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Cytoskeletal Drugs Prevent Posterior Capsular Opacification in Human Lens Capsule *in vitro*

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Abstract

Background—To determine whether the cytoskeletal drugs H-7 and Latrunculin B (LAT-B) inhibit posterior capsular opacification (PCO) in the cultured human lens capsular bag.

Methods—Following extracapsular cataract (lens) extraction in human donor eyes, the capsular bag was prepared and cultured by standard techniques. Forty-eight capsular bags were studied, in which 13 were treated with H-7 (50, 100 or 300μM), 12 with 1% BSS (vehicle of H-7), 11 with LAT-B (2, 5 or 10μM), and 12 with 0.25% DMSO (vehicle of LAT-B). Forty out of the 48 capsular bags were from paired eyes of 20 donors with 1 bag being treated with H-7/LAT-B and the other with BSS/DMSO for each pair, including 20 for the H-7-BSS protocol and 20 for the LAT-B-DMSO protocol. The medium with the cytoskeletal drug/vehicle was replaced every 3–4 days for 4 weeks. PCO was assessed daily using inverted phase-contrast microscopy and scored on a 4-point scale.

Results—In all cultures with BSS or DMSO, residual lens epithelial cells (LECs) on the anterior capsule migrated to and proliferated on the posterior capsule by 3–7 days and apparent LEC growth on the posterior capsule with severe capsular wrinkling (PCO Grade 3) was seen by 2–3 weeks. When treated continuously with H-7 or LAT-B, the migration and proliferation of LECs and the capsular wrinkling were inhibited in a dose-dependent manner, with the inhibition being complete (PCO Grade 0) in the 300μM H-7 (n=8, p<0.001) or 10μM LAT-B culture (n=3, p=0.002).

Conclusion—H-7 and LAT-B dose-dependently inhibited PCO formation in the cultured human lens capsular bags, suggesting that cytoskeletal drugs might prevent PCO formation after surgery in the human eye.

Keywords

Actin cytoskeleton; H-7; human eye; latrunculin B; lens epithelial cells; posterior capsule opacification

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Introduction

Age-related cataract is a major cause of visual impairment throughout the world. Population based surveys in recent years have reported that the prevalence of blindness in the Indian population among the 50 year age group ranges from 6% to 11.9% and that cataract is the identifiable cause of blindness in 55% to 70% of the blind [1–3]. The preferred means of treatment is to implant a synthetic intraocular lens (IOL) into the lens capsular bag after surgical removal of the opaque lens tissue. Though millions of people benefit from IOL implantation, posterior capsular opacification (PCO)-induced dysopsia is not uncommon. It is possible to treat PCO by Nd:YAG laser, but this is not free from the risk of complications. Moreover, laser capsulotomy may not be compatible with the next generation of accommodating IOLs that need an intact posterior capsule to function [4].

It has been established that PCO is caused by proliferation, migration and epithelial-mesenchymal transition (EMT) of residual lens epithelial cells (LECs) [5]. Several attempts have been made to completely remove the residual LECs by mechanical and pharmaceutical methods without sacrificing the lens capsule integrity [6–10], but none has proved satisfactory. It remains important to develop novel methods or agents to prevent PCO, both for current clinical practice and for future use with accommodating IOLs.

The cellular processes of proliferation, migration and EMT are related to actin cytoskeleton dynamics. Down-regulation of the actin cytoskeleton dynamics in the residual LECs after surgery by cytoskeletal drugs may prevent PCO via inhibiting these cellular processes. Integrin antagonists (e.g., RGD peptide or salmosin), which inhibit focal adhesions [11,12], reduced PCO formation in the rabbit eye [13,14], suggesting that the inhibition of cytoskeleton associated proteins may prevent PCO. However, it remains to be determined if agents that disorganize the actin cytoskeleton and associated proteins indirectly by inhibiting cellular contractility or directly by disrupting the actin microfilaments will prevent PCO more effectively.

H-7, a broad-spectrum serine-threonine kinase inhibitor, reduces actomyosin-driven contractility, leading to cellular relaxation, deterioration of the actin microfilaments and perturbation of their membrane anchorage, and loss of stress fibers and focal adhesions in many types of cultured cells probably by inhibiting myosin light chain kinase and Rho kinase [15–21]. Latrunculin B (LAT-B), a macrolide isolated from the marine sponge *Latrunculia (Negombata) magnifica*, is a specific and potent actin-disrupting agent that directly sequesters monomeric G-actin, leading to destructions of actin bundles and associated proteins and separations of cell-cell adherens junctions in a variety of cultured cells [22–25]. Both H-7 and LAT-B seem to have stronger and more rapid inhibitory effects on the actin cytoskeleton network than RGD peptide, and less toxicity to corneal endothelium than ethylenediaminetetraacetic acid (EDTA) or antimetabolites that had been confirmed to inhibit PCO formation in the literature [26,27]. Therefore, H-7 and LAT-B might have potential as PCO inhibitors.

Thus, the current study was undertaken to determine the effects of H-7 and LAT-B on PCO following extracapsular cataract (lens) extraction (ECCE) in donated human eyes.

Materials and Methods

After the removal of corneo-scleral discs for transplantation, donated human eyes were obtained from the Rotary Aravind International Eye Bank, located at Aravind Eye Hospital, Madurai, India. Eyes were from both sexes and ages from 8 months to 84 years, and were used within 24 hrs of death. In addition to 1 donor eye that was used for Fig. 1a to show

residual LECs on the anterior capsule immediately after ECCE, 48 donor eyes were included in the study (see below).

Extracapsular Lens Extraction

The globe was fixed in a holder to restrict its movement during surgery, and the iris was removed such that the lens-zonular complex was well visualized. A 5-6mm continuous curvilinear anterior capsulorhexis was performed using a 26-gauge needle. Cortical cleaving hydrodissection and hydrodelineation were then performed. One pole of the nucleus was prolapsed out of the capsular bag using a Sinskey hook and the rest wheeled out gently. Manual irrigation/aspiration was performed using the Simcoe cannula, taking care to remove all visible lens fibers. No drug or vehicle was used during surgery. The capsular bag is randomly selected for drug or vehicle treatment after surgery.

Lens Capsular Bag Culture

Following surgery, the capsular bag was dissected free from the zonules and secured on a sterile polymethylmethacrylate petri dish (W120; Bioscience tools, USA) as described by Liu et al [28]. Six to eight entomological pins (D1, Watkins and Doncaster (Kent, UK)) were inserted through the edge of the capsular bag to retain its circular shape. Cultures were maintained at 37°C in a 5% CO₂ atmosphere in Eagle's minimum essential medium (Sigma-Aldrich, St.Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 50mg/L gentamycin (Sigma-Aldrich, St.Louis, MO) for 4 weeks [28]. Different concentrations of H-7 or LAT-B (Sigma-Aldrich, St. Louis, MO) or corresponding vehicles (see below), based on the previous reports [15,18,21,26,29], were added to the medium that was replaced once in 3 to 4 days.

PCO Evaluation

Forty-eight capsular bags were cultured as above, in which 13 were treated with H-7 (50, 100 or 300µM), 12 with 1% balanced saline solution (BSS) (vehicle of H-7), 11 with LAT-B (2, 5 or 10µM), and 12 with 0.25% dimethyl sulfoxide (DMSO) (vehicle of LAT-B). In the 48 capsular bags, 40 were from paired eyes of 20 donors with 1 bag being treated with H-7 or LAT-B and the other with BSS or DMSO for each pair, including 20 for the H-7-BSS protocol and 20 for the LAT-B-DMSO protocol. The other 8 capsules were from 6 donors, including 4 capsules from paired eyes of 2 donors with 2 being used in the BSS group and 2 in the DMSO group, and 4 capsules from single eyes of 4 donors with 3 being used in the H-7 group and 1 in the LAT-B group (Table 1). Observations of proliferation and migration of LECs and capsular wrinkling were performed every day using inverted phase microscope for 28-30 days, and appearance of each capsular bag was recorded. Photographic (Nikon Coolpix E8400; 8 Megapixels) images were collected at least once weekly. PCO was graded on a four-point scale at 28th day of culture: 0-clear, no visible migrating cells on the peripheral and central posterior capsule; 1-mild, migrating/proliferating cells in the periphery only; 2-moderate, proliferating cells on both peripheral and central capsules with sparse wrinkling; 3-dense, apparent LEC growth and severe wrinkle formation on the entire capsule [30, 31]. Treatment of the lens capsular bag with drug or vehicle was masked to the person who maintained the cultures, recorded the microscopic images and carried out the grading. H-7 and LAT-B treated groups were compared with BSS and DMSO control groups respectively. Fisher Exact Test was used to analyze the PCO data with STATA 11.1 (Stata corp, TX, USA). Additionally, during PCO evaluation, floating cells in the medium and viability of remaining cells on the anterior capsule of drug- or vehicle-treated cultures were also observed or evaluated by inverted phase microscopy.

Results

Following ECCE, donor lens capsular bags contained a large population of viable cells on the anterior capsule and these cells showed a typical epithelial morphology (Fig. 1a).

PCO Formation on Control Capsules

In capsular bags treated with 0.25% DMSO, residual LECs on the anterior capsule began to migrate to and proliferate on the posterior capsule through the rhexis region on day 3 to 5 (Fig. 1b). Within 6 to 14 days the cells became confluent on the posterior capsule, resulting in capsular wrinkles by day 14 (Fig. 1c). Subsequently there was an increase in the number of wrinkles along with a confluent monolayer of cells on the posterior capsule (PCO Grade 3). Similarly, capsular bag cultures with 1% BSS also showed severe wrinkle formation and a confluent monolayer of cells on day 28 (Table 1). No floating cells were seen in the medium of the control cultures, and no dead cells were observed on the capsule treated with 1% BSS or 0.25% DMSO during the entire period of culture.

Effect of H-7 on PCO

Although a large population of LECs remained on the anterior capsule of the human lens capsular bag after ECCE, PCO formation was significantly inhibited by H-7 in a dose-dependent manner (Table 1). Of special note, in 2 cultured capsular bags obtained from the same donor aged 8 months with 1 being treated with 1% BSS and the other with 300 μ M H-7, the capsule treated with BSS showed highly aggressive cell proliferation and severe wrinkle formation (PCO Grade 3, Fig. 2a, b), while the capsule treated with 300 μ M H-7 showed total absence of cell proliferation and wrinkling (PCO Grade 0, Fig. 2c, d). Additionally, per observations by inverted phase microscopy during PCO evaluation, floating cells were seen in the medium of all cultures containing H-7, and remaining LECs on the anterior capsule of 300 μ M H-7-treated capsular bags were not viable at the 4th week (Fig. 3a).

Effect of LAT-B on PCO

All 3 capsular bags treated with 2 μ M LAT-B showed apparent LEC growth on the posterior capsule and severe capsular wrinkling (PCO Grade 3). Out of the 5 capsular bags treated with 5 μ M LAT-B, 2 showed complete inhibition of PCO, while 3 showed dense PCO (Table 1). All 3 capsular bags treated with 10 μ M LAT-B showed total absence of migrating and proliferating LECs on the posterior capsule after 4 weeks in culture (PCO Grade 0), in contrast to dense PCO formation in 0.25% DMSO-treated cultures (Fig. 4, Table 1). Additionally, more LECs were retained on the anterior capsule after LAT-B (Fig. 4b; Fig. 3b) than after H-7 (Fig. 2c, d; Fig. 3a), consistent with floating cells being seen in the H-7- but not the LAT-B- treated culture. Remaining LECs on the anterior capsule of 10 μ M LAT-B-treated capsular bags were not viable at the 4th week (Fig. 3b).

