The Presence and Properties of Myocilin in the Aqueous Humor

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Purpose. To determine whether myocilin is present in the aqueous humor (AH) and to examine certain properties of this protein.

Methods. Human AH was obtained at the time of either glaucoma surgery or cataract extraction. Monkey AH was obtained at the time of death, and bovine aqueous was obtained from eyes delivered from an abattoir. Column chromatography was performed on aqueous samples to determine the approximate size of the myocilin present. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were performed using antibody prepared against a peptide sequence in myocilin. Analysis of the bovine proteins present in AH that were retained by a microporous filter was also performed using western blot analysis.

Results. By western blot analysis, myocilin was present in human, monkey, and bovine AH. The apparent molecular size of the myocilin present in the AH were greater than 250,000 Da, when quantified with a gel filtration column. Myocilin appeared to be hydrophobic and was one of the proteins that was retained on microporous filters that were obstructed by AH.

Conclusions. Myocilin is a constituent in the AH. It appears that myocilin is a hydrophobic protein that may exist in an oligomeric state or in association with other proteins. Myocilin is retained by microporous filters and may be involved in the obstruction of these filters that occurs when AH is perfused through them. (*Invest Ophthalmol Vis Sci.* 2001;42:983–986)

Aqueous humor (AH) flows through the chambers of the eye, and there is a circadian modulation of its formation.[1] There have been many studies concerning the ion, amino acid, and protein composition of AH.[2–4] Although the bulk of the proteins probably enters the AH through the root of the iris, they can also be released from other tissues in the eye.[5,6] Recently, mutations in a gene on human chromosome 1 have been genetically linked to primary open-angle glaucoma.[7] The protein encoded by this gene had been studied as the trabecular meshwork inducible glucocorticoid response protein (TIGR).[8,9] Expression of this protein was also present in other tissues in the eye, and the protein was named myocilin.[10–12] Because immunohistochemistry had indicated localization of myocilin in cells of the nonpigmented ciliary epithelium, corneal endothelium and iris stroma,[12] it was of interest to determine whether the myocilin was present in the AH and the apparent molecular size of the protein.

Initial results indicated that this protein was very hydrophobic, and additional studies were therefore undertaken. Previous studies had suggested that there is a hydrophobic component in the AH that is able to obstruct flow through microporous polycarbonate filters with pore sizes similar to those that are found in the juxtacanalicular connective tissue. This component appeared not to be present in serum, because serum at concentrations equivalent to those found in AH would not impede the flow through the filter to the extent that AH would.[13,14] To determine whether myocilin is associated with obstruction of the microporous filters, filters were perfused with AH, and the proteins tightly bound to those filters were examined.

Methods

All procedures in this study conformed to the tenets of the Declaration of Helsinki, the National Institutes of Health guidelines on the care and use of animals in research and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Human AH was obtained at the time of either glaucoma surgery or cataract removal. An institutionally approved human protