Modulation of Factors Affecting Optic Nerve Head Astrocyte Migration

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PURPOSE. The authors investigated the role of myosin light chain kinase (MYLK) and transforming growth factor beta (TGF β) receptor pathways in optic nerve head (ONH) astrocyte migration. They further investigated how the expression of these genes is altered by elevated hydrostatic pressure (HP).

METHODS. PCR was used to determine the isoforms of MYLK expressed in ONH astrocytes. siRNAs against MYLK (all isoforms) and TGF β receptor 2 (TGFB2) were prepared and tested for effects on the migration of cultured ONH astrocytes. Finally, the effects of elevated HP (24–96 hours) on the expression of MYLK isoforms and selected TGF β pathway components were measured.

RESULTS. Multiple isoforms of MYLK are present in ONH astrocytes from Caucasian (CA) and African American (AA) donors. Both populations express the short form (MYLK-130) and the long form (MYLK-210) of MYLK and a splicing variant within MYLK-210. MYLK-directed siRNA decreased MYLK expression and cell migration compared with control siRNA. siRNA directed against TGF β receptor 2 also decreased cell migration compared with control and decreased extracellular matrix genes regulated by TGF β signaling. Elevated HP increased the expression of MYLK-130 and MYLK-210 in both populations of astrocytes. However, TGFB2 was uniquely upregulated by exposure to elevated HP in CA compared with AA astrocytes.

CONCLUSIONS. Differential expression of TGF β pathway genes and MYLK isoforms observed in populations of glaucomatous astrocytes applies to the elevated HP model system. MYLK may be a new target for intervention in glaucoma to alter reactive astrocyte migration in the ONH. (*Invest Ophthalmol Vis Sci.* 2010;51:4096–4103) DOI:10.1167/iovs.10-5177

Migration of astrocytes occurs during normal development, in neurodegenerative diseases, after injury, and during tumor invasion in the CNS. Migration of reactive astrocytes is an important component in the remodeling of the optic nerve head (ONH) in glaucoma.^{1,2} Astrocyte migration occurs in response to neuronal injury through the actions of growth factors,³ cytokines,⁴ and other mediators such as ATP.⁵ In glaucoma, reactive astrocytes migrate from the cribriform plates into the nerve bundles² and synthesize neurotoxic mediators such as nitric oxide (NO) and TNF- α , which may be released near the axons causing neuronal damage.^{6,7}

In previous work, microarray analysis comparing glaucomatous astrocytes from African American (AA) and Caucasian (CA) donors with the corresponding healthy samples identified differentially expressed genes involved in astrocyte migration.⁸ These include myosin light chain kinase (MYLK), a calmodulin-activated protein kinase that phosphorylates serine 19 on the myosin regulatory light chain, and MYPT1, the regulatory subunit of myosin-light chain phosphatase that dephosphorylates the myosin light chain.

Another signaling pathway family altered in glaucomatous astrocytes includes TGF β , whose isoforms are differentially expressed, the TGFB2 receptor, and downstream protein SMAD3.⁸ These proteins are also coupled to the Rho, CDC42, and Rac1 signaling pathways that control astrocyte polarity and process formation.⁹ TGF β also induces the expression of extracellular matrix proteins,¹⁰ proteases,¹¹ and other enzymes that modify matrix components¹² in ONH astrocytes.

Previous work from the Hernandez¹³ laboratory demonstrated that human ONH astrocytes in vitro respond to elevated hydrostatic pressure with an increase in cell migration to remodel the cell monolayer in a way that may be relevant to the tissue remodeling observed in glaucomatous optic neuropathy.^{2,14} Thus, in the current work, we examined the roles of myosin-associated proteins and the TGF β pathway in cell migration and response to elevated hydrostatic pressure.

MATERIALS AND METHODS

Human Eyes

Twenty-one eyes from 21 healthy age-matched CA (age 62 $\pm$ 12) and 16 eyes from 16 healthy AA (age 60 $\pm$ 11) donors were used in this study to generate primary cultures of optic nerve head (ONH) astrocytes. In addition, we used six eyes from CA and AA glaucoma donors to generate cultures for MYLK expression experiments. These are designated CAG and AAG cells, respectively. Healthy donors did not have a history of eye disease, diabetes, or chronic CNS disease, as confirmed by paraphenylenediamine staining of myelinated nerves, as described previously.¹⁵ Eyes were obtained from the local eye banks and from the National Disease Research Interchange. Eyes were enucleated shortly after death and maintained at 4°C. Optic nerve heads were dissected within 24 hours of death and were processed to generate ONH astrocytes.

Astrocyte Cultures

Cultures of human ONH astrocytes were generated as previously described.^{16,17} Briefly, explants from each lamina cribrosa were dissected and placed in 25-cm² tissue culture flasks (Falcon, Lincoln Park, NJ). Explants were maintained in DMEM/F-12 supplemented with 10% FBS (BioWhittaker, Walkersville, MD), and 10 μ L/mL antibiotic mixture containing (10,000 U/mL penicillin, 10,000 μ g/mL streptomycin, and 25 μ g/mL amphotericin B [30–004 CL; CellGrow, Herndon, VA]). Cells were kept in a 37°C, 5% CO₂ incubator. After 2 to 4 weeks, primary cultures were purified by using a modified immunopanning procedure.¹⁸ Immunopanning results in astrocytes of 95% purity, as

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TABLE 1. Primers Used for qRT-PCR

mRNA	Primer 5'–3'	Product Length (bp)	Accession No.
TGFB2 forward	TGTTGAGTCCTTCAAGCAGACCGA	86	NM_003242
TGFB2 reverse	ACTTCTCCCACTGCATTACAGCGA		
TGFB1 forward	TGGGACCCACTTCCATTTCCTTCA	85	NM_004612
TGFB1 reverse	TCCCAAGCCTCATCTGCTCAATCT		
TGFB1 forward	TATCAACGGGAAGGCGATCA	79	NM_000358
TGFB1 reverse	GTGTCTTGGCTGAGTCTGGAAT		
MYLK total forward	TCTCCGACGATGCCAAGGATTTCA	134	NM_053025
MYLK total reverse	TGGAGAGTTTCTTGGCTCCATGT		
MYPT1 forward	CGCCGACATCAATTACGCCAATGT	157	NM_001143885
MYPT1 reverse	AGGAAGCTGCTGCATGTAGTGGTA		
MYLK-210 forward	TGAGCAAGGCTGCTAACAGGAGAA	133	NM_053025
MYLK-210 reverse	GGCAAGCCCTTCACATCTGAACCT		
COL4A1 forward	ATTTTCGACTTGGGGTCAAAGGTG	145	NM001845
COL4A1 reverse	TTTCAATCCTACAGAACCCTGGCGA		
ELN forward	CCAAGCTGCCTGGTGGC	106	NM_000501
ELN reverse	TGTTGGGTAACCAGCCTTGC		
COL15A1 forward	CTTTTGGAGGATGAGCAAGCCAGT	103	NM_001855
COL15A1 reverse	TCATCACCAGGACCAGAAGTGA		
MMP1 forward	GAGATGAAGTCCGCTTTTTCAAAG	100	NM_002421
MMP1 reverse	GAAGCCAAAGGAGCTGTAGATGTC		

determined by flow cytometry.¹⁸ Purified cells were expanded after characterization by immunostaining for astrocyte markers GFAP and NCAM, as described.¹⁷ Second-passage cell cultures were stored in RPMI 1640 with 10% DMSO in liquid nitrogen until use. For each set of experiments, cells were thawed and cultured for one more passage so that a sufficient number from the same batch was available for each set of experiments.

Real-Time Quantitative RT-PCR

Independent confirmation of differential expression was conducted using 17 CA astrocyte cultures and 13 AA astrocyte cultures from all from age-matched donors. Cytoplasmic RNA was isolated from cultured ONH astrocytes (passage 3) using reagent (Trizol; Invitrogen, Carlsbad, CA), as previously described.¹⁹ cDNA was synthesized using a synthesis system (SuperScript III First-Strand; Invitrogen) in accordance with the manufacturer's protocol. qRT-PCR was performed by monitoring the increase in fluorescence of SYBR-Green using a real-time PCR detection system (MyIQ Single-Color Real-Time PCR Detection; Bio-Rad, Hercules, CA). Serial dilutions (1:5, 1:10, 1:40, 1:160, 1:640) of the mixed cDNA from all samples were used for standard curves. Individual sample cDNA was used at 1:20 dilution. All experiments were carried out in triplicate, and astrocytes derived from one eye of each donor were used. The relative amounts of mRNA for target genes were normalized to reference gene RNA (18S) in each sample. The means of relative expression values were considered significantly different when $P < 0.05$ (unpaired *t*-test). Primer sequences are shown in Table 1.

Detection of MYLK-210 Splice Variants

mRNA from four CA and three AA cultures and six each of the CAG and AAG cells was used. To detect the deletion variants in exon 11, we used primer pair A (forward, 5'-GAGCCAAGATGTTGTGAGCA-3'; reverse, 5'-ATTCAGCAGCCAAGTGATCC-3'), which gives either a 480- or a 273-bp product. For exon 11 variants, PCR was performed with 50 ng cRNA template using the following program steps: 95°C, 2 minutes; 95°C, 30 seconds; 55°C, 30 seconds; 72°C, 1 minute. The second through fourth steps were repeated for 35 total cycles.

To detect the deletion variants in exon 30, we used primer pair B (forward, 5'-GCTGTGTTGAGCGCATCATGACGA-3'; reverse, 5'-TG-GAGAGTTTCTTGGCTCCATGT-3'). This pair gives either a 562- or a 409-bp product. PCR for the exon 30 variants was performed in the

same fashion as for exon 11 except that the annealing temperature was 59°C.

Elevated Hydrostatic Pressure Treatments

Confluent cells (six-well plates) were transferred to HP chambers equipped with a pressure gauge. Plates were placed on a supporting rack in the chamber with a water reservoir below the rack to maintain 98% humidity. Elevated HP was created by compression of the gas phase in the closed chamber, as described previously.^{20,21} Briefly, pressure was raised to 60 mm Hg (8.6 kPa) above ambient pressure by filling the chambers with a mixture of 92% air and 8% carbon dioxide. The elevated CO₂ concentration helps to maintain the pH at approximately 7.3 to 7.4.²² Partial oxygen pressure is not altered in the conditions of these experiments, as documented earlier.^{22,23} After filling, the sealed chambers were placed in a tissue culture incubator and maintained at 37°C for the desired treatment time while control groups were cultured at ambient pressure.

Western Blot Analysis

Western blot analysis was carried out as previously described.^{8,19} Briefly, protein lysates were generated from at least four AA cultures and four CA cultures. Lysates were prepared using 1× protein lysis buffer (Cell Signaling Technology, Beverly, MA). Extracted protein (3–20 μg) was electrophoresed on 4% to 12% bis-Tris gels (Invitrogen) in MES running buffer (Invitrogen). Gels were blotted to polyvinylidene membranes (Millipore, Billerica, MA). Membranes were washed in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and then were incubated with anti-MYLK monoclonal antibody (M7905; Sigma-Aldrich, St. Louis, MO) in TBS-T containing 5% nonfat dry milk for 2 hours at room temperature or for 16 hours at 4°C. Membranes were then washed in TBS-T and incubated with secondary antibody (HRP-conjugated anti-mouse; Bio-Rad) for 1 hour at room temperature. For detection we used a bone marrow chemiluminescence detection system (Roche, Indianapolis, IN). Membranes were stripped and reprobed with anti-β-actin antibody as the loading control.

ML-7 and siRNA Experiments

ML-7 (1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazapine hydrochloride; Sigma-Aldrich), a small molecule inhibitor of MYLK, was dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 10 μM (0.1% DMSO). ONH astrocytes were seeded in six-well plates containing coverslips and grown to a monolayer in astrocyte

growth media (AGM; CC4123; Clonetics, Walkersville, MD). Media were aspirated, ML-7 in serum-free DMEM/F12 media was added, and cells were incubated for 24 hours at 37°C, 5% CO₂.

MYLK siRNA was a smart pool reagent (L005351-00-0005; Dharmacon, Lafayette, CO) containing four siRNA duplexes that are targeted across the human MYLK mRNA. TGFBR2 siRNA was a targeted duplex with the sequence UCCUGCAUGAGCAACUGCA.²⁴ The siRNA was terminated at the 3' ends with two dT bases as synthesized by Dharmacon. siRNAs were dissolved in RNase-free water to a stock concentration of 100 μ M. siRNAs were transfected into ONH astrocytes using transfection reagent (TKO; Mirus, Madison, WI) according to the manufacturer's protocol. Pilot experiments determined that 2.5 μ L reagent (TKO; Mirus) was optimal for each well of a six-well plate. Thus, 1.5 mL transfection cocktail contains 15 μ L TKO reagent, 9 μ L TGFBR2, or 4.5 μ L MYLK siRNA (100 μ M) or control siRNA (same amount as targeted siRNA) in serum-free media (DMEM/F12). ONH astrocytes were grown to 80% confluence. The media were aspirated and replaced with 1.25 mL media (AGM; CC4123; Clonetics). Then 250 mL of the desired transfection cocktail was added dropwise to each well. The plate was covered and gently swirled to mix the reagent and media. Cells were incubated at 37°C, 5% CO₂, for 48 hours.

Migration Assays

ONH astrocytes were seeded into six-well plates (50,000 cells/well) containing a glass coverslip and grown in media (AGM; CC4123; Clonetics) until a monolayer formed on the coverslip. For the siRNA-transfected cells, 48 to 54 hours after transfection was the time window chosen for the migration assay because most wells had cells near confluence. Two scratches were made on each coverslip with a 200- μ L plastic pipette tip, and the wells were washed once with PBS. One coverslip for each condition was reserved for making a control scratch to define the wound area. The cells were reincubated with 25% media (AGM; CC4123; Clonetics) diluted with serum-free DMEM-F12 to promote migration and slow cell proliferation.²⁵ Cells were incubated at 37°C for 16 to 18 hours. For ML-7-treated cells, the media were serum-free DMEM/F12 containing 10 μ M ML-7, and the incubation time was 24 hours. The scratch control was made in one well, washed twice with PBS, and fixed as described.

After incubation, the wells were washed with PBS, and the coverslips were removed and fixed in 4% paraformaldehyde in PBS and then processed for standard indirect immunofluorescence. Fixed coverslips were washed in PBS with 0.5% bovine serum albumin (BSA) and permeabilized with 1% Triton X-100 in PBS. After permeabilization, Texas Red phalloidin (1:40; Invitrogen Molecular Probes, Eugene, OR) diluted in PBS was applied to the coverslips for 30 minutes at 37°C. After washing with 0.5% BSA/PBS, coverslips were mounted on slides using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Scratch areas were imaged on a fluorescence microscope (Eclipse 80i; Nikon, Tokyo, Japan), and 10 to 15 images were created from each scratch.

Images of the scratch areas were stored as TIFF files. Cell migration was calculated using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) from the recorded images as the distance (in micrometers) covered by the cells from the edge of the original scratch to the edge of the scratch after incubation. Determinations were made from five different ONH cultures from either CA or AA donors.

RESULTS

MYLK Isoform Characterization in ONH Astrocytes

Our previous studies demonstrated higher expression of MYLK in AA compared with CA ONH astrocytes. MYLK has two major isoforms, MYLK-130 and MYLK-210, with the latter uniquely containing an N-terminal extension.²⁶ At the protein level,

FIGURE 1. Expression of MYLK-210 in ONH astrocytes. (A) MYLK-210 mRNA is increased in glaucomatous astrocytes. The ratio of MYLK-210 to the total MYLK increases 3.6-fold in AAG compared with AA astrocytes ($P < 0.01$; $n = 6$) and 2.9-fold in CAG astrocytes ($P < 0.01$; $n = 9$). (B) There are four MYLK splice variants. (C) RT-PCR products from exon 11 splice variants. Upper band: 480 bp; lower band: 273 bp, corresponding to the 207-bp deletion. These do not vary among ONH astrocyte donors.

astrocytes from donors with glaucoma expressed more MYLK-210 than normal astrocytes.⁸ Other studies have shown that MYLK-210 displays enhanced interaction with the actin cytoskeleton compared with MYLK-130.²⁷ In the current work, we confirmed that the difference in isoform preference between glaucomatous and normal astrocytes also occurs at the mRNA level (Fig. 1A). The fraction of MYLK-210 mRNA is significantly higher in glaucomatous astrocytes than in those from healthy donors. MYLK-210 has additional sequence variants (MYLK-1, MYLK-2, MYLK-3A, MYLK-3B) that result from alternative splicing²⁸ (Fig. 1B). These splicing sites are at exon 11 and exon 30. Alternative splicing at exon 11 results in a 207-bp deletion found in variants 2 and 3B, whereas splicing at exon 30 results in a 153-bp deletion giving rise to variants 3A and 3B. We designed primers outside these two exons (Fig. 1B). By using PCR, we have identified that alternative splicing at exon 11 occurs in all 30 of the ONH astrocyte cell line cultures tested (Fig. 1C). Alternative splicing at exon 30 does not occur in any of the ONH astrocyte cell lines tested because the only PCR product detected was 562 bp (not shown). None of the 409-bp product expected for a deletion variant was

detected. Thus, MYLK-1 and MYLK-2 are the two variants expressed in ONH astrocytes.

Effects of Elevated Hydrostatic Pressure on MYLK Expression in Normal ONH Astrocytes

To determine whether the difference in MYLK isoforms expression between glaucomatous astrocytes and normal astrocytes is an adaptation to stress initiated by elevated IOP, we subjected ONH astrocytes to elevated hydrostatic pressure to simulate this effect. In our pressure chamber system, pressure is elevated to 60 mm in a closed vessel that maintains cell culture conditions without a significant change in dissolved oxygen content, as described previously.²⁰ Astrocytes were exposed to elevated hydrostatic pressure for 6 hours, 24 hours, 72 hours, or 96 hours and then harvested for mRNA and protein analysis. Astrocytes under ambient pressure were harvested at time 0 and 5 days and were used as controls. qRT-PCR was performed to monitor the effect of elevated pressure on MYLK mRNA expression. Western blot analysis was used to study effects on protein levels. Figure 2 illustrates that higher expression of total MYLK and MYLK-210 mRNA occurs after extended HP treatment (>24 hours; $P < 0.05$) for both AA and CA astrocytes. In total MYLK expression, the increases were 2.25 ± 0.78 -fold ($n = 3$; CA astrocytes) and 1.93 ± 0.46 -fold (AA astrocytes; $n = 3$, $P < 0.05$). MYLK-210 exhibits 2.13 ± 0.61 -fold (CA) and 2.74 ± 0.04 -fold (AA) increases ($n = 3$, $P < 0.05$) in expression. Consistent with these qRT-PCR data, protein levels of MYLK-130 protein increase after 5 days of exposure to elevated pressure (Figs. 2C, 2D). Although it caused a significant increase in MYLK-210 mRNA, elevated HP alone did not appear to initiate the switch between MYLK-130 and MYLK-210 proteins that was seen in glaucomatous astrocytes.⁸