Discussion

In this study, we tested whether the cytoskeletal drugs H-7 or LAT-B could prevent PCO formation in the cultured human lens capsular bag. Although it may be more relevant to study PCO clinically or in living animals, details of LEC migration and proliferation and capsular wrinkling are difficult to determine in those situations. Additionally, PCO in patients and experimental animals can be influenced by postoperative inflammation. Therefore, an in vitro model of the human lens capsular bag from donor eyes after ECCE may have advantages [28]. We have cultured the human lens capsular bag in vitro primarily to follow the dynamics of cell growth and capsular wrinkling after cytoskeletal drug (H-7/

LAT-B) or vehicle (BSS/DMSO) treatment. The four-point scale for grading PCO in vitro [30,31] was used to quantify the effectiveness of the drugs.

H-7 disorganizes the actin cytoskeleton by reducing actomyosin-driven contractility, primarily through its inhibitory effects on myosin light chain kinase and Rho kinase [15–21]. H-7 inhibits proliferation, migration and EMT of several non-tumor and tumor cell lines [32–34], affects collagenase production and matrix metalloproteinase release in cultured human keratinocytes and airway smooth muscle cells [35,36], and blocks wound healing in organ cultured rat corneas [37]. Such findings support H-7 as a potential PCO inhibitor. In the present study, H-7 dose-dependently prevented PCO formation and accompanying capsular wrinkling in cultured human lens capsular bags (Table 1). As shown in Figure 2, a number of residual LECs on the anterior capsule migrated to and proliferated on the posterior capsule by day 3 in the BSS-but not 300 μ M H-7- treated culture (Fig. 2a, c), and a confluent monolayer of cells on the posterior capsule was seen in the former but not in the latter by day 30 (Fig. 2b,d), suggesting that H-7 might have inhibited LEC migration and proliferation. Additionally, capsular wrinkling was observed in the culture containing BSS but not in that containing H-7. Since the contractile property of EMT cells was believed to be the basis of capsular wrinkling [38], the extensive wrinkling of the posterior capsule in the BSS-treated culture might indicate the transdifferentiation of epithelial cells into myofibroblasts or EMT. However, more experiments are needed to clarify if H-7 may prevent PCO by inhibiting migration, proliferation, and EMT of residual LECs.

PCO formation is more aggressive in cultured capsules from younger donors [39,40] as well as in younger patients [41]. The complete PCO prevention in the young (8 months) donor by 300 μ M H-7 in this study (Fig. 2b, d) suggests that this concentration of H-7 is substantially effective. The data are also consistent with a recent study where multiple intravitreal injections (twice weekly for 10 weeks) with 50 μ l of 36 mM H-7 (the peak drug concentration in the vitreous or anterior chamber is ~1,200 or 300 μ M) inhibited post-surgical secondary cataract in the rabbit eye [42].

Unlike H-7, LAT-B directly disrupts the actin microfilaments, leading to separations of cell-cell adherens junctions [22–25] and cellular relaxation [43]. In a previous study, LAT-B significantly inhibited proliferation and migration of human mast cells [44]. In the present study, 10 μ M LAT-B completely inhibited PCO formation in the cultured human capsular bags from old donors (65–75 years; Table 1). Similar to H-7, LAT-B might also inhibit LEC migration, proliferation and EMT as shown in Figure 4. Additionally, it remains to be clarified if 10 μ M LAT-B may also inhibit PCO formation in lens capsules from younger donors, as does 300 μ M H-7. In 5 capsular bags, 5 μ M LAT-B completely inhibited PCO in 2 capsules (26, 62 years) but had no effect in the others (3, 57, 75 years). No moderate PCO was observed in the 5 μ M LAT-B-treated capsules. Clearly the lack of the evident gradient regarding the effect of LAT-B on PCO in this group is not due to the donor age because both young and old donors are included in the 2 subgroups that have completely different responses to the drug. Different amounts of residual LECs remaining on the anterior capsule after ECCE could be responsible for this variability in PCO formation. However, similar size of the capsulorhexis and carefully controlled surgery procedures might have minimized the difference in the amount of residual LECs among eyes. Small samples and other unknown causes may be also responsible. Nevertheless, the data show that the effect of 5 μ M LAT-B on PCO is between the effects of 2 μ M and 10 μ M LAT-B and supports an overall dose-response relationship.

