Published in final edited form as:

Invest Ophthalmol Vis Sci. 2009 February ; 50(2): 752–759. doi:10.1167/iov.08-2322.

Prevention of Posterior Capsular Opacification through Aldose Reductase Inhibition

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Abstract

Purpose—The purpose of this study was to evaluate the effect of aldose reductase (AR) inhibition on Posterior capsular opacification (PCO) using pig eye capsular bag model.

Methods—Pig eye capsular bags were prepared by capsulorhexis and cultured in medium without or with AR inhibitors for 7 days. Immunostaining was performed in paraformaldehyde-fixed capsular bags to determine the expression of proliferating cell nuclear antigen (PCNA), α -smooth muscle actin (SMA), β -crystallin, and intercellular adhesion molecule (ICAM)-1. We examined the effect of AR inhibition on basic fibroblast growth factor (b-FGF) -induced mitogenic signaling in cultured human lens epithelial cells (HLEC). Cell growth was assessed by MTT assay and cell counting, the expression of α -SMA, β -crystallin and ICAM-1 by Western Blot and immunocytochemical analysis, protein kinases by Western blot analysis, and NF- κ B activation by Gel shift and reporter assays.

Results—During culture of pig eye capsular bags, residual cells on both the anterior and posterior capsule showed vigorous growth. Treatment with AR inhibitors significantly prevented the lens epithelial cell growth in capsular bags and expression of α -SMA, β -crystallin and ICAM-1. HLEC showed a dose-dependent response to b-FGF, proliferation at lower (<20 ng/ml) and differentiation/transdifferentiation at higher (>50 ng/ml) concentrations. Inhibition of AR also prevented the b-FGF-induced activation of ERK1/2, JNK and NF- κ B in HLEC.

Conclusions—Our results suggest that AR is required for lens epithelial cell growth and differentiation/transdifferentiation in the capsular bags indicating that inhibition of AR could be a potential therapeutic target in the prevention of PCO.

Keywords

Aldose reductase; secondary cataract; capsular bag; lens epithelial cells

Introduction

Posterior capsular opacification (PCO), also called secondary cataract, is the most common postoperative complication of cataract surgery. Decrease in visual acuity due to PCO occurs in greater than 25% of subjects who undergo phacoemulsification surgery,^{1,2} with younger

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patients being at higher risk. After cataract surgery PCO develops due to proliferation and differentiation and/or transdifferentiation of residual lens epithelial cells (LEC) at the equator and beneath the anterior lens capsule. The residual lens epithelial cells proliferate and migrate on top of the posterior capsule underneath the intraocular lens causing the obstruction in the vision. These cells may keep proliferating, or undergo normal fiber differentiation or transdifferentiation, a change in phenotype from an epithelial to myofibroblastic/fibroblastic phenotype leading to opacification.³ Therefore post-surgical pharmacological inhibition of LEC proliferation, migration, and transdifferentiation is a potential opportunity to prevent PCO. In this context, several drugs that can block LEC proliferation and differentiation/transdifferentiation have been studied, but to date none of them have been shown to be clinically effective.⁴⁻⁷

Many studies have suggested the role of several cytokines and growth factors such as IL-6,⁸ transforming growth factor (TGF)- β ,^{3,9} fibroblast growth factor (FGF)-2,^{10,11} hepatocyte growth factor (HGF),^{12,13} and epidermal growth factor (EGF)¹⁴ in the development of PCO. Subsequent to cataract surgery, the levels of these cytokines and growth factors are elevated in aqueous humor influencing proliferation, migration and differentiation/transdifferentiation of LEC. Basic FGF or FGF-2 (b-FGF), an important growth factor that establishes and maintains normal lens physiology, is constantly present in the normal lens milieu.¹⁵ Basic FGF regulates proliferation, and promotes the differentiation of lens epithelial cells to lens fiber cells.¹⁶ Studies have shown that b-FGF induces LEC proliferation at low doses and differentiation at higher doses, which may contribute to the development of PCO.³ Though the exact mechanism involved in b-FGF-induced LEC differentiation is not clear, studies show the role of b-FGF-stimulated activation of ERK1/2 and the expression of differentiation markers such as β -crystallin and filensin in LEC.^{19,20} On the other hand TGF- β induces an epithelial mesenchymal transition (EMT) causing the residual LEC to transdifferentiate into spindle like myofibroblastic cells which express α -smooth muscle actin (α -SMA).^{3,17} Hales et al showed that TGF- β -induced opacification in the cultured lens was morphologically and biochemically similar to cataract.¹⁸ It has been suggested that immediately after cataract surgery, when levels of active TGF- β are low, elevated levels of b-FGF may induce LEC to proliferate. It could be speculated that, when the level of active TGF- β increases it inhibits b-FGF-induced proliferation and stimulates PCO-specific changes such as EMT and transdifferentiation.⁸