Role of MYLK in Astrocyte Migration

An earlier report showed that ONH astrocytes treated with ML-7, an MYLK inhibitor, migrated more slowly through a collagen matrix than did control astrocytes.¹⁹ In the current work, we used a different migration assay that measures the closure of a cell-free area (scratch) by migrating astrocytes.²⁹ We found that although ML-7 (10 μ M) inhibited migration, there was some loss of cells after treatment (Fig. 3). In the current work, we used siRNA, an alternative method of inhibiting MYLK, to study the role of MYLK in astrocyte migration. Astrocytes were treated with an siRNA (SMARTpool MYLK; Millipore), which inhibits all MYLK mRNA isoforms, and control siRNA 2 as nontargeting control. Twenty-four hours after transfection there was a 95% reduction in total MYLK mRNA level when cells were treated with the pooled MYLK siRNA (MYLK_i), whereas the control siRNA has no effect on MYLK mRNA level (Fig. 4A). MYLK protein (MYLK-210 and MYLK-130) was also decreased in both AA and CA MYLK_i-transfected astrocytes. A representative Western blot is shown in Figure 4B. We then performed the migration assay on siRNA-treated astrocytes. Figures 5A and 5C illustrate the effects of the control siRNA on CA and AA astrocytes. Figures 5B and 5D show the inhibition of migration of CA and AA astrocytes, respectively. Control siRNA did not impede the migration of astrocytes compared with untreated cells, but the migration of the MYLK_i-treated cells was slowed. Overall, astrocytes treated with MYLK_i migrate significantly more slowly ($P < 0.001$; average 40% decrease) than do control siRNA-transfected astrocytes (Fig. 6).

Other Factors Affecting ONH Astrocyte Migration

TGF β modulates cellular migration or motility in corneal fibroblasts.²⁴ Our previous report on gene expression among healthy and glaucomatous ONH astrocytes found differences in

FIGURE 2. Expression of MYLK induced by elevated hydrostatic pressure. (A) Total MYLK mRNA is increased by elevated hydrostatic pressure at 72 to 96 hours of treatment. The increase is significant ($P < 0.05$; $n = 3$) in both CA (white bars) and AA astrocytes (black bars) at 96 hours. (B) MYLK-210 mRNA is also increased by elevated hydrostatic pressure at 96 hours ($P < 0.05$; $n = 3$). All expression data are normalized to 18S RNA levels. (C) Representative changes in MYLK-130 protein expression in AA astrocytes, with elevated hydrostatic pressure for 0 to 96 hours. (D) Representative changes in MYLK-130 protein expression in CA astrocytes with elevated hydrostatic pressure for 0 to 96 hours. The C96 band in both groups is from untreated cells cultured for the same time as the 96-hour-treated group. The lower band is β -actin.

the expression of TGF β or TGF β receptors among these populations of ONH astrocytes.⁸ In these studies, more TGF β was secreted by cultured AA astrocytes than by CA cells, and TGF β receptors were differentially expressed between cultured AA and CA astrocytes from donors with glaucoma.⁸ Therefore, we investigated how the inhibition of TGF β signaling affects the migration of ONH astrocytes. siRNA to TGFBR2 was used to inhibit TGF β signaling in ONH astrocytes. Figure 7A shows that siRNA treatment decreased the expression of TGFBR2 by an average of 80% compared with control siRNA. Other genes

FIGURE 3. Effects of ML-7 treatment on the migration of ONH astrocytes. (A) A time 0 scratch area is shown. (B) Migration of ONH astrocytes 24 hours after scratch. (C) Migration of ONH astrocytes 24 hours after scratch in the presence of 10 μ M ML-7.

regulated by TGF β signaling in ONH astrocytes¹⁰ are also affected. These include elastin (ELN), matrix metalloprotease (MMP1), and collagens (Col4A2, Col15A1; Fig. 7A). Forty-eight hours after siRNA treatment, ONH astrocytes were tested for migration using the scratch assay, as described for MYLK. Figure 7B illustrates that TGFBR2 siRNA significantly ($P < 0.001$) inhibits migration by 17% and 34% in AA and CA astrocytes, respectively.

Elevated HP and Expression of TGF β Signaling Components in CA and AA ONH Astrocytes

As with MYLK, we also tested the effects of elevated HP on the expression of selected components of the TGF β signaling pathway and the regulatory subunit of myosin light chain phosphatase (MYPT1). We selected these components because of their

FIGURE 4. Knockdown of MYLK expression in ONH astrocytes by siRNA treatment. (A) Relative expression of MYLK mRNA (all isoforms) by qRT-PCR ($P < 0.01$; $n = 5$). (B) Representative Western blots of MYLK-210 and MYLK-130 protein decrease caused by siRNAs after 48-hour treatment in CA or AA ONH astrocytes. SCR/Scramble, nontargeted siRNA 2; MYLKsi, siRNA pool.

