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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 outflow and Intraocular Pressure (IOP). It has been suggested that TM relaxation may increase conventional outflow by increasing tissue porosity and lead to decline 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 (rescovitine), rho and Ca^{2+}-dependent protein kinase (Y-27632), tyrosine kinase (tyrphostin AG879), phosphatidylinositol 3-kinase (bisindolylmaleimide I, BIM I), and Ca^{2+}/calmodulin kinase (chelerythrine), strongly inhibited collagen gel contraction. This contraction was also inhibited by ethacrynic acid, by inhibitors of Na^+, K^+-ATPase (ouabain), Ca^{2+}-ATPase (thapsigargin), and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, by a non-selective phosphodiesterase inhibitor (papaverine), and by adenosine A_2 (metrifudil), and cannabinoid receptor (CP-55940) agonists. In addition, BIM I, simvastatin, BQ-123, and CP-55940 demonstrated partial cytotoxicity. The inhibitory activities of chelerythrine and thapsigargin were also attributed to 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-1; 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 Ca^{2+}-dependent Protein Kinase; TGF-1β: Ttransforming Growth Factor-β1; TIMP: Tissue Inhibitors of Metalloprotease; TM: Trabecular Meshwork; TMCs: Trabecular Meshwork Cells; 3-D: Three-Dimensional

Introduction

Glaucoma 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 ethacrynic 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

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decrease IOP in monkey and rabbit [10,13]. 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 epithelium [20], vitreous body hyalocytes [21], and TM [22]. Fetal Bovine Serum (FBS) [23] and various growth factors [24] such as Transforming Growth Factor-β1 (TGF-β1), Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF)-BB, Basic Fibroblast Growth Factor (bFGF), fibronectin, and vitronectin all can induce collagen gel contraction [25]. Nakamura et al. [22] reported that TGF-β1 induced collagen gel contraction in bovine TM cells through activation of Rho and Ca^{2+}-dependent Protein Kinase (ROCK) and Myosin Light-Chain Kinase (MLCK), and they showed that these signaling molecules contributed to rearrangement of the actin cytoskeleton, cell spreading, and cell motility.

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.

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; tyrphostins AG879, AG82, AG490, AG494, AG527, AG555, and AG1296; papaverine hydrochloride; H-7, bisindolylmaleimide I (BIM I); chelerythrine chloride; ML-9; thapsigargin; LY294002; ouabain; BQ-123; BQ-788; N6-cyclohexyladenosine; metrifudil; N6-(2-amino-phenyl) ethyladenosine; CGS15943; 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 were from Invitrogen Corp (Carlsbad, California, USA). The 15-deoxy prostaglandin J2 was obtained from BIOMOL International L.P. (Plymouth Meeting, PA, USA). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-acetyl LDL was from Biomedical Technologies Inc (Stoughton, MA, USA). N-(6-Aminohexyl)-5-chloro-1- naphthalenesulfonamide hydrochloride (W-7), ethacrynic acid, nitrendipine, sodium pravastatin, simvastatin, genistein, Hoechst 33258, and 4% paraformaldehyde solution were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cellmatrix (acid solubilized porcine type-I collagen) was from Nitta Gelatin Inc. (Osaka, Japan). Hank's Balanced Salt Solution (HBSS) was from Nussui Pharm Co, Ltd. (Tokyo, Japan). Porcine eyeballs were obtained from Hokkaido Livestock Corp (Hokkaido, Japan), a local abattoir.

Porcine eyeballs were bisected along the equator, and TM tissue was isolated from the anterior segment. Explant pTM cultures were initiated in a 6-well multiwell plate, in DMEM medium that contained 10% FBS, 100 μg/ml kanamycin sulfates, and 1.25 μg/ml amphotericin B, under 5% CO_2 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 cm^2 cell culture flasks. For the current study, we used confluent second- and third-passage cells.