Several reports also suggest that growth factors-induced reactive oxygen species (ROS) formation mediates lens epithelial cell growth that may cause PCO,^{14,21} and antioxidants such as caffeic acid and retinoic acid have been shown to prevent PCO.²²⁻²⁴ Further, in a recent study redox-sensitive transcription factor NF- κ B has been implicated in LEC proliferation and PCO.²⁵ We have shown that aldose reductase (AR), a polyol pathway enzyme that reduces glucose and various lipid peroxidation-derived aldehydes and their glutathione conjugates, mediates ROS signals initiated by growth factors, cytokines, high glucose and bacterial endotoxin (Lypopolysaccharide; LPS) via activation of NF- κ B.²⁶⁻²⁹ Furthermore, we have shown that pharmacological inhibition or genetic (siRNA and antisense) ablation of AR prevents activation of ROS-sensitive transcription factors such as NF- κ B and AP-1 in human lens epithelial cells and macrophages.²⁶⁻²⁸ We have also shown that LPS induced the expression of tumor necrosis factor (TNF)- α , inducible nitric oxid synthase (iNOS), cyclooxygenase (Cox)-2 in human lens epithelial cells (HLEC) and rat eyes and inhibition of AR prevented it.^{27, 30} Indeed, the association of AR with ROS-mediated signaling is evident by the observations that AR inhibition attenuates high glucose-induced oxidative stress and superoxide production in LEC and retinal pericytes.^{26,31} Further, AR has also been implicated in the development of diabetic cataractogenesis due to 1) increased flux of glucose through the polyol pathway that increases osmotic pressure, 2) decreased NADPH/NADH ratio that could increase oxidative stress, 3) activation of PKC which activates ROS signals and 4) increased

advanced glycation end products formation leading to protein alteration and tissue damage.³² We and others have shown that inhibition of AR could prevent DC in rodents.³³⁻³⁵ Further, AR is a growth response protein that is over-expressed during oxidative stress, diabetes, cancer and in the cells undergoing proliferation *in vitro* as well as *in vivo*.^{36,37} The studies discussed above thus suggest that AR may play a role in the development of PCO and inhibition of AR could be a therapeutic target to prevent PCO. Here we have investigated the role of AR in LEC growth and differentiation/transdifferentiation in cultured pig eye capsular bags and cultured HLEC. Our results show that inhibition of AR could significantly prevent LEC growth and differentiation/transdifferentiation in pig eye capsular bags explants as well as in HLEC suggesting a potential therapeutic use of AR inhibitors in the prevention of PCO.

Material and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine and 1 g/l glucose, phosphate-buffered saline (PBS), gentamicin solution, 0.25% trypsin/EDTA solution, and heat-inactivated fetal bovine serum (FBS) were purchased from Invitrogen-Gibco (Grand Island, NY). Antibodies against β -crystallin, filensin, intercellular adhesion molecule (ICAM)-1 and glyceraldehyde phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against α -smooth muscle actin (SMA) from research diagnostic Inc (Concord, MA) and antibodies against proliferating cell nuclear antigen (PCNA), phospho-p38, phospho-ERK1/2 and phospho-SAPK/JNK and total- p38, -ERK1/2 and -SAPK/JNK were from Cell Signaling Inc. (Beverly, MA). AR inhibitors Sorbinil and Zopolrestat were gift from Pfizer (Groton, CT) and Fidarestat was from Sanwa Kagaku Kenkyusho Co. Ltd, Japan. The transfection reagent (LipofectAMINE Plus) and reduced-serum cell-culturing medium (OptiMEM) were obtained from Invitrogen-Life Technologies (Gaithersburg, MD). Consensus oligonucleotides for NF- κ B (5'-AGTTGAGGGGACTTTCCAGGC-3') transcription factor was from Promega Corp. (Madison, WI); Basic fibroblast growth factor (b-FGF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and other reagents used in the electrophoretic mobility shift-gel assay (EMSA) and Western blot analysis were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents used were of analytical grade.

Explant culture of Pig eye capsular bags

Pig eye capsular bags were prepared by phacoemulsification followed by capsulorhexis and incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin and streptomycin without or with AR inhibitors, sorbinil (50 μ M) and fidarestat (10-30 μ M) for 7 days in 5% CO₂ condition at 37°C. Medium was replaced with fresh medium without or with AR inhibitors every day. At the end of incubation, capsular bags were examined under the dissection microscope, cut into four pieces and flat mounted on glass slide with cellular side facing up. The pieces of capsular bags were fixed with 4% paraformaldehyde for 3-4 h at 4°C. After fixation, they were washed, transferred to 70% alcohol and stored at 4°C until used for immunohistochemical analysis.

Immunohistochemical analysis of pig eye capsular bag specimens

The capsular bag specimens flat mounted on slides were washed thrice with PBS and incubated with blocking solution containing 2% BSA, 0.1% TritonX-100, 5% normal goat serum for 1h at room temperature. Subsequently specimens were washed, incubated with antibodies against α -SMA, β -crystallin, PCNA, and ICAM-1 for 1 h at room temperature. The slides were stained with either LSAB-HRP system (Dako, Carpinteria, CA) or incubated with respective fluorescein isothiocyanate (FITC) labeled secondary antibodies. The samples were examined

by using Nikon epifluorescence microscope (EPI-800 microscope) connected to a computer with Metamorph software.

Determination of lens epithelial cell number in pig eye capsular bags

The pig eye capsular bags were incubated for 7 days as described above. The lens epithelial cells were detached from capsular bags by incubating with 0.25% trypsin-EDTA solution. The trypsinized cells were harvested, washed with PBS and re-suspended in PBS. To determine cell growth, equal volumes of cell suspension and trypan blue were mixed and the cells which excluded trypan blue dye were counted manually by using a hemocytometer.

Cell culture of HLEC

Adenovirus SV-40 viral DNA-transformed human lens epithelial cells (HLE-B3; HLEC) were purchased from ATCC. Cells were maintained in DMEM supplemented with 20% heat-inactivated FBS, and 50 µg/ml gentamicin. The cells were grown in a humidified incubator at 37°C and 5% CO₂. For the assessment of cell viability and expression of α -SMA, β -crystallin and ICAM-1, the HLEC were pretreated with or without 20 µM of AR inhibitors sorbinil or zopolrestat overnight in medium containing 0.5% FBS then, without change of medium, stimulated with various concentrations of b-FGF (0 - 100 ng/mL) for 24 h. In experiments to investigate the effect of b-FGF on NF- κ B and MAPK activation, HLEC were starved in medium containing 0.5% FBS with or without AR inhibitors for 16 h. The cells were washed with serum-free medium followed by incubation in serum-free medium containing 50 ng/mL b-FGF, with or without AR inhibitor as before, for various time intervals.

Cell Viability Assays

The HLEC were grown to confluence, harvested and plated at 5000 cells/well in 96-well plates. The cells were growth-arrested by incubation in 0.5% FBS medium without or with AR inhibitor (20 µM) for 24 h. After 24 h, the cells were stimulated with b-FGF (0, 10, 20, 50, and 100 ng/mL), and incubated for an additional 24 h. Cell viability was determined by MTT assay as described by us earlier.²⁶ Briefly, after incubation of HLEC, 10 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in PBS were added to each well and incubated further for 2 h at 37°C. The medium was removed and formazan granules obtained were dissolved in 100% dimethyl sulfoxide (DMSO), and absorbance at 562 nm was detected with an ELISA plate reader. The cell viability was also determined by cell counting with a hemocytometer as described above.

Western Blot Analysis

HLEC incubated under various conditions described above were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer containing 50 mM HEPES [pH 7.6], 10 mM KCl, 0.5% NP-40, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), and 1:100 dilution of protease inhibitor cocktail (Sigma, Saint Louis, MO) for 15 min at 4°C. The crude lysates were cleared by centrifugation at 12,000 g for 10 min at 4°C. Aliquots of the lysates containing equal amount of protein (40 µg) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were then incubated in blocking solution containing 5% wt/vol dried fat-free milk and 0.1% vol/vol Tween-20 in tris-buffered saline. Subsequently, the membranes were incubated with antibodies against α -SMA, β -crystallin, ICAM-1, phospho-p38, phospho-ERK1/2, phospho-SAPK/JNK, and total-p38, -ERK1/2 and -SAPK/JNK. The membranes were washed and probed with the respective HRP-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL) and visualized by chemiluminescence (Pierce biotechnology, Rockford, IL). All blots were probed with GAPDH or β -actin as a loading

control and protein band intensities were determined by densitometric analysis by using Kodak Image station 2000R.