During PCO evaluation by inverted phase-contrast microscopy, we had also seen floating cells in the medium of all H-7- but not BSS- treated cultures. The floating cells are most likely residual LECs detached from the anterior capsule by H-7, which suggests that cell

detachment may also play an important role in the H-7-induced PCO inhibition in vitro. Interestingly, no apparent floating cells were seen in the medium of LAT-B-treated cultures. Since focal adhesions are more sensitive to H-7 while β -catenin-rich intercellular adherens junctions are more sensitive to latrunculins [21,45], the different results in cell detachment after H-7 and LAT-B may suggest that focal adhesions are more important than cell-cell adherens junctions for LEC attachment onto the lens capsule. In previous studies, 2 μ M LAT-B had shown a greater effect on outflow facility in monkeys than 300 μ M H-7 [15,29], suggesting that the former may induce greater cytoskeleton disorganization in trabecular meshwork cells. However, in the current data, 300 μ M H-7 had a much greater inhibitory effect on PCO formation than 2 μ M LAT-B. This suggests that inhibition of focal adhesions may play a more important role than separation of cell-cell adherens junctions or microfilament depolymerization in the prevention of PCO.

Previous studies had shown that the effect of H-7 or LAT-B (or the analogue LAT-A) on the actin cytoskeleton was reversible in vivo and in vitro [21, 25–27,45–48], suggesting that these drugs might have no permanent toxicity to cells. However, it also suggests that a single dose of these drugs might not permanently prevent PCO. In the present study, the 4-week exposure of the cultured human lens capsule to 300 μ M H-7 or 10 μ M LAT-B completely inhibited PCO formation, accompanied by dead residual LECs on the anterior capsule (Fig. 3). This indicates that cell death may also be involved in the mechanism of the PCO inhibition, especially when a high concentration of either drug is used for a long term. Additionally, the current data also indicate that a long-term constant drug concentration in the lens capsular bag seems necessary for the cytoskeletal drugs to effectively inhibit PCO. A possible method to meet this condition could be the implantation of a drug-coated IOL [49] after cataract surgery, since a controlled drug release from the coated IOL into the capsular bag might be maintained for 4 weeks or longer. Although the cell toxicity of the drugs may facilitate a permanent prevention of PCO, it also generates an ocular safety concern. However, the drug toxicity might not affect the adjacent tissues in a live eye that has undergone ECCE and implantation of a drug-coated IOL, since the large volume of the anterior chamber and the continuous aqueous humor outflow may significantly decrease the drug concentration when the coated drug slowly diffuses from the lens capsular bag to the anterior chamber. This hypothesis needs to be confirmed in future studies of H-7 or LAT-B in the live non-human primate eye. Nevertheless, the present study indicates that pharmacological perturbation of the actin cytoskeleton in the residual LECs might prevent PCO and accompanying capsular wrinkling in the human eye after cataract surgery. Therefore, it may be worthwhile to test analogs of H-7 (such as rho kinase inhibitors) or LAT-B (agents that directly disrupt actin microfilaments) that are less toxic to cells to clarify whether these cytoskeletal drugs are viable candidates for PCO inhibitors.

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Fig. 1.

a. Residual lens epithelial cells (LECs) on the anterior capsule of the human donor eye immediately after ECCE surgery. The image shows a large number of LECs on the anterior capsule with typical epithelial morphology. The cells were stained with hematoxylin, and were confirmed to be viable by trypan blue dye exclusion test. Arrow indicates capsulorhexis. Scale bar = 100µm. Inner box (enlarged view; scale bar=20µm) shows the hexagonal morphology of LECs. b, c. Phase micrographs of lens capsule in culture with vehicle 0.25% DMSO. Panel (b) shows migrated LECs on the posterior capsule (PC) on day 5 (PCO Grade 2). Scale bar=200 µm. Panel (c) shows severe wrinkling of the PC and migrated LECs on day 14 (PCO Grade 3). Scale bar=200 µm. The asterisk in (b) and (c) (*) indicates capsulorhexis.