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 (5×10^3 cells/100 μl/well), cultured overnight, and the culture medium was discarded. Then, 5 μg/ml DiI-Acetyl LDL, dissolved in DMEM containing 10% FBS, was added to the wells, and cells were incubated at 37°C for 5 hrs. The pTM cells were then washed twice with HBSS. Cell viability was evaluated based on uptake of 200 μM Hoechst 33258 dye, which was added to the same multiwell plate and incubated at 37°C for 5 minutes. The pTM cells were then washed twice with HBSS, and fixed in 4% paraformaldehyde at room temperature for 10 minutes. Both probes, DiI and Hoechst 33258, were observed by fluorescence microscopy (Olympus, Tokyo, Japan). The DiI staining was evaluated based on red fluorescence with excitation 550 nm / emission 565 nm. The Hoechst 33258 staining was observed using a blue fluorescence filter with excitation 365nm / emission 465 nm.

The pTM cells were harvested and gently mixed in an ice-bath with type I collagen obtained from porcine tails. The collagen-pTM-cell suspension (final concentration of type I collagen, 1 mg/ml; final volume, 500 μl; final cell density, 1×10^5 cell/ml) was added to each well of a 24-well multiwell plate and polymerized at 37°C for 1 h. Various concentrations of agents (10^{-8} – 10^{-5} M), prepared with DMEM containing 0.5% or 2% FBS, were overlaid onto the collagen gels. Collagen gels were freed using a microspatula and cultured in 5% CO_2 at 37°C for 48 hrs. After cultivation, collagen gels were stained with 0.1% crystal violet solution and their areas were calculated based on measurements obtained with NIH image software version 1.6.2.

The pTM cells were seeded in 96-well multiwell plates (final volume, 100 μl; final cell density, 5×10^3 cell/ml) and cultured overnight. The effects of drug exposure on cell viability were measured in 0.5% FBS, without dispersing the pTM cells, in a collagen-containing medium. The culture medium was discarded and various concentrations of agents (10^{-8} to 10^{-5} M), prepared with DMEM containing 0.5% FBS, were added in a 100 μl volume and the cells were cultured under 5% CO_2 at 37°C. After 48 hrs, the medium was replaced with DMEM containing 20 μM resazurin and the cells were incubated for a further 2 hrs. Fluorescence intensity at 590 nm was then measured by excitation at 560 nm (GEMINI EM, Molecular Devices, Sunnyvale CA, USA). Cell viability was determined based on reduction of resazurin, since resazurin acts as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor.

Various agents were evaluated based on their ID_{50} and LD_{50}. ID_{50} is the dose needed to cause a 50% inhibition in gel contractile activity induced by 0.5% or 2% FBS, while LD_{50} is the dose needed to cause 50% cytotoxicity. If the ID_{50} value was lower than the LD_{50} value, collagen gel

contraction was considered to have been inhibited without cytotoxicity. If the ID_{50} value was somewhat less than the LD_{50} value, collagen gel contraction was considered as weakly inhibited without or with partial cytotoxicity. If the ID_{50} value was equal to the LD_{50} value, collagen gel contraction was considered to be inhibited with cytotoxicity.

Data were analyzed by the Student's *t*-test. P-values of <0.05, <0.01, and <0.005 were considered to be statistically significant.

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). DiI 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_1 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^{-7} 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^{-6} to 10^{-5} 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^{-5} 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 Ca^{2+}-calmodulin-dependent phosphodiesterase (W-7), were not effective in this assay.

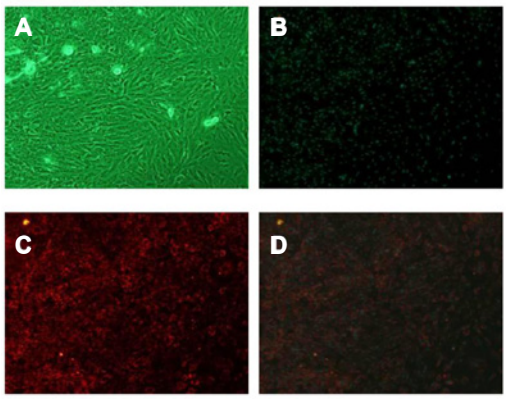

Figure 1: Identification of Porcine Trabecular Meshwork Cells (pTMCs) by detection of acetyl Low Density Lipoprotein (LDL) receptors and nuclei; pTMCs were cultured in multi-plate wells. (A) Shows spindle shapes by phase microscopy. (B) and (C) show fluorescence micrographs of nuclei and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate DiI-labeled LDL, respectively, (D) merged image of both fluorescence pictures.