Electrophoretic Mobility Shift Assay (EMSA)

The HLEC were pretreated with or without AR inhibitors for 24 h in serum free medium, followed by treatment with b-FGF (50 ng/ml) for additional 1 h. The nuclear extracts were prepared as described earlier.²⁶ The Consensus oligonucleotides for NF- κ B transcription factors were 5'-end labeled using T4 polynucleotide kinase. EMSA was performed as described earlier.²⁶ The specificity of the assay was examined by competition with an excess of unlabeled oligonucleotide and supershift assays with antibodies to p65.

NF- κ B-Dependent Secretory Alkaline Phosphatase (SEAP) Expression Assay

To examine NF- κ B promoter activity in HLEC in response to b-FGF treatment, cells (1×10^5 cells/well) were plated in 24-well plate. The cells were starved for 16 h in 0.5% FBS medium without or with AR inhibitors and transfected with pNF- κ B-SEAP2-construct and pTAL-SEAP control plasmid (Clontech, USA) using Lipofectamine plus (Invitrogen, Carlsbad, CA) transfection reagent following suppliers instructions. After 6 h of transfection, cells were stimulated with b-FGF (50 ng/ml) for 48 h. The cell culture media were harvested and centrifuged at 5000 rpm and supernatants were stored at -80°C . The medium was thawed and used for chemiluminescent secretory alkaline phosphatase (SEAP) assay using Great EscAPETM SEAP reporter assay system according to protocol essentially as described by the manufacturer, (BD Biosciences, Palo Alto, CA) using a 96-well chemiluminescence plate reader. All the suggested controls by manufacturers were used in the assay.

RNA interference ablation of AR

HLEC were grown to ~60% confluence in DMEM supplemented with 20% FBS in 6-well plate. The cells were incubated with OptiMEM medium containing the AR-siRNA (AATCGGTGTCTCCAACCTTCAA) or scrambled siRNA (AAAATCTCCCTAAAT CATACA; control) to a final concentration of 100 nM and the RNAiFectTM transfection reagent (Qiagen) as described by us earlier.³⁸ Briefly, for each well, 2 μg AR siRNA was diluted in serum-free medium to give a final volume of 100 μl and incubated with 6 μl RNAiFect[®] for 15 min at room temperature. The transfection mixture was added to the respective wells, each containing 1900 μl complete medium (20% fetal bovine serum), and incubated for 24 h. After 24 h, the medium was replaced with fresh DMEM (serum free) for another 24 h before stimulation with b-FGF. Changes in the expression of AR were estimated by Western blot analysis using anti-AR antibodies.

Immunocytochemical analysis

For immunocytochemical analysis, HLEC were plated in chambered slides and grown to ~70% confluence. The cells were growth arrested by incubating in 0.5% FBS medium for 24 h without or with AR inhibitors. After treatment with b-FGF for 24 h, the cells were washed and quickly fixed in methanol (pre-chilled at -20°C) for 10 min. The slides were air dried and washed three times with PBS and blocked in the blocking solution containing 2% BSA, 0.1% Triton-X100, and 5% normal goat serum for 1 h at room temperature. Slides were washed twice and incubated with antibodies against α -SMA, β -crystallin, filensin, and ICAM-1 for 1 h at room temperature. Subsequently, the slides were washed thrice with PBS and stained using FITC labeled secondary antibodies, washed and mounted using flour-savie mounting medium with DAPI (Vector laboratories Inc, Burlingame, CA). The sections were examined under Nikon epifluorescence microscopy (EPI-800 microscope).

Statistical analysis

Data presented as mean $\pm$ SD and p values were determined by unpaired Student's t test using Microsoft Office Excel 2003 software. $p < 0.05$ was considered as statistically significant.

Results

Prevention of Epithelial Cell Growth in Pig Eye Capsular Bags by AR Inhibition

Phacoemulsification technique for cataract surgery in humans was used to operate on enucleated pig eyes. After removing the lens, a capsular tension ring was inserted in the capsular bag. The capsular bag was then cultured in complete growth-medium without or with AR inhibitors. The lens epithelial cells first appeared after 2-3 days in culture near the equator then migrated towards anterior as well as posterior regions of the capsule covering it entirely in 7 days and the capsule showed distinct folding in the central region (data not shown). As shown in Fig. 1A, lens epithelial cells showed massive growth in the complete medium. Epithelial cells counted from three capsular bags in three subsequent batches of cultures showed a significant ($\sim 74\%$) inhibition of cell growth by sorbinil (Fig. 1A). Similar results were obtained with another structurally distinct AR inhibitor, fidarestat, which showed a dose-dependent response in inhibiting the growth of LEC in pig eye capsular bags (Fig. 1B). The most effective dose at which fidarestat significantly ($p < 0.001$) inhibited the LEC growth was $30 \mu\text{M}$ (Fig. 1B).

