecular cells in culture.²³⁻²⁵

RESPONSE TO HYDRAULIC PRESSURE

Hydraulic pressure exerted on human trabecular cells was elevated continuously from 0 to 100 mm Hg. About 10% of the cells derived from three eyes of two patients (Table 1) showed transient elevation (transients) or oscillations (a group of transients) of intracellular calcium concentration at a hydraulic pressure over 20 mm Hg (Figs 4 and 5). The threshold levels of hydraulic pressure to induce transients of intracellular calcium varied from cell to cell and ranged between 20 and 30 mm Hg. These transients of intracellular calcium lasted for 40 to 80 seconds and then returned to the same baseline level as before the transients. The peaks of these transients as fluorescent intensity were at least 1.5-fold higher than the baseline level. Downward trends of the baseline level of fluorescent intensity as a lapse of time seen in Figure 5 were due to defocusing of the images caused by expansion of the flask.

The baseline levels of intracellular calcium in the responding cells were similar to those in the non-responding cells (Fig 4). The responding cells belonged morphologically to trabecular cells rather than fibroblasts or keratocytes. The morphological difference between the responding and non-responding cells was not evident. Spindle-shaped cells growing in superimposed layers as small foci considered to be fibroblasts or keratocytes did not show any response to the elevation of hydraulic pressure.

The number of responding cells did not increase at levels of hydraulic pressure higher than 20 mm Hg. Non-responding cells remained non-responsive at the higher levels of pressure up to 150 mm Hg. The mainten-

ance of hydraulic pressure at 0 mm Hg for 10 minutes, as a control, did not induce transients or oscillations of intracellular calcium. Cells derived from one patient (Table 1) did not show any response to hydraulic pressure.

Discussion

In the system we developed, hydraulic pressure exerted on cells in culture could be changed both at a level of mm Hg and in a time span of seconds, and monitored continuously with an electric pressure gauge. Systems used by others simply involved putting a load on the layer of cells growing at the bottom of culture dishes³⁶ or elevating atmospheric pressure in the culture chamber resulting in the transfer of air pressure to fluid pressure.³⁷ In these systems, real hydraulic pressure to which cells were exposed could not be measured or monitored, although rough values for pressure could be calculated.

Our system utilised a closed chamber filled with fluid which was not circulated or exposed to air. Therefore, the limited supply of oxygen to the cells was a major drawback. We finished the experiments within 30 minutes after the flasks with cultured cells in medium were taken out of an incubator and filled with fluid for measuring intracellular calcium. These flasks had a volume of 60 ml, and this contained enough oxygen for a small number of cells during this short period.

Intracellular calcium has been shown to play a role as a second messenger in bovine trabecular cells and mediate the stimuli by cholinergic and adrenergic ligands as well as neuropeptides.³⁸⁻³⁹ The oscillations of intracellular calcium in human trabecular cells in this study could be observed only under the change of hydraulic pressure and would be mediated by mechanosensitive (stretch activated) ion channels.⁴⁰⁻⁴¹ These channels would be gated directly for calcium ions or other ions,⁴² and calcium oscillations could result from a combination of calcium flowing from the calcium storage in the cells and outside the cells.⁴³⁻⁴⁴ The elevation of intracellular calcium, as a second messenger, then causes a variety of cellular responses such as synthesis and secretion of biological signals, proliferation, and differentiation.⁴⁵ The calcium oscillations might also induce contraction of trabecular cells since some cells in the human trabecular meshwork were shown by immunohistochemistry to have smooth muscle myosin and actin.⁴⁶⁻⁴⁷

The threshold of hydraulic pressure to induce the transients of intracellular calcium ranged from 20 to 30 mm Hg in human trabecular cells used in this study. In contrast, the threshold of pressure for intracellular calcium response in human periodontal ligament fibroblasts examined in the same system ranged widely from 20 to 50 mm Hg.⁴⁸ In these two types of cells, the elevation of pressure did not lead to such increased responses as more frequent transients of intracellular calcium and their higher peaks. In another study, human gingival fibroblasts did not show oscillations of intracellular calcium at 1% stretch of cells, but

Figure 5 Time course of intracellular calcium concentration as shown by fluorescence intensity and the level of hydraulic pressure. Transient elevation of the intracellular calcium concentration (A, transient) is observed at 20 mm Hg in one trabecular cell, while a group of transients (B, oscillations) started at 20 mm Hg in another cell (corresponding to the cell (arrow) shown in Figure 4).

did show oscillations at 2.8% stretch.⁴⁹ These facts suggest that different types of cells have different ranges of the threshold for the response to pressure and that pressure within these ranges generates the constant response. Further studies are necessary to obtain a definite value for the threshold, based on molecular characteristics of putative mechanosensitive ion channels involved in the response to hydraulic pressure in human trabecular cells.

It should be noted that only a limited number of human trabecular cells responded to the elevation of hydraulic pressure. This can be explained by the presence of different subpopulations in the trabecular cells as reported previously.³⁻⁵ In addition, the cultured cells used in this study would contain different types of cells, since these cells grew out of the trabeculum tissue excised during trabeculectomy, which consisted not only of trabecular cells but also of scleral fibroblasts, keratocytes, and endothelial cells of the Schlemm's canal. However, the present study demonstrated that the responding cells belonged morphologically to trabecular cells, and that fibroblasts or keratocyte-like cells did not show any response to hydraulic pressure. Another possibility is that the non-responsive cells might utilise a different second messenger system other than calcium in their responses to pressure, which naturally could not be detected as a positive response in this study.

Our present finding that trabecular cells themselves could respond to the change in hydraulic pressure within a physiological range suggests another way for regulation of the IOP, in addition to its regulation by nerve endings in the trabecular meshwork and scleral spur.¹²⁻¹³ The trabecular cells could sense the change in the IOP and modify their function to facilitate outflow at the trabecular meshwork.⁵⁰ They might also secrete paracrine signals which diffuse in the aqueous to the ciliary epithelium. Our previous result that cyclic mechanical stretching increased the production of prostaglandin $F_{2\alpha}$ by trabecular cells²¹ gave us a hypothesis—namely, that trabecular cells responding to the elevation of IOP secrete prostaglandin $F_{2\alpha}$ as a paracrine signal which facilitates the uveoscleral outflow.⁵¹

The response of intracellular calcium to mechanical stress has been well studied, especially in vascular endothelial cells which are exposed in vivo to the changing stress of blood flow.⁵²⁻⁵⁷ The vascular endothelial cells in culture were subjected to mechanical shearing force or stretching. Trabecular cells in the eye are also exposed to the aqueous flow and hydraulic pressure as well as mechanical stretching induced by the deformation of trabecular meshwork. The architecture of meshwork is more easily deformed by subtle changes in the flow and hydraulic pressure than the rigid structure. This may be the reason why the outflow site at the angle is formed as a meshwork in the eye.

In conclusion, human trabecular tissue contained a small group of cells capable of responding to the elevation of hydraulic

pressure, suggesting that these cells play a role in sensing the level of IOP. Abnormalities or loss of these pressure sensitive cells might be related to the development of glaucoma. The number of cells in the trabecular meshwork decreases with age and this decrease is more marked in patients with primary open angle glaucoma.⁵⁸ Such loss of cells⁵ including pressure sensitive cells might underlie the absence of response to hydraulic pressure in trabecular cells derived from the one eye in this study with primary open angle glaucoma.

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