altered expression in glaucomatous ONH astrocytes.⁸ Figures 8A and 8B summarize changes in selected mRNA expression induced by elevated HP in CA and AA astrocytes, respectively. In both groups, the expression of TGF β 1, TGF β receptors 1

FIGURE 5. Effect of MYLK siRNA compared with control siRNA on ONH astrocyte migration. (A) Representative scratch areas after 18 hours of control siRNA treatment and (B) 18 hours of MYLK siRNA treatment of CA astrocytes. (C) Representative scratch area after 18 hours of control siRNA treatment and (D) 18 hours of MYLK siRNA treatment of AA astrocytes.

FIGURE 6. Bar graph of migration data for ONH astrocytes treated with MYLKi or control siRNA. Shown are the mean migration distances for ONH astrocytes treated with siRNAs. The decrease in migration distance is significant ($P < 0.01$; $n = 16-20$) for both AA (gray bars) and CA (black bars) astrocytes. Scramble, nontargeted control siRNA; MYLKi, MYLK siRNA pool.

and 2, and MYPT1 decrease with time of exposure to elevated HP. However, only in CA astrocytes did we find that TGF β 2 expression increased (Fig. 8A) with elevated HP.

DISCUSSION

ONH astrocytes from glaucoma donors exhibit altered cell migration and adhesion properties.³⁰ Gene expression analyses revealed that MYLK and TGF β pathways relevant to astrocyte migration were altered in glaucomatous astrocytes.⁸ Here we found that ONH astrocytes express two splice variant isoforms of MYLK that are not different among astrocytes from AA or CA donors or altered in glaucomatous astrocytes from either population. The significance of the deletion in exon 11 within the N-terminal region of MYLK-210 is unclear. In experiments conducted in cultured cells, the exon 11 deletion removes an SRC-kinase tyrosine phosphorylation site.³¹ MYLK-210 that can be phosphorylated by SRC-kinase at this site exhibits higher specific activity than the kinase species containing the deletion.³¹ Because the relative amount of deletion product does not change at the mRNA level in different populations of astrocytes (Fig. 1), the presence or absence of the deletion may have no particular relevance in ONH astrocytes. Genetic studies will be necessary to determine whether single nucleotide polymorphisms in MYLK are associated with susceptibility to glaucoma in either the AA or the CA population.

Combined with the current results on the change in expression of MYLK on extended (48- to 96-hour) treatment with elevated hydrostatic pressure, it appears that ONH astrocytes react to this stress in a two-phase process. In the first phase (<24 hours), there is increased synthesis of cyclic AMP,³² increased Rho GTPase,³³ and nitric oxide synthase 2 activity³⁴ but a decrease in gap junctional communication.³⁵ In the second phase (24-96 hours), changes in extracellular matrix proteins synthesis (elastin³⁶), cyclooxygenase,¹³ cell adhesion (NCAM²⁰), heat shock proteins,²¹ intermediate filament (vimentin, GFAP²¹), and migration (MYLK, this study) emerge. Interestingly, many of these proteins are also increased in the optic nerve head of patients^{1,37} or experimental animals with glaucoma.³⁸ Thus, in terms of gene expression, elevated HP for periods greater than 24 hours appears to induce what we call a preglaucomatous phenotype. The evidence for this phenotype is limited. For example, earlier work using preexposure to elevated HP for 48 hours showed that ONH astrocytes exhibited fourfold to sixfold higher migratory ability compared with untreated cells.³⁹ In this work, our finding that specific inhibi-

tion of MYLK or TGF β signaling decreases migration, indicating that these are two key components of migratory capacity in ONH astrocytes.