Subsequent to observation that AR inhibition could block the cell growth, we examined the status of the residual cells i.e. whether they were still in cell division mode, by using anti-proliferating cell nuclear antigen (PCNA) antibodies. As shown in supplementary Figs. IA and IB, in capsular bags cultured without AR inhibitor, a large number of cells were PCNA positive indicating that they were still dividing, whereas in AR inhibitor - treated capsular bags, fewer PCNA positive cells were present. These results suggest that AR inhibition could block the growth/proliferation of residual cells after phacoemulsification of lens.

Prevention of Differentiation/Transdifferentiation of LECs in Pig Eye Capsular Bags by AR Inhibition

We next examined the effect of AR inhibition on the transdifferentiation of LEC in pig eye capsular bags. As shown in Fig. 2A and B, lens epithelial cells from pig eye capsular bags cultured without AR inhibitors showed 3-5 fold increased expression of α -SMA and β -crystallin compared to those cultured with AR inhibitors. Similarly, including the AR inhibitors with FGF reduced ICAM-1 expression by ~ 2 fold.

Immunohistochemical studies on flat-mounted capsular bags after 7 days of culture showed that a large number of cells in control capsular bags expressed α -SMA and β -crystallin in the absence of AR inhibitor, sorbinil (supplementary Fig. IIA, and IIC), whereas in AR inhibitor-treated capsular bags, only few cells expressed α -SMA and β -crystallin. Treatment with fidarestat also showed similar inhibition in the expression of α -SMA and β -crystallin (Supplementary Fig. IIB and IID). Nishi et al., have suggested that cell adhesion molecules such as ICAM-1 could be involved in adhesion of LEC to extra-cellular matrix and play a crucial role in cell growth and migration.³⁹ Therefore, we examined the expression of ICAM-1 in the pig eye capsular bag and found that in control capsular bags the epithelial cells expressed ICAM-1 protein at both anterior and posterior capsule. In AR inhibitors- treated capsular bags, the expression of ICAM-1 was markedly inhibited (supplementary Fig. IIE and IIF). Our results thus indicate that AR inhibition could be successfully used to block the differentiation/transdifferentiation of epithelial cells subsequent to phacoemulsification of lens in cataract surgery which could prevent PCO.

Prevention of BFGF-Induced HLEC Growth by AR Inhibition

In order to confirm our findings in the pig eye capsular bag model and to understand the molecular mechanisms by which AR inhibition prevents lens epithelial cell proliferation, we examined the effect of AR inhibitors on b-FGF -induced proliferation of cultured HLEC. As shown in Fig. 3A, b-FGF induced significant increase in proliferation, compared to controls, at all doses greater than 10 ng/ml and the response at 20 ng/ml b-FGF was greater than at higher doses. Inhibition of AR by two structurally distinct inhibitors significantly ($p<0.01$) blocked the cell growth induced by 20 ng/ml b-FGF as determined by cell counting and MTT assay (Fig. 3A-B).

Prevention of expression of α -SMA, β -crystallin and ICAM-1 in HLEC by AR inhibition

We next examined the effect of AR inhibition on b-FGF-induced expression of β -crystallin and α -SMA in HLEC. As shown in Fig. 4A, treatment of HLEC with 50 ng/ml b-FGF caused ~4-fold increase in the expression of β -crystallin, and α -SMA and ~2.5-fold increase in the expression of adhesion molecule ICAM-1 and AR inhibitors significantly (~90%) inhibited the increase (Fig. 4A). However, treatment with AR inhibitors alone did not cause any significant change in the expression of these proteins. Although sorbinil and zopolrestat are specific inhibitors of AR, to rule out any nonspecific effect of pharmacological inhibitors, we ablated AR message by transfecting the HLEC with AR-siRNA. As shown in Fig. 4B, b-FGF-induced increase in the expression of α -SMA and β -crystallin in untransfected (C) and scrambled siRNA oligo (Csi) transfected HLEC but not in the AR-siRNA transfected cells suggesting that AR plays a crucial role in the aberrant expression of α -SMA and β -crystallin in HLEC. Further, immuno-cytochemical analysis also demonstrates that AR inhibition significantly blocked b-FGF-induced expression of α -SMA, β -crystallin, filensin and ICAM-1 in HLEC (Supplementary Fig. IIIA-D).

