A Magnetic Bead-Based Method for Mouse Trabecular Meshwork Cell Isolation

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PURPOSE. Mice have been used widely for glaucoma research. However, due to the small size of the mouse eye, it is difficult to dissect mouse trabecular meshwork (MTM) tissues and establish MTM cell strains. To circumvent this problem, we took advantage of the phagocytic property of trabecular meshwork (TM) cells, and developed a novel magnetic bead-based method that enables us to isolate pure MTM cells.

METHODS. After anesthesia, up to 2 μ L of fluorescent or magnetic microbeads were injected intracamerally into the mouse eyes. To study the distribution and localization of the beads, mice were sacrificed 1 to 7 days after injection, and eyes were enucleated for fluorescent or transmission electron microscopy (TEM) study, respectively. To isolate MTM cells, anterior segments injected with magnetic beads were dissected from 10 to 15 sterilized mouse eyes 7 days after injection. The tissues were digested with collagenase A and purified by using a magnetic field as well as repeated washing.

RESULTS. TEM studies showed that the magnetic beads were located in the mouse TM, but not in corneal or scleral fibroblast cells. Cultured MTM cells were similar morphologically to human TM cells. MTM cells expressed TM markers, including collagen IV, laminin, and α -smooth muscle actin. Also, MTM cells treated with 100 nM dexamethasone showed increased formation of cross-linked actin networks and induction of myocilin expression.

CONCLUSIONS. The magnetic bead-based method is efficient for isolating MTM cells with minimal microdissection techniques required. It will be a useful approach for isolating TM cells from small animals for glaucoma research.

Keywords: trabecular meshwork, mouse models, cell isolation, glaucoma, magnetic beads

Glaucoma is a leading cause of visual impairment worldwide with primary open-angle glaucoma (POAG) being the most prevalent type. Although the exact disease mechanism of POAG is not clear, elevated IOP has been found to be the primary risk factor and a causative factor.^{1–4} IOP is determined by aqueous humor (AH) production, AH outflow resistance, and episcleral vein pressure.⁵ In POAG patients, increased aqueous humor outflow resistance is the key contributor to IOP elevation.⁵

The majority of the AH outflow resistance is generated at the trabecular meshwork (TM),⁵ a circular, thin layer of tissue located at the inner surface of the limbus between the iris and cornea. This meshwork-like tissue consists of TM cells and TM beams. The AH flows through the TM and enters the Schlemm's canal, where it is directed into aqueous veins and then episcleral veins. Glaucomatous insults to the TM alter TM structure, damage TM cells, impair TM functions, and eventually cause IOP elevation. In the glaucomatous TM (GTM), a set of pathologic changes, including loss of TM cells, formation of excessive cross-linked actin networks (CLANs), and thickened TM beams, have been observed.⁵ Because lowering IOP is an effective therapy for preventing optic nerve damage, there is an increasing interest in elucidating the pathogenesis in the TM, as well as restoring TM function and cellularity.

The most frequently used TM cells in glaucoma research are isolated from human, bovine, and porcine eyes. However,

one major drawback of using these cell strains is their mixed genetic background. In recent years, the mouse glaucoma model has been introduced into glaucoma research.⁶ Different from human, cows, and swine, lab mice have stable and clearly defined genetic backgrounds. In addition, their fast proliferation, and the availability of transgenic as well as knockout mice make them a powerful tool for glaucoma research.

Although mouse glaucoma models have many advantages, one key issue is the difficulty in isolating mouse TM cells. Due to the size of the mouse eye, it is a challenge to dissect pure mouse TM (MTM) cleanly without non-TM tissue/cell contamination. To our knowledge, the only published study of establishment of MTM cell strains was reported by Tamm et al., who microdissected and cultured MTM cells.⁷ However, that method is time-consuming and technically demanding.

To circumvent this problem, we developed a magnetic bead-based method for MTM cell isolation. TM cells have the unique property of phagocytosis that other ocular cell types (except macrophages) in the anterior segment do not possess.⁸ Therefore, when magnetic beads are injected into the anterior chamber, they will be phagocytized by MTM cells. The MTM cells with intracellular magnetic beads can be dissociated and separated from non-TM cells by applying a magnetic field.

METHODS

Animals

All animal procedures performed in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all protocols and regulations established by the Animal Care and Use Committee of the University of North Texas Health Science Center (UNTHSC). Adult female BALB/cJ mice (20–25 g body weight, 3–6 months old; Jackson Laboratories, Bar Harbor, ME) were housed in transparent plastic rodent boxes under 12-hour light/dark cycle with lights on starting at 6 AM. Mouse chow and water were available *ad libitum*.