Earlier studies showed that pharmacologic inhibition of other signaling systems, such as phosphoinositide kinase and protein kinase C,³⁰ slows ONH astrocyte migration induced by elevated HP or inflammatory agents. Inhibition of these signaling systems also decreases the ability of ONH astrocytes to respond to wounding by slowing migration into the cell-free area.¹³ Mathematical modeling of MYLK activation suggests that Ca²⁺ levels are highly correlated with MYLK activation as a result of altering the binding of MYLK to calmodulin, the Ca²⁺ sensor/activator for MYLK.⁴⁰ Based on these studies and our current results, signals originating with phosphoinositide hydrolysis and protein kinase C can affect MYLK activity as a result of modulating Ca²⁺ levels.⁴¹ Furthermore, protein kinase C and other kinases activated through lipids or phospholipids may also modulate myosin light chain phosphatase activity through phosphorylation of its regulatory subunit.⁴⁰ Additionally, the nonkinase activity of MYLK, particularly the amino-terminal tail of MYLK-210,⁴² may function to integrate

FIGURE 7. Effects of TGFBR2 siRNA on gene expression and migration in ONH astrocytes. (A) Relative expression of TGFBR2, ELN, MMP1, Col15A1, and Col4A2 in AA (white bars) and CA (black bars) ONH astrocytes that were treated with TGFBR2 siRNA. Gene expression is normalized to 18S RNA. (B) Bar graph showing the migration of AA and CA ONH astrocytes in the presence of control siRNA (white bars) or TGFBR2 siRNA (black bars). The decrease in migration is significant ($P < 0.05$; $n = 10$) for both CA and AA astrocytes.

FIGURE 8. Effect of elevated HP on the expression of TGF β -related and myosin light chain phosphatase genes in ONH astrocytes. **(A)** Bar graph of the expression of TGFB1, TGFB2, TGFBR1, TGFBR2, and MYPT1 in AA ONH astrocytes at 0, 24, 72, and 96 hours of elevated HP treatment. **(B)** Bar graph of the expression of TGFB1, TGFB2, TGFBR1, TGFBR2, and MYPT1 in CA ONH astrocytes at 0, 24, 72, and 96 hours of elevated HP treatment. Bars indicate increasing time of HP treatment. White bars: 0 hour; light gray bars: 24 hours of treatment; dark gray bars: 72 hours of treatment; black bars: 96 hours of elevated HP treatment. For TGFB2, the difference in expression between CA and AA astrocytes is significant ($P < 0.05$; $n = 4$) at 72 and 96 hours of elevated HP.

signals that result in changes in cell morphology⁴³ and chemotactic responses.⁴⁴

TGF β has been studied extensively in normal and glaucomatous optic nerve head astrocytes⁸ and optic nerve head.⁴⁵ Both TGF β 2 and TGF β 1 are expressed in cultured astrocytes.⁸ However, in the normal optic nerve head, TGF β 1 and TGF β 2 proteins levels are barely detectable, whereas TGF β 2 is increased in glaucomatous optic nerve head and colocalizes with GFAP-positive astrocytes.⁴⁵ Thus, our finding that TGF β 2 expression is upregulated by elevated HP in CA astrocytes suggest that it may be an important indicator of both preglaucomatous astrocytes in vitro and glaucoma in the optic nerve head. TGF β also modulates expression of extracellular matrix components in trabecular meshwork (TM) cells.^{46,47} Similarly, TGF β 1 or genes regulated by TGF β signaling are also upregulated by cyclic mechanical stress in TM cells.^{48–50} Thus, altered TGF β signaling may be a common feature of ONH astrocytes and TM

cells when they are subjected to mechanical or elevated hydrostatic pressure as stressors.

Myosin light chain kinase, particularly the MYLK-210 isoform, is elevated and a potential drug target for conditions such as inflammatory bowel disease,^{51,52} airway hypersensitivity in asthma,^{53,54} and inflammation-induced lung injury.^{55,56} Interestingly, TGF β signaling is also altered in asthma⁵³ and inflammatory bowel disease.⁵⁷ Although these diseases are a consequence of epithelial barrier dysfunction, altered MYLK and TGF β signaling are features shared with ONH astrocytes in glaucoma. Thus, targeting TGF β with vaccines⁵⁸ and myosin light chain kinase with inhibitors⁵⁹ may have application to glaucoma by inhibiting the migration of ONH astrocytes.

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