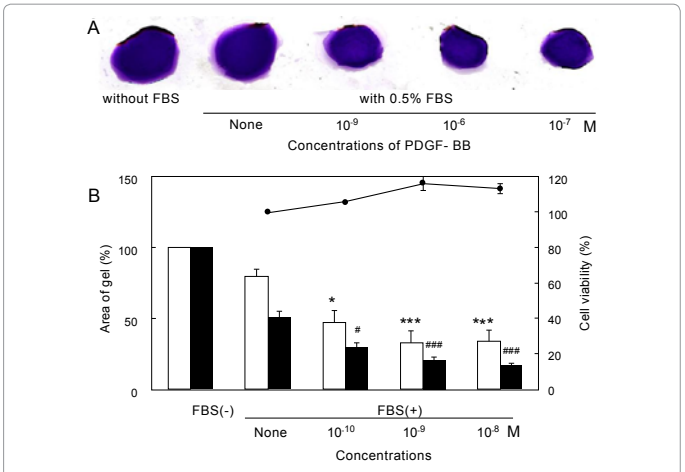

Figure 2: Effect of Fetal Bovine Serum (FBS) and additive Platelet-derived Growth Factor (PDGF)-BB on contraction of collagen gels containing Porcine Trabecular Meshwork Cells (pTMCs). (A) Representative photographs of a collagen gel stained by crystal violet after 48 h culture. (B) Effect of PDGF-BB 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.

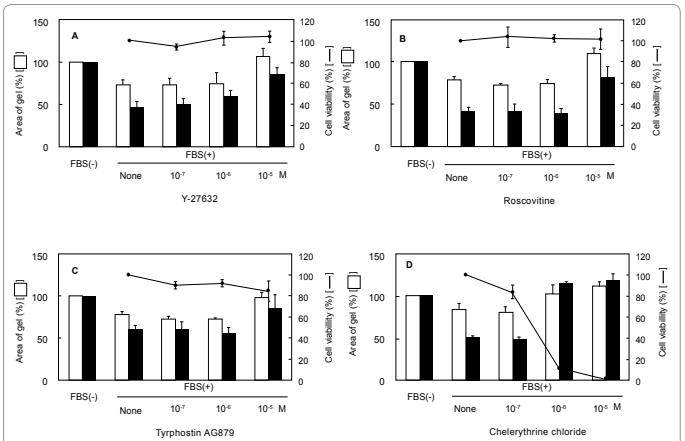

Figure 3: Effect of Y-27632 (A), roscovitine (B), tyrphostin AG879 (C), and chelerythrine chloride (D) on collagen gel contraction induced by 0.5% or 2% Fetal Bovine Serum (FBS) and on cell viability. Various concentrations of inhibitors were added and cells were cultured on collagen gels in the presence of FBS at a concentration of 0.5% or 2%. The areas of collagen gels were measured and data are shown as % of collagen gel area without FBS. Open bars indicate 0.5% FBS-stimulated contraction and solid bars indicate 2% FBS. Symbols, "-" or "+" indicate absence or presence of FBS. Dotted line indicates cell viability (%). Data represent the mean ± SE of triplicate measurements. *P<0.05, *P<0.01 versus 0.5% FBS alone, #P<0.05, ###P<0.005 versus 2% FBS alone.

The thiol group inhibitor ethacrynic acid inhibited collagen gel contraction at a concentration of 10^{-5} M (Figure 4A). The Ca^{2+}-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^{-8} M to 10^{-5} 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 5B) and the endothelin receptor A antagonist BQ-123 (Figure 5C) inhibited contraction at a concentration of 10^{-5} M. However,