Prevention of b-FGF-induced activation of NF- κ B in HLEC by AR Inhibition

Since activation of NF- κ B mediates expression of various marker proteins and cytokines, we next examined the effect of AR inhibition on b-FGF-induced NF- κ B activation in HLEC. Treatment with b-FGF caused ~-fold increase in the activation of NF- κ B as indicated by DNA binding capacity determined by EMSA (Fig. 5A). Inhibition of AR by sorbinil or zopolrestat significantly (>90%) prevented the b-FGF-induced NF- κ B DNA binding capacity. However, sorbinil or zopolrestat alone did not affect the basal activity of NF- κ B. We further confirmed our results with more sensitive NF- κ B-dependent reporter assay. As shown in Fig. 5B, b-FGF caused >5-fold activation in the NF- κ B dependent reporter SEAP activity which was significantly (~60%; $p<0.01$) inhibited by AR inhibitors. These results suggest that inhibition of AR could modulate b-FGF-induced activation of redox-sensitive transcription factors, which could be responsible for preventing the growth and differentiation/transdifferentiation of LEC and thereby prevent PCO.

Prevention of b-FGF-induced phosphorylation of MAPKs by AR Inhibition

MAPKs are known to regulate the phosphorylation and activation of NF- κ B; therefore, we next examined the effect of AR inhibition on b-FGF-induced activation of MAPKs. As shown in Fig. 6, treatment with b-FGF enhanced phosphorylation of ERK1/2- MAPK, and SAPK/JNK in HLEC and pre-treatment with AR inhibitor prevented it. There was no change in the expression of total ERK1/2 and SAPK/JNK proteins. We did not observe any change in phosphorylation of p38-MAPK in response to b-FGF treatment (Data not shown) suggesting that ERK1/2 MAPK and SAPK/JNK are the main mediators of growth factor -induced NF- κ B activation in HLEC.

Discussion

Subsequent to cataract surgery the residual lens cells undergo proliferation and transdifferentiation and/or differentiation resulting in PCO (see Introduction). ROS-mediated processes and stimulation by growth factors including TGF- β and/or FGF appear to play key roles in PCO development.⁹⁻¹¹ Studies suggest that redox-sensitive transcription factor such as NF- κ B plays a crucial role in trauma-induced oxidative stress that could result in cataract formation.⁴⁰⁻⁴¹ Various factors including growth factors, cytokines, endotoxins, high glucose, and injury are known to activate NF- κ B.^{26-30,40} We have shown earlier that NF- κ B activation is regulated by AR-catalyzed reduced glutathione-lipid aldehyde conjugates generally formed due to changes in the cellular redox status and ROS levels.²⁸ Inhibition of AR prevents the AR-catalyzed reduction of glutathione-lipid aldehydes which is required for the activation of signaling pathway that leads to the phosphorylation and nuclear translocation of NF- κ B and resultant inflammatory gene expression that promotes growth/proliferation.³⁶⁻³⁸

Based on our previous studies,^{26-30,35-38} we reasoned that blocking the initial burst of inflammation in post-surgery eye by inhibiting AR would prevent the development of PCO. We therefore, used pig eye capsular bag model to study the effects of AR inhibition on post-surgical events leading to PCO. Two different inhibitors of AR, namely sorbinil and fidarestat, were used in the experiments with pig eye capsular bags. While the control capsular bags cultured without AR inhibitors, showed robust proliferation, differentiation and/or transdifferentiation of lens epithelial cells, the AR inhibitors-treated capsular bags showed significantly less cell growth. The cell growth event was similar in many batches of capsular bag culture showing a consistent pattern. Because proliferating nuclei express PCNA and incorporate Brdu during DNA replication, PCNA and Brdu positive signal would indicate that cells are in division phase. We observed that in capsular bags without AR inhibitor large percentage of cells were positive for PCNA while in AR inhibitor groups fewer cells were PCNA positive suggesting that LEC continue to divide even after 7 days in culture and AR inhibitor blocked the cell division of LEC in capsular bags. We have shown earlier that in colon cancer cells (Caco-2) AR inhibition caused cells to exit cell cycle and accumulate in G-phase.²⁹ Similarly, our earlier studies show that inhibition of AR could prevent cytokine- and hyperglycemia-induced cyto-toxicity as well as activation of redox-sensitive transcription factors in HLEC.²⁶ In addition to cell growth, transdifferentiation of the LEC in myofibroblast-like cells is important for PCO.³ We observed that inhibition of AR prevented the expression of α -SMA, β -crystallin and intercellular adhesion molecule, ICAM-1. We have shown earlier that inhibition of AR prevents oxidative stress-induced signals that cause activation of NF- κ B leading to cell growth or death.^{26,27} Thus, our current and previous results strongly support our hypothesis that AR could regulate cell growth,³⁶ and its inhibition can attenuate PCO formation.