Intracameral Injection

Animals were anesthetized with ketamine and xylazine, as well as with topical ocular Alcain (Alcon Laboratories, Inc., Fort Worth, TX). To drain aqueous humor, a paracentesis was performed at the limbal region using a 30-gauge syringe needle. During this procedure, caution was exercised to avoid damaging the iris or lens. Fluorescent Yellow Low Intensity beads (1.7–2.2 μm in diameter) or Magnetic Polystyrene Smooth Surface beads (2.0–2.9 μm in diameter; Spherotech, Inc., Lake Forest, IL) were sterilized with 70% ethanol (ETH) and suspended in PBS. Up to 2 μL 1% (wt/vol) sterile fluorescent or magnetic beads were injected slowly (over 30 seconds) intracamerally into the left eye by using a 33-gauge syringe needle and a Hamilton syringe. The contralateral eye was not injected according to UNTHSC Institutional Animal Care and Use Committee (IACUC) policy. After injection, buprenorphine and topical bacitracin were administered to minimize pain and prevent infection, respectively.

Imaging of Fluorescent Beads

After euthanasia, mouse eyes injected with or without fluorescent beads were enucleated and fixed with 4% paraformaldehyde in PBS. After PBS washing, mouse eyes were imaged using the Nikon Ti Epifluorescent microscope (Nikon, Inc., Melville, NY) or the ChemiDoc XRS+ System (Bio-Rad Laboratories, Inc., Hercules, CA) imaging system.

Transmission Electron Microscopy (TEM)

Mouse eyes injected with magnetic beads were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in PBS. Fixed mouse eyes were stained with osmium solution, dehydrated, and embedded in Polybed 812 with or without propylene oxide washing. We found that the morphology of the beads was well-preserved in samples without propylene oxide washing. Ultrathin sections were prepared and stained with uranyl acetate/lead citrate for imaging. Images were taken using the Zeiss EM910 (Carl Zeiss, Thornwood, NY) or the FEI Tecnai 12 (Walkersville, MD) transmission electron microscope.

MTM Cell Isolation

Mice were sacrificed 7 days after injection. Mouse eyes were enucleated, soaked in Betadine (Purdue Pharma LP, Stamford, CT) for 2 minutes, and rinsed in PBS at room temperature. Those eyes were dissected carefully under a surgical microscope. After trimming extraocular tissues, the eyes were cut at approximately 0.5 mm posterior to the equator (Fig. 1A). The retina, choroid, vitreous, and lens were removed carefully (Fig. 1B). The remaining anterior segment was cut radially at the limbal region twice (e.g., at 12 and 6 o'clock) to facilitate enzymatic digestion (Fig. 1C). Such tissues were pooled from

13 to 15 mice, and transferred to a 1.5 mL test tube containing 4 mg/mL collagenase A (Worthington Biochemical Corporation, Lakewood Township, NJ) and 4 mg/mL BSA dissolved in PBS. After 2 or 4 hours of incubation at 37°C, the tube was attached to a magnet on the tube hinge side. Medium containing cells that did not phagocytize the beads were aspirated carefully from the tube. With the magnet detached, 1 mL medium containing serum was added to the tube to resuspend the cell pellet. Cells with beads were pelleted again with the magnet. The cell pellet was resuspended in 5 mL medium and filtered through a 100 μm cell strainer (Thermo Scientific, Worcester, MA) to remove any tissue fragments. Alternatively, this step and the following centrifugation could be performed right after enzymatic digestion. The flow through was centrifuged at 600g for 10 minutes. With the help of the magnet, culture medium was removed carefully. The cell pellet was resuspended in 0.5 to 1 mL culture medium, and seeded into a 96-well plate with approximately 200 μL cell suspension per well.

MTM Cell Culture

MTM cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-low or high glucose medium supplemented with 10% fetal bovine serum as well as glutamine and antibiotics (Thermo Scientific). Medium was changed every other day. When cells were approximately 90% confluent, they were trypsinized and passaged at a ratio of 1:2 or 1:3.