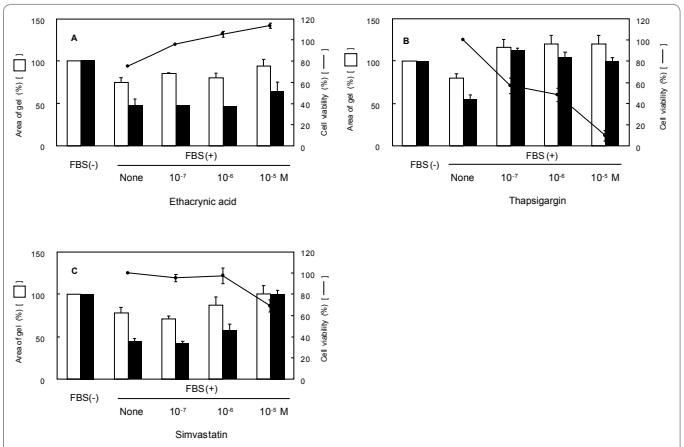

Figure 4: Effects of ethacrynic acid (A), thapsigargin (B), and simvastatin (C) on collagen gel contraction induced by 0.5% or 2% Fetal Bovine Serum (FBS) and on cell viability. Various concentrations of inhibitors were added and cells were cultured on collagen gels in the presence of FBS at a concentration of 0.5% or 2%. The areas of collagen gels were measured and data are shown as % of collagen gel area without FBS. Open bars indicate 0.5% FBS-stimulated contraction and solid bars indicate 2% FBS. Symbols, "-" or "+" indicate 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<0.01, ###P<0.001 versus 2% FBS alone.

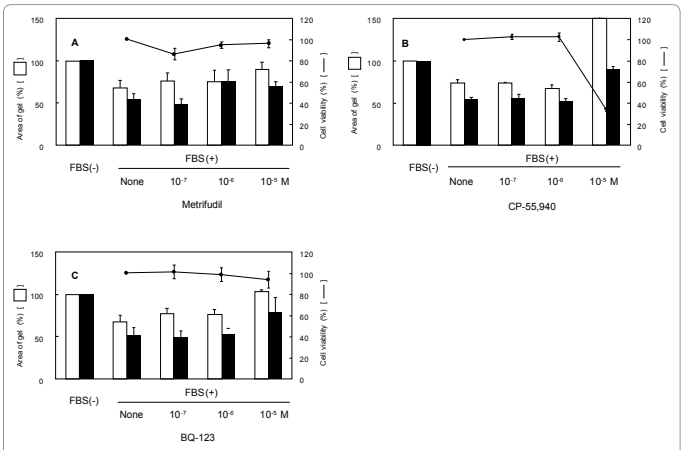

Figure 5: Effects of metrifudil (A), CP-55,940 (B), and BQ-123 (C) on collagen gel contraction induced by 0.5% or 2% Fetal Bovine Serum (FBS) and on cell viability. Various concentrations of inhibitors were added and cells were cultured on collagen gels in the presence of FBS at a concentration of 0.5% or 2%. The areas of collagen gels were measured and data are shown as % of collagen gel area without FBS. Open bars indicate 0.5% FBS-stimulated contraction and solid bars indicate 2% FBS. Symbols, "-" or "+" indicate 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<0.005 versus 2% FBS alone.

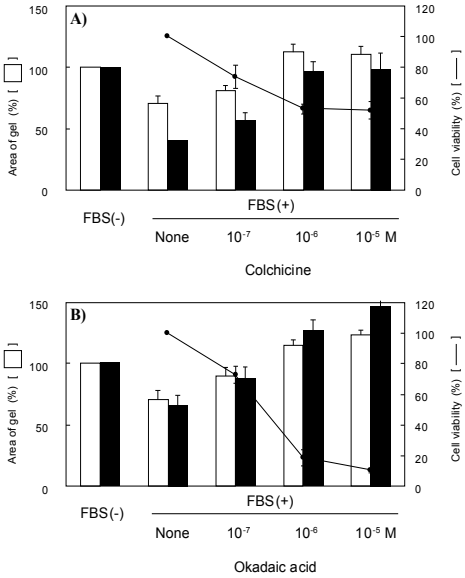

Figure 6: Effects of colchicine (A) and okadaic acid (B) on collagen gel contraction induced by 0.5% or 2% FBS and on cell viability. Various concentrations of inhibitors were added and cells were cultured on collagen gels in the presence of FBS at a concentration of 0.5% or 2%. The areas of collagen gels were measured and data are shown as % of collagen gel area without FBS. Open bars indicate 0.5% FBS-stimulated contraction and solid bars indicate 2% FBS. Symbols, "-" or "+" indicate absence or presence of FBS. Dotted line indicates cell viability (%). Data represent the mean ± SE of triplicate measurements. ***P < 0.005 versus 0.5% FBS alone. #P < 0.05, ###P < 0.005 versus 2% FBS alone.

both agents showed partial cytotoxicity. The K-ATP channel blocker glibenclamide and the Ca^{2+} channel blocker nitrendipine showed a weaker inhibition of contraction when compared to the other kinase and enzyme inhibitors (data not shown).

