A Three-Dimensional Collagen Gel Contraction Monitoring System that Uses A Porcine Trabecular Meshwork for Screening of Anti-Intraocular Pressure Agents

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**Abstract**

Trabecular Meshwork (TM) contractile state is thought to affect aqueous humor flow and Intraocular Pressure (IOP). It has been suggested that TM relaxation may increase conventional outflow by increasing tissue porosity and lead to decrease in IOP; therefore, it could be a therapeutic target for glaucoma. Accordingly, we investigated the effects of various agents on the contractility of cultured Porcine Trabecular Meshwork (pTM) cells using a three-dimensional (3-D) collagen gel assay, in order to develop a screening method for identifying novel anti-IOP agents. We identified pTM cells, obtained from porcine eyeballs, by cell shape and expression of Low Density Lipoprotein (LDL) receptors. The pTM cells, when embedded in collagen gels, showed contractile activity dependent on concentration of Fetal Bovine Serum (FBS). Various kinase inhibitors, especially inhibitors of cell cyclin-dependent kinase (rescovitoline), rho and Ca2+-dependent protein kinase (Y-27632), tyrosine kinase (tyrophostin AG879), phosphatidylinositol 3-kinase (bisindolylmaleimide I, BIM I), and Ca2+/calmodulin kinase (chelerythrine), strongly inhibited collagen gel contraction. This contraction was also inhibited by ethacrynic acid, by inhibitors of Na+, K+-ATPase (ouabain), Ca2+-ATPase (thapsigargin), and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, by a non-selective phosphodiesterase inhibitor (papaverine), and by adenosine A1 (metrifudil), and cannabinoid receptor (CP-55940) agonists. In addition, BIM I, simvastatin, BQ-123, and CP-55940 demonstrated partial cytotoxicity. These findings indicate that this in vitro 3-D collagen gel contraction monitoring system could be used as a rapid and sensitive screening method for identifying novel agents that induce pTM cell relaxation and could simultaneously detect both main and side effects.

**Keywords:** Cell cyclin-dependent kinase; Contraction; Cytotoxicity; 3-D collagen gel; Relaxation; Trabecular meshwork

**Abbreviations:** bFGF: Basic Fibroblast Growth Factor; BIM I: Bisindolylmaleimide I; CB: Cannabinoid; CDK: Cyclin-Dependent Kinase; CM: Ciliary Muscle; DMEM: Dulbecco’s Modified Eagle Medium; EGF: Epidermal Growth Factor; ET-1: Endothelin-I; FBS: Fetal Bovine Serum; HBSS: Hank’s Balanced Salt Solution; HMG-CoA: 3-Hydroxy-3-Methylglutaryl Coenzyme A; IOP: High Intraocular Pressure; LDL: Low Density Lipoprotein; MLCK: Myosin Light-chain Kinase; MMPs: Matrix Metalloproteinases; PDGF: Platelet-derived Growth Factor; pTM: Porcine Trabecular Meshwork; ROCK: Rho and Ca2+-dependent Protein Kinase; TGF-β: Ttransfoming Growth Factor-β1; TIMP: Tissue Inhibitors of Metalloproteinase; TM: Trabecular Meshwork; TMCs: Trabecular Meshwork Cells; 3-D: Three-Dimensional

**Introduction**

Glucoma is a term used to denote a group of eye diseases that gradually diminish eyesight, often without warning or symptoms. Loss of vision results from optic nerve damage, which was once attributed primarily to high Intraocular Pressure (IOP).