Immunocytochemistry

MTM cells were cultured on coverslips in 24-well plates. Cells were fixed in 4% paraformaldehyde at 4°C for 30 minutes. After PBS washing, cells were incubated with 0.5% Triton X-100 (Fisher Scientific, Pittsburgh, PA) in PBS at room temperature for 30 minutes, and then blocked with Superblock (Thermo Scientific). Cells were incubated with the primary antibody at room temperature (RT) for 2 hours or 4°C overnight, and the corresponding secondary antibody at RT for 1 hour. After PBS washing, cells were mounted with Anti-fade Prolong Gold containing 4',6-diaminido-2-phenylindole (DAPI; Invitrogen Corporation, Carlsbad, CA).

The following antibodies were used for immunostaining.

Primary antibodies: rabbit anti-collagen IV (Col IV, 1:100; Sigma-Aldrich Corporation, St. Louis, MO), rabbit anti-Laminin (1:100; Sigma-Aldrich Corporation), and mouse anti- α -smooth muscle actin (α -SMA) conjugated with FITC (1:100; Sigma-Aldrich Corporation).

The secondary antibody was: goat anti-rabbit alexa-488 (1:200 or 500; Invitrogen Corporation)

Assessment of CLANs

Confluent MTM cells cultured on coverslips in 24-well culture plates were treated with 0.1% ETH as a vehicle control or 100 nM dexamethasone (DEX; Sigma-Aldrich Corporation) for 10 days. Cells were processed as described in "Immunocytochemistry" except that phalloidin-Alexa-488 (1:100; Lonza, Walkersville, MD) or 568 (1:100; Invitrogen Corporation) was used to stain actin stress fibers at RT for 4 hours or 4°C overnight. CLANs were defined as web-like structures containing at least three triangles.⁹ CLAN formation rate was expressed as the ratio of CLAN-positive cells, that is, CLAN-positive cells/total number of DAPI-stained cells. For each coverslip, 5 regions were counted with each region containing 80 to 200 cells. CLANs were counted in a masked manner.

FIGURE 1. Dissection of the mouse anterior segment. (A) A side view of the mouse eye. *Dotted line*: the equator. (B) The anterior segment of the mouse eye with the lens removed. (C) The mouse anterior segment was cut radially at 12 and 6 o'clock (*dotted lines*) for tissue dissociation and TM cell isolation.

Western Immunoblotting

MTM cells cultured in a 12-well plate were treated with 0.1% ETH or 100 nM DEX for 10 days. After PBS washing, MTM cells were lysed in 100 μ L M-PER lysis buffer (Thermo Scientific). Protein concentration was measured with the Protein DC kit (Bio-Rad Laboratories, Inc.). Total protein (30 μ g) was mixed with Laemmli buffer and boiled for 5 minutes before loading. Proteins were separated by 10% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was blocked with 10% dry milk, and probed with rabbit anti-myocilin antibody (H-130; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and goat anti-rabbit secondary antibody conjugated with HRP (Cell Signaling

Technology, Inc., Denver, MA). Signals were developed using the SuperSignal West Femto Substrate (Thermo Scientific) and detected by the FluoroChem digital imaging system (Cell Biosciences, Inc., Santa Clara, CA). The membrane was stripped, reblocked, and reprobed with mouse anti- β -actin antibody (Millipore) and goat anti-mouse secondary antibody conjugated with HRP (Santa Cruz Biotechnology, Inc.). Signals were developed using the same method as described previously.

Statistical Analysis

Student's *t*-test was used for data analysis. *P* values less than 0.05 were considered significant.

FIGURE 2. Distribution of fluorescent beads in the mouse eye. Fluorescent microbeads were injected into the anterior chamber of the mouse eyes. (A) A side view of a mouse eye. *Green dots*: fluorescent beads. *Arrow*: the anterior chamber angle. *Asterisk*: the iris. *Triangle*: the pupil. (B) A front view of the same mouse eye. *Arrow*: the anterior chamber angle. (C) Another pair of mouse eyes. *Arrow*: fluorescent beads at the anterior chamber angle.

FIGURE 3. Localization of magnetic beads in the mouse eye. Mouse eyes injected with magnetic beads were used for TEM. (A) A low magnification view of the MTM. Triangle: Schlemm's canal. Asterisks: magnetic beads. S, sclera; AC, anterior chamber. (B) A medium magnification view of the MTM, showing phagocytized beads (asterisks). (C) A high magnification view of (B). Note that the beads have a low electron density core and high electron density coating under TEM. (D) Magnetic beads also were present inside macrophages infiltrating into the anterior chamber (arrows).

RESULTS

Distribution and Localization of the Magnetic Beads in the Anterior Segment

We first studied the distribution of the beads in the anterior segment. Because magnetic beads are difficult to image *ex vivo*, we injected fluorescent beads intracamerally and dissected mouse eyes for imaging. We found that the majority of the beads were located at the anterior chamber angle and the anterior surface of the iris, with a few beads attached to the inner surface of the cornea (Fig. 2).

Seeing the global distribution of fluorescent beads in the anterior chamber, we injected magnetic beads intracamerally into mouse eyes. The mouse eyes were collected at different time points for histology studies. Because thick sections (e.g., cryosection or paraffin sections, which are 1–10 μm thick) may lead to false results (i.e., the beads may appear to be inside TM cells, but actually are on the cell surface), we prepared ultrathin sections (~ 90 nm) of mouse eyes for TEM studies. At this thickness, it should be possible to differentiate the localization of the beads to TM cells because the diameter of the beads is 2 to 2.9 μm , which is 20 to 30 times thicker than our ultrathin sections.

On day 1 after injection, we did not find any mouse TM cells that had phagocytized the beads (data not shown). However, 7 days after injection, we observed quite a few TM cells with beads in their cytoplasm (Fig. 3). By TEM examination, the beads appeared as round or oval in shape, with different sizes due to different section angles and position. The polystyrene core of the beads was visible clearly as a homogeneous, low electron density material. In the periphery of the beads, the magnetic particle coating appeared as a ring of high electron density dots. The diameter of the beads measured by TEM nicely matched their actual size. We did not observe any beads inside the cells of the cornea or sclera except macrophages (Fig. 3D), the infiltration of which was due to injection-related inflammation. We did not find clinically significant ocular inflammation (e.g., red eyes, hypopyon) *in vivo*, and macrophages should not impose a threat of non-TM cell contamina-

tion, because macrophages do not proliferate in cultures and will diminish after several passages.

Morphology of MTM Cells

After confirming the localization of magnetic beads in TM cells, we injected those beads into mouse eyes and isolated MTM cells from 13 to 15 mice 7 days after injection. Usually <20 cells 24 hours after seeding were observed. Our cell cultures demonstrated typical TM cell morphology: spindle-like cells that did not grow into “vortex”-like structures that often are seen in fibroblast cell cultures (Fig. 4C). The doubling time of MTM cells was approximately 24 hours at early passages. Initially, the isolated TM cells had multiple magnetic beads in their cytoplasm (Fig. 4A). With proliferation, the beads eventually were diluted and diminished, leaving bead-free MTM cell cultures (Figs. 4A–E).

Characterization of MTM Cells

The difficulty in TM cell identification arises from the lack of TM-specific cell markers. Therefore, a number of criteria, including the expression of Col IV, laminin, α -SMA, as well as DEX-induced formation of CLANs, and DEX-induced expression of MYOC, were used to characterize isolated MTM cells.^{10–12}

We first studied the expression Col IV (Fig. 5A), laminin (Fig. 5E), and α -SMA (Fig. 6C) in MTM cells by immunocytochemistry. All three proteins were expressed in our MTM cell cultures.

FIGURE 4. Morphology and growth of MTM cells *in vitro*. Isolated MTM cells were cultured and imaged using Hoffman modulus optics (Nikon, Inc.). (A) An MTM cell the next day after plating (arrowhead). Magnetic beads were visible inside the cell (arrows). (B–D) The same area as in (A). (B) 3 days after plating. (C) 5 days after plating. (D) 16 days after plating. (E) Another MTM cell strain at passage number 12. Beads were diluted as MTM cells proliferated (C–E).

FIGURE 5. The expression of Col IV and laminin in MTM cells. MTM cell cultures were stained with anti-Col IV (A) or anti-laminin (E) antibody. (C, G) Cell cultures stained with only secondary antibody as a negative control. (B, D, F, H) Nuclear staining with DAPI.

FIGURE 6. The formation of CLANs in MTM cells. Confluent MTM cell cultures were stained with phalloidin-Alexa-488/568 (A, B) or anti-SMA-FITC (C). CLANs are visible as web-like structures with spokes and hubs (circled areas in [A]). (B, C) High magnification views of CLANs. (D) MTM cell cultures treated with DEX for 10 days showed significantly more CLAN-positive cells compared to ETH (vehicle)-treated controls. Columns and error bars represent means and standard deviations, respectively. *** $P < 0.001$.

FIGURE 7. DEX induced the expression of myocilin in MTM cells. MTM cell cultures were treated with ETH or DEX for 10 days, and whole cell lysate was used for WB. β -Actin was used as a loading control. MYOC, myocilin. $N = 3$.

Second, we compared the formation of CLANs in MTM cells treated with 0.1% ETH (vehicle control) or 100 nM DEX for 10 days. CLANs are web-shaped structures consisting of spokes and hubs¹¹ (Figs. 6A–C). After 10-day DEX treatment, the percentage of CLAN-positive cells increased by approximately 3-fold ($9.8\% \pm 4.5\%$ vs. $30.7 \pm 7.4\%$, $n = 5$ or 6 , $P < 0.001$, Fig. 6D).

Finally, we compared the expression of *MYOC*, a glucocorticoid-inducible gene in the TM,^{12,13} in our MTM cell cultures treated with 0.1% ETH (vehicle control) or 100 nM DEX for 10 days. Western immunoblotting showed that there was a significant elevation in *MYOC* expression upon DEX treatment (Fig. 7).

DISCUSSION

We took advantage of the phagocytic feature of MTM cells and used magnetic beads for MTM cell isolation. Our MTM cell cultures showed TM characteristics, including the expression of Col IV, laminin, α -SMA, as well as DEX-induced CLAN formation, and *MYOC* expression. All these findings supported that our cells isolated from mouse eyes were TM cells. Compared to traditional methods that are based on microdissection of the TM tissue, our method is less technically challenging. Therefore, we believe that this method is suitable for TM cell isolation from small animals, for example, mice and rats. For animals with large eyes, direct dissection may be a better option.

The magnetic beads that we used have a polystyrene core coated with magnetic particles. These beads have a “smooth” surface and, therefore, are less toxic to cells, according to manufacturer’s instructions. We did not observe significant ocular inflammation after bead injection. However, whether other “nonsmooth” types of beads also are suitable for TM isolation is not clear and must be investigated. Theoretically, fluorescent beads also may be used in combination with fluorescence-activated cell sorting (FACS). However, the low yield of MTM cells makes this option impractical.

A major issue in TM cell isolation is non-TM cell contamination, and the cells that line the inner surface of the anterior chamber are the potential sources of contamination. These cells include fibroblast cells, corneal endothelial cells, iridal cells, ciliary epithelial cells, and lens epithelial cells. Among these cell types, corneal endothelial cells do not proliferate, while the lens is removed completely before processing the TM. Thus, these two cell types should not cause contamination. Cells from the iris or ciliary body do not proliferate well in TM culture medium. Therefore, fibroblast cells from either the cornea or sclera are the potential sources of contamination. Nevertheless, there have not been any reports of the phagocytic feature of corneal or scleral fibroblast cells to our knowledge. Second, our TEM studies did not show beads localized in non-TM tissues. Third, the cornea fibroblast

cells and scleral fibroblast cells are shielded from the beads by the Descemet’s membrane as well as the TM and Schlemm’s canal, respectively. Based on these facts, we reasoned that our MTM cell strains are of high purity.

The optimal time to harvest MTM cells is on day 7. It seems that MTM cells need more time to “ingest” sufficient beads for magnetic isolation. However, whether leaving beads in mouse eyes for more time will produce better results is not clear. Longer times may decrease the overall yield because the beads may be cleared from TM cells by exocytosis.

Although the yield of this method was low (usually <20 cells 24 hours after seeding), MTM cells proliferate rapidly with a doubling time of approximately 1 day at early passages. These cells could be passaged at least 25 times without significant signs of senescence. In another study, we grew p15 MTM cells into confluency in twelve 100 mm culture dishes, which suggests that these cells still are actively proliferating. However, all primary cells will lose the ability of proliferation. A potential solution to this problem is to isolate TM cells from transgenic mice carrying the telomerase or SV40 gene,⁷ which is worthy of further investigation.

Being able to isolate TM cells has a number of exciting advantages. We now will be able to dissect molecularly a number of pathogenic signaling pathways using the power of mouse genetics. The cells can be isolated from transgenic and knockout mice to test specific pathways. This approach has distinct advantages because primary TM cells traditionally are difficult to be transfected efficiently.

In summary, our magnetic bead-based method enables researchers to isolate pure MTM cells. We believe that this method will be a powerful tool in obtaining TM cells from wild-type, transgenic, and knockout mice as well as other small experimental animals for glaucoma research.

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