Growth factors, especially TGF- β and b-FGF, have been implicated in the development of PCO.^{9-11, 44} Indeed, Nishi et al., have shown that cytokines such as IL-1, IL-6, and b-FGF may be produced *in-vivo* by residual LEC,⁴⁵ causing postoperative inflammation and proliferation of residual lens epithelial cells. We observed that in HLEC, b-FGF caused increased growth at all doses ≤ 20 ng/ml and differentiation/transdifferentiation at the dose ≥ 50 ng/ml as evident by approximately 4-fold enhanced expression of β -crystallin and α -SMA, respectively. The low level expression of β -crystallin and α -SMA even in control HLEC not treated with b-FGF (Fig 4A, B) may suggest the presence of mixed population of cells which could include differentiating and transdifferentiating cells as well as normal LEC. Consistent with this possibility we also observed the presence of immunoreactivity for filensin in at least some cells in controls (Supplementary Figure IIIC(C)), which occurs only in lens fiber cells and not in normal LEC.⁴² A range of cell types such as this may be present in the capsular bag after surgery. However, it is significant to note that inhibition as well as siRNA ablation of AR

prevented the expression of these marker proteins in b-FGF- treated HLEC. Moreover, AR inhibition also blocked the phosphorylation of MAPKs, including that of ERK1/2 thereby preventing the activation of a molecular cascade that ultimately activates the transcription factor NF- κ B. These observations are highly significant because HLEC have been shown to be dependent on ERK signaling for growth factor-induced cell proliferation.^{13,14;19,20} Besides, TGF- β has also been implicated in LEC transdifferentiation and expression of α -SMA,^{17,46} and induced cataract in cultured lens.¹⁸ Studies by other investigators have shown that b-FGF exacerbated TGF- β -induced cataract in cultured lens.⁴⁷ However, it is not clear how α -SMA is induced by b-FGF in LEC. We predict that our preculture system with 10 to 20% FBS may contain various growth factors including TGF- β which could be responsible observed increase in α -SMA induction and FGF per se may not be responsible for expression of α -SMA in LEC.

Several pharmacological agents have been investigated for the possible use in the prevention of PCO, including nonsteroidal anti-inflammatory drug (NSAID) diclofenac which has been shown to inhibit proliferation of subconfluent cultures of LEC in a dose-dependent manner.⁵ Although use of antioxidants (such as caffeic and retinoic acids) and corticosteroids has also been suggested,^{22-24,48} their effectiveness is marred either by toxicity and/or only partial prevention of the PCO. Our results suggest that AR inhibitors block signaling events that lead to both LEC proliferation and differentiation/transdifferentiation indicating that inhibition of AR could be a novel therapeutic approach to prevent PCO. Over the years AR inhibitors have been clinically tested for the potential treatment of diabetic complications such as diabetic neuropathy and retinopathy. Initial studies showed hepatic and renal toxicity.⁴⁹ However, structural modifications of AR inhibitors and dosage studies have led to reduced toxicity and in fact AR inhibitor epalrestat is currently marketed in Japan for the treatment of diabetic neuropathy.⁵⁰ Furthermore, AR inhibitors such as fidarestat, zopolrestat, zenarestat, and minalrestat have been found to be safe and have already passed in FDA's phase-1 and phase-2 clinical trials and some of them are in ongoing phase-3 clinical trials for diabetic complications.⁵⁰⁻⁵²

Although the precise series of events through which AR activates NF- κ B remain unclear, we have shown earlier that AR-catalyzed reduced product of glutathione-4-hydroxynonenal (GS-HNE) i.e. glutathionyl 1,4-dihydroxynonene (GS-DHN) could be the main causative factor for the activation of NF- κ B via a number of kinases which eventually cause cytotoxicity including development of PCO subsequent to cataract surgery. Therefore, AR inhibitors could be potential therapeutic drugs for the prevention of PCO. Further studies are required to ascertain this hypothesis.