The aqueous humor outflow pathway consists of both conventional and uveoscleral outflow routes. In human and primate eyes, 80 to 90% of the outflow of aqueous humor, which is produced in the ciliary body, occurs via drainage through the conventional route comprised of Schlemm’s canal and the Trabecular Meshwork (TM). The TM has several unique properties: (i) it is the site of biosynthesis and secretion of various extracellular matrix materials (types I, III, IV, V, and VI collagen) and glycoproteins (laminin and fibronectin) [1]; (ii) it is associated with the production of Matrix Metalloproteinases (MMPs) -1, -2, -3, and -9 and Tissue Inhibitors of Metalloprotease (TIMP)-1 [2]; (iii) it displays macrophage-like phagocytic activity [3]; and (iv) it has smooth-muscle-like properties [4]. The IOP is regulated by a balance between aqueous inflow and outflow, and is modulated by changes in the contractile state of the TM. If aqueous outflow decreases, IOP may gradually increase, resulting in hypertensive glaucoma. At present, only a limited number of agents are available which have the capability of modulating outflow through the conventional outflow route. Identification of new and improved agents that can improve outflow is therefore important.

Cytoskeletal changes occur during exposure to the cytoskeletal inhibitors ethacrynlic acid [5,6], cytochalasin B or D [7,8], and latrunculin-A or B [9], as well as to various intracellular signal transduction modulators, such as H-7[10], Y-27632[11] and ML-9[12]. These mediators have been proposed to enhance outflow and to...
it. Therefore, modulation of cytoskeletal occurs. Contractile tension is one approach for increasing aqueous humor outflow.

The three-dimensional (3-D) collagen gel assay is a simple and widely used method for evaluating contractile activity [14]. For example, it has been used with fibroblasts that (i) are a major source of extracellular interstitial connective tissue matrix material, (ii) play an important role in wound healing, and (iii) are important in the development of fibrosis of the skin [14], liver [15], heart [16], and smooth muscle [17]. The 3-D collagen gel assay has been also used to characterize changes in corneal stromal tension [18], tenon capsule fibroblasts [19], retinal pigment epitheliunm [20], vitreous body hyalocytes [21], and TM [22].

Materials and Methods

The following reagents were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA): Dulbecco’s Modified Eagle Medium (DMEM); amphotericin B; recombinant human PDGF-BB; recombinant human TGF-β1; PMA; roscovitine; tyrophostins AG879, AG82, AG490, AG494, AG257, AG555, and AG1296; papaverine hydrochloride; H-7, bisindolylmaleimide I (BIM I); chelerythrine chloride; ML-9; thapsigargin; LY294002; ouabain; BQ-123; BQ-788; N6-cyclohexyladenosine; metrifulidil; N6-(2-amino-phenyl) ethyladenosine; CGS19343; and resazurin. Calbiochem Novabiochem Corp. (San Diego, CA, USA) provided Y-27632. CP-55,940 was obtained from Tocris Cookson Ltd. (Bristol, UK). FBS was obtained from Sanko Junyaku Co., Ltd. (Tokyo, Japan). Kanamycin sulfate and cell dissociation buffer, and were subsequently cultured in 225 cm² cell culture flasks. For the current study, we used confluent second- and third-passage cells.

In this study, using a 3-D gel assay, we investigated (i) the inhibitory effects of a variety of agents on contractility of isolated porcine TM (pTM) cells and (ii) whether any of the observed inhibitory activity was due to cytotoxicity in order to develop a rapid and sensitive screening method for identifying novel agents that can induce pTM cell relaxation.

The pTM cells were identified by characterizing cell shape when viewed with a light microscope, and by determining Low Density Lipoprotein (LDL) receptor-expression using DiI-labeled acetyl LDL. The pTM cells were seeded in 96-well multiwell plates (5 × 10⁴ cells/well) on the collagen matrix and cultured under 5% CO₂ atmosphere in DMEM medium that contained 10% FBS, 100 μg/ml kanamycin sulfates, and 1.25 μg/ml amphotericin B, under 5% CO₂ at 37°C. Several days after initiation of cultures, the pTM cells had migrated off the explant. When cultures became confluent, pTM cells were detached from the multiwell plate by incubation with cell dissociation buffer, and were subsequently cultured in 225 cm² cell culture flasks. For the current study, we used confluent second- and third-passage cells.