Fig. 2.

Phase micrographs of 2 capsular bags from an 8-month donor in culture. Panels (a, b) are images taken on day 3 and 30 respectively from the capsule treated with 1% BSS, showing migrated LECs (a) and severe wrinkle formation (b)(PCO Grade 3). Inner box in b (scale bar=20 μ m) shows the cells behind the wrinkles. Panels (c, d) are images taken on day 3 and 30 respectively from the other capsule of the same donor treated with 300 μ M H-7, showing no migrated cells or wrinkling on the posterior capsule (PCO Grade 0). AC- anterior capsule, PC- posterior capsule. Arrow indicates capsulorhexis. Scale bar = 200 μ m.

Fig. 3.

Phase micrographs of remaining lens epithelial cells (LECs) on the anterior capsule of lens capsular bags after 4 weeks in culture. Panel (a) shows only a few remaining LECs on the anterior capsule of a capsular bag cultured in 300 μ M H-7 (sample from Table 1). Panel (b) shows more remaining LECs on the anterior capsule of a capsular bag cultured in 10 μ M LAT-B (sample from Table 1). The remaining cells (*) on the anterior capsule in (a) and (b) were dead based on their appearance (see viable remaining LECs immediately after surgery in Fig. 1a). Scale bar=100 μ m.

Fig. 4.

Phase micrographs of 2 lens capsular bags from a 65 years old donor in culture. Images were taken on day 28. Panel (a) shows severe wrinkle formation and migrated lens epithelial cells (LECs) on the posterior capsule (PC) of the capsular bag cultured in 0.25% DMSO. Panel (b) shows clear PC and remaining LECs on the anterior capsule (AC) of the other capsular bag from the same donor cultured in 10 μ M LAT-B in 0.25% DMSO. Arrow indicates capsulorhexis. Scale bar =100 μ m.

Table 1
PCO Scores of Human Lens Capsules Cultured in the Presence or Absence of Cytoskeletal Drugs

Cytoskeletal Drug or Vehicle (†)	n	Age in Years [Median (Range)]	Number of Eyes in 4 PCO Grades				p value
			Grade 0 (Clear)	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	
1% BSS [‡]	12	57.5 (0.67–84)	0	0	0	12	-
50μM H-7	2	46 (17–75)	0	0	2	0	≠0.011
100μM H-7	3	66 (64–84)	0	3	0	0	≠0.002
300μM H-7	8	40.5 (0.67–73)	8	0	0	0	<0.001
0.25% DMSO [‡]	12	63.5 (3–77)	0	0	0	12	-
2μM LAT-B	3	72 (53–77)	0	0	0	3	-
5μM LAT-B	5	5 (3–75)	2	0	0	3	≠0.074
10μM LAT-B	3	67 (65–75)	3	0	0	0	≠0.002

PCO was graded on a four-point scale for 48 capsules on the 28th day of culture. Out of the 48 capsules, 40 were from paired eyes of 20 donors with 1 being treated with H-7 or LAT-B and the other with BSS or DMSO for each pair, including 20 for H-7-BSS protocol and 20 for LAT-B-DMSO protocol. The other 8 capsules were from 6 donors, including 4 from paired eyes of 2 donors with 2 being used in the BSS group and 2 in the DMSO group, and 4 from single eyes of 4 donors with 3 being used in the H-7 group and 1 in the LAT-B group. The difference in PCO score between the H-7-treated capsule and the BSS-treated capsule or between the LAT-B-treated capsule and the DMSO-treated capsule was evaluated using the Fisher Exact Test with STATA 11.1 (Stata Corp TX, USA).