In summary, our results suggest that AR plays an important role in the development of PCO. Since this enzyme mediates the molecular signaling events leading to differentiation and transdifferentiation of LEC in porcine capsular bags as well as in in-vitro cell culture studies, our results suggest that AR inhibition could be a novel pharmacological approach to prevent the characteristic changes that cause PCO in humans subsequent to cataract surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by NIH grants GM71036 (KVR) and DK36118 (SKS).

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Figure 1. AR inhibition prevents the proliferation of lens epithelial cells in the pig eye capsular bag Pig eye capsular bags were prepared by capsulorhexis and incubated in DMEM with 10% FBS and 1% penicillin/streptomycin with or without (A) sorbinil (50 μM) and (B) fidarestat (10-30 μM) for 7 days in humidified incubator at 37°C with 95% O₂ and 5% CO₂. The lens epithelial cells from capsular bags were harvested, washed, re-suspended in PBS and trypan blue positive cells were counted by using a hemocytometer. The bars represent Mean ± SD (n=9). [#]P<0.03; ^{##}P<0.001 Vs Control. Sorb, sorbinil; fida, fidarestat.

Figure 2. AR inhibition prevents the expression of various markers in the lens epithelium of pig eye capsular bag

Pig eye capsular bags were prepared by capsulorhexis and cultured with or without AR inhibitor, (A) sorbinil (50 μ M) and (B) fidarestat (30 μ M) for 7 days. The lens epithelial cells were harvested from capsular bag, pooled together from each batch, washed and lysed in RIPA lysis buffer. Equal amounts of protein were subjected to Western blot analysis using specific antibodies against α -SMA, β -crystallin, and ICAM-1. The blots were stripped and re-probed with antibodies against β -actin as loading control. Numbers below the blots show the fold change in the expression of proteins. A representative blot from 3 individual analyses is shown.

Figure 3. AR inhibition prevents b-FGF-induced proliferation of HLEC

Growth-arrested HLEC without or with AR inhibitors, sorbiniil and zopolrestat (20 μ M) were stimulated with (A) b-FGF (0, 10, 20, 50, and 100 ng/mL) or (B) 20 ng/ml for 24 h. Cell viability was determined by MTT assay (A) or by cell counting (B). The bars represent mean $\pm$ SD (n=4). [#]p<0.01 vs. control; ^{##}p<0.01 and ^{*}p<0.05 vs. b-FGF only. Sorb (S), sorbiniil; zopol (Z), zopolrestat; FGF (F), basic fibroblast growth factor.

Figure 4. AR inhibition prevents the b-FGF-induced expression of differentiation markers in HLEC

(A) Growth-arrested HLEC without or with AR inhibitors (sorbinil and zopolrestat; 20 μ M) and (B) untransfected (C), AR siRNA (ARsi) or control siRNA (Csi) transfected were stimulated with b-FGF (50 ng/ml) for 24 h. After incubation the cells were harvested, washed and lysed in RIPA lysis buffer. Equal amounts of protein (40 μ g) were subjected to Western blot analysis using specific antibodies against α -SMA, β -crystallin, ICAM-1 and GAPDH. A representative blot from 3 individual analyses is shown. Sorb, sorbinil; zopol, zopolrestat; FGF, basic fibroblast growth factor.

Figure 5. AR inhibition attenuates the b-FGF-induced activation of NF-κB in HLEC

(A) Growth-arrested HLEC without or with AR inhibitors (sorbinil and zopolrestat; 20 μ M) were stimulated with b-FGF (50 ng/ml) for 1 h and equal amounts of nuclear proteins were subjected to EMSA. (B) HLEC were transiently transfected with the pNF-κB-SEAP reporter vector. The cells treated without or with AR inhibitors (sorbinil and zopolrestat; 20 μ M) were incubated with 50 ng/mL of b-FGF. After 48 hours, the culture supernatants were assayed for SEAP activity with a chemiluminescence kit according to the supplier's instructions. Bars represent Mean $\pm$ SD (n=4). #p<0.001 Vs. Control and **p<0.01 vs. b-FGF alone. Sorb, sorbinil; zopol, zopolrestat; FGF, basic fibroblast growth factor.

Figure 6. AR inhibition prevents b-FGF-induced activation of MAPK in HLEC

Growth-arrested HLEC without or with AR inhibitor, zopolrestat (20 μ M), were treated with b-FGF (50 ng/ml) for indicated time periods. Cytosolic extracts containing equal amounts of protein were subjected to Western blot analysis using antibodies against phospho- and total-ERK1/2, -JNK/SAPK and GAPDH. The representative blots from 3 individual analyses are shown.