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Effect of Fetal Bovine Serum (FBS) and additive Platelet-derived Growth Factor (PDGF)-BB on contraction of collagen gels containing Porcine Trabecular Meshwork Cells (pTMCs). (A) Representative photographs of a collagen gel stained by crystal violet after 48 h culture. (B) Effect of PDGF-BB on both concentration of FBS-stimulated collagen gel contraction and on cell viability. Various concentrations of PDGF-BB solution were added and the cells were cultured on collagen gels in the presence of FBS at a concentration of 0.5% or 2%. The areas of collagen gels were measured and data were shown as % of collagen gel area without FBS. Open columns are data for 0.5% FBS-stimulated contraction and solid columns are data for 2% FBS. Symbols, "*" or "**" indicated absence or presence of FBS. Dotted line indicates cell viability (%). Data represent the mean ± SE of triplicate measurements. *P<0.05, **P<0.005 versus 0.5% FBS alone, P<0.05, ***P<0.005 versus 2% FBS alone.

Results

We confirmed pTM cell identity by observing the characteristic cell shape and by ascertaining the presence of acetyl-LDL receptors. The pTM cells were spindle-shaped (Figure 1A) when observed by light microscopy. Nuclei were detected as blue fluorescence using Hoechst 33258 (Figure 1B). Dil uptake by acetyl LDL receptors was evident as red fluorescence in whole cells (Figure 1C). When the fluorescence micrographs were merged, the fluorescent images overlaid to reveal identical localization of TM cells (Figure 1D).

We investigated the effect of FBS concentration on the contraction of the pTM cells in a collagen-containing gel. Without FBS, no collagen gel contraction occurred. In contrast, as seen in Figure 2A and 2B, addition of 0.5% FBS resulted in contraction. Contraction was greater with 2% FBS than with 0.5% FBS. With PDGF-BB alone, contraction increased in a dose-dependent manner. Both TGF-β, and the PKC activator PMA had similar effects to those observed with PDGF-BB over the same concentration range (data not shown).

We investigated the effects of various protein kinase inhibitors over a concentration range from 10⁻² to 10⁻⁵ M on collagen gel contraction in the presence of 0.5% FBS. Various agents inhibited collagen gel contraction at concentrations ranging from 10⁻² to 10⁻³ M. However, this inhibitory activity was attenuated if the contraction was induced by 2% FBS. The ROCK inhibitor Y-27632 inhibited collagen gel contraction and showed no cytotoxicity (Figure 3A). At 10⁻³ M, no FBS-induced contraction was observed. The cell Cyclin-dependent Kinase (CDK) inhibitor roscovitine (Figure 3B) and tyrosine kinase inhibitor tyrphostin AG879 (Figure 3C) showed the same patterns as Y-27632. The PKC inhibitor, chelerythrine chloride, was also an effective inhibitor, but showed cytotoxicity (Figure 3D). Other kinase inhibitors, MLCK (ML-9) and Ca²⁺-calmodulin-dependent phosphodiesterase (W-7), were not effective in this assay.

The thiol group inhibitor ethacrynic acid inhibited collagen gel contraction at a concentration of 10⁻³ M (Figure 4A). The Ca²⁺-ATPase inhibitor thapsigargin (Figure 4B) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor simvastatin (Figure 4C)
inhibited gel contraction in a concentration-dependent manner from 10^{-4} M to 10^{-1} M. However, as was the case with chelerythrine chloride, both agents also exhibited cytotoxicity. The phosphodiesterase inhibitor papaverine had no effect on collagen contraction (data not shown).

The adenosine A2 agonist metrifudil caused inhibition at a concentration of 10^{-6} M (Figure 5A). The adenosine A3 agonist N^6-(2-amino-phenyl) ethyladenosine and the non-selective adenosine agonist cyclohexyladenosine showed the same pattern as did metrifudil (data not shown). The Cannabinoid (CB) receptor agonist CP-55,940 (Figure 5C) inhibited contraction at a concentration of 10^{-3} M. However,

**Discussion**

The aim of this study is to investigate the effect of various different TM functional inhibitors on contractility and cytotoxicity using 3-D
collagen gel contraction monitoring system. The contractility activity was determined by the ability of these modulators to induce relaxation of a FBS (0.5 and 2%) contracted state. We selected viable candidates by comparing their ID<sub>S</sub> and LD<sub>S</sub> values with respect to relaxation and cytotoxicity. Compounds that effectively inhibited contraction without showing cytotoxic effects could be valuable in the selective induction of increases in aqueous humor outflow without inducing cytotoxicity.

In the present study, collagen gels containing pTM cells contracted in the presence of FBS, and this contractile activity was enhanced with PDGF-BB in a concentration-dependent manner. Addition of PMA and TGF-β also stimulated FBS-induced contraction (data not shown). Because previous report revealed that some cytokines activate the PKCs in pTM cells [26], these results suggested that some cytokine receptors, possibly through PKC activation, induce TM contraction. PKC is a serine/threonine kinase and has several functions, including permeability, contraction, migration, proliferation, differentiation, hypertrophy, tumor metastasis, apoptosis, and secretion [27,28]. PKC isoforms are classified into three classes: conventional or classical PKCs, including PKC-α, -β1, -β2, and -γ; novel PKCs, including PKC-δ, -ɛ, -η, and -θ; and atypical PKCs, including PKC-ζ, -τ, -μ, and -υ [29,30]. However, the nature of the PKC isoforms that may have participated in collagen gel contraction was not determined. Calcium-dependent PKC-α and calcium-independent PKC-ε, are both located in TM and Ciliary Muscle (CM), but are expressed to a greater extent in TM than in CM [5,6,31,32]. Specific inhibition of PKC isoforms may represent a new approach for research into and development of anti-glaucoma drugs.

The ROCK inhibitor (Y-27632), serine/threonine kinases (H-7 and BIM I), tyrosine kinases (tyrphostin AG879, PI3-K), and CDK (roscovitine) inhibited contraction very effectively and about Y-27632 and tyrophostin AG879, the results in this study agree with other previous researches [13,33-35]. Y-27632 causes reversible changes in cell shape [36], decreases MLC phosphorylation [37], disrupts actin bundles, impairs focal adhesion formation [11], and inhibits in vitro calbachol-induced contraction of isolated bovine CM strips [12], whereas PMA induces contraction in isolated bovine TM strips [37]. Y-27632 is also capable of enhancing outflow and decreasing IOP [11,36,37], which suggests that it may inhibit collagen gel contraction under similar conditions. In the present study, Y-27632 was not cytotoxic at the dosages used, which indicates that this compound might be an excellent new candidate for anti-glaucoma drug therapy.

Serine/threonine PKC inhibitors (e.g., H-7 and BIM I) also induced changes in cell shape and cytoskeletal organization [6,32,38]. These changes induced outflow and decreased IOP in organ culture and animal models [39]. While these inhibitors exhibited cytotoxicity at high concentrations (>10<sup>-6</sup> M), this phenomenon may have been due to prolonged exposure (for 48 h). Another PKC inhibitor, chelerythrine chloride, was a somewhat effective inhibitor of contractility, but showed strong cytotoxicity. The ability of this agent to decrease IOP is unclear, but Wiederhort et al. reported that it could relax TM strips [13]. These results support the involvement of PKC in the TM contraction and suggest that PKC inhibitors may be a good candidate as an anti-glaucoma drug therapy.

The existing literature reveals no reports of effects of tyrophostin AG 879 on outflow or IOP. Two other tyrosine kinase inhibitors, tyrphostin AG879 and genistein, inhibited calbachol-induced contraction of isolated bovine TM strips [13]. However, Volberg et al. reported that the tyrophostins did not increase outflow [40]. Therefore, the usefulness of tyrosine kinase inhibitors for glaucoma therapy remains unconfirmed. In the present study, apart from tyrphostin AG879, no effect was observed for the other tested tyrophostins (genistein, AG82, AG490, AG494, AG527, AG555, or AG1296).

To date, the CDK inhibitor roscovitine has not been used in glaucoma experiments. CDKs have been suggested to regulate contraction-relaxation of TM; therefore, they might be valuable as new anti-glaucoma agents. The PI3-K inhibitors wortmannin and LY294002 showed both weak and strong inhibition of contractility, but LY294002 was partially cytotoxic. In addition, since association of PI3-K with TM has not been demonstrated, we suggest that little, if any, relationship exists between PI3-K and contractility.
Inhibition of other enzymes, e.g., Na⁺, K⁺-ATPase (ethacrynic acid and ouabain), Ca²⁺-ATPase (thapsigargin), and HMG-CoA reductase (sodium pravastatin and simvastatin), by inhibitor concentrations at 10⁻⁵ M also inhibited the collagen gel contraction of pTM cells. Ouabain was a more effective inhibitor than ethacrynic acid, but was cytotoxic. No reports have yet appeared regarding inhibition of contraction by ethacrynic acid and ouabain, although they have been reported to induce cytoskeletal changes and to decrease IOP [7,41,42]. Thapsigargin had the same inhibitory efficacy as ethacrynic acid, but was cytotoxic. HMG-CoA reductase inhibitors (sodium pravastatin and simvastatin) inhibited contractility at 10⁻⁵ M.

The lipophilic statins (e.g., simvastatin and sodium fluvastatin) were more effective at inhibiting gel contraction than was their hydrophilic analogue, sodium pravastatin (data not shown). However, the more lipophilic statins had greater cytotoxicity. In recent years, several studies have shown a relationship between statins and the eye. For example, statins have been reported to show anti-angiogenic side effects on choroidal neovascularization [43], maculopathy [44], risk for cataract [45], and ocular hemorrhage [46]. A recent report indicated that the risk for developing glaucoma was decreased when statins were taken continuously [47]. However, the relationship between statins and IOP regulation is not known. Statins have been suggested to have possible effects on IOP regulation through indirect suppression of rho activation induced by isoprenylation [48]. Statins may have capabilities similar to those seen for kinase inhibitors for inducing cytoskeletal changes and decreasing IOP.

Receptor agonists/antagonists for endothelins, adenosines, and CB inhibited contractility at a concentration of 10⁻⁵ M, in accordance with previous reports [49,50]. Endothelin-1 (ET-1) induced a biphasic IOP response [51] and TM contraction [52]. The ET-1 receptor antagonists (BQ-123 and BQ-788) might possibly inhibit contraction induced by ET-1 or ET-1-like agonists containing FBS.

Adenosine reportedly affects IOP and ocular blood circulation. Adenosine agonists subtype A1 and A2a decreased IOP as result of a decline in aqueous humor production and increase in outflow [53,54]. However, the precise mechanism underlying the increased outflow in response to these agonists is not known. MMP-2-induced secretion by an adenosine agonist has been reported previously [55] and was suggested to result in a direct change in TM cells contraction/relaxation through a mechanism other than that involving an increase in MMP-2 secretion.

Reports indicate that CB agonists decrease IOP by an as yet undetermined mechanism, although the decrease in IOP in response to CB agonists is thought to be due to change in outflow [56]. Development of CB agonists for use in the treatment of glaucoma will be difficult because of the problem of separating their IOP and central nervous system effects [57]. CB agonists have been suggested to inhibit contraction of pTM cells and reduce resistance through the conventional route by activation of CB receptors in the TM.

Ion channels (L- and T-types of calcium channels, and the KATP channel); adrenergic receptors α and β; GABA receptors A, B, and C; prostaglandin FP receptors; dopaminergic receptors; and phosphodiesterases do not appear to mediate contractility of pTM cells in this 3-D collagen gel assay system.

Conclusion

In summary, the 3-D collagen gel contraction monitoring system described here provides a rapid and sensitive screening method for identifying novel agents that can induce pTM cell relaxation. This technique identifies crucial determinant pathways that affect increases in conventional outflow through increases in pTM cell relaxation, and will be useful in identifying new anti-glaucoma drugs.

References