The cytoskeletal agent colchicine (Figure 6A) and the phosphatase inhibitor okadaic acid (Figure 6B) inhibited collagen gel contraction in a concentration-dependent manner; however, cytotoxicity also occurred along with this inhibition.

Table 1 summarizes the inhibitory activity and cytotoxicity seen in response to these various agents and shows their relationships to ID_{50} and LD_{50}. As previously mentioned, the compounds are categorized based on differences between their ID_{50} and LD_{50} values. In group A, roscovitine, Y-27632, and tyrphostin AG879 were the most effective at inhibiting collagen gel contraction without inducing cytotoxicity in the presence of either 0.5% or 2% FBS. Ethacrynic acid inhibited contraction in the presence of 0.5% FBS, but this effect was attenuated in the presence of 2% FBS. Metrifudil, cyclohexyladenosine, nitrendipine, and wortmannin showed much weaker inhibition than did ethacrynic acid. On the other hand, compounds in group B, which included simvastatin BQ-123, BQ-788 (endothelin receptor B antagonist), LY294002 (PI3-K inhibitor), and CGS15943 (non-selective adenosine antagonist), inhibited contraction, but showed partial cytotoxicity. Compounds in group C, which included chelerythrine chloride, ouabain, and okadaic acid, effectively inhibited contraction, but it was associated with substantial cytotoxicity.

Discussion

The aim of this study is to investigate the effect of various different TM functional inhibitors on contractility and cytotoxicity using 3-D

Compounds	ID_{50} with 0.5% FBS	ID_{50} with 2% FBS	LD_{50}
ID_{50}<<LD_{50}			
Roscovitine	1.23	7.50	–
Y-27632	4.40	6.06	–
Tyrphostin AG879	5.91	8.19	–
Ethacrynic acid	2.64	10.0	–
Metrifudil	6.09	10.0	–
N^6-(2-Amino-phenyl)ethyladenosine	9.65	10.0	–
N^6-Cyclohexyladenosine	10.0	10.0	–
Nitrendipine	9.65	10.0	–
Wortmannin	10.0	10.0	–
ID_{50}<LD_{50}			
H-7	5.78	9.65	>10.0
Sodium pravastatin	10.0	10.0	>10.0
Simvastatin	2.07	4.04	>10.0
BQ-123	3.56	8.93	>10.0
BQ-788	3.05	7.88	>10.0
LY294002	3.98	10.0	>10.0
CGS15943	2.28	3.00	>10.0
15-Deoxy prostaglandin J_2	3.21	7.33	>10.0
ID_{50}>LD_{50}			
CP55940	2.51	6.80	7.84
BIM I	2.57	3.40	7.21
Chelerythrine chloride	0.49	0.44	0.52
Ouabain	0.06	0.36	0.07
Okadaic acid	0.08	0.07	0.05
			(x10^{-6} *M*)

− : not determined

Table 1: ID_{50} and LD_{50} Values for Various Inhibitors of Collagen Gel Contraction by pMA Cells.

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_{50} and LD_{50} 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_1 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 -λ; novel PKCs, including PKC-δ, -ε, -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 tyrphostin 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^{-6} 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 tyrphostin AG 879 on outflow or IOP. Two other tyrosine kinase inhibitors, tyrphostin 51 and genistein, inhibited calbachol-induced contraction of isolated bovine TM strips [13]. However, Volberg et al. reported that the tyrphostins 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 tyrphostins (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 P13-K with TM has not been demonstrated, we suggest that little, if any, relationship exists between P13-K and contractility.

Inhibition of other enzymes, e.g., Na^+, K^+-ATPase (ethacrynic acid and ouabain), Ca^{2+}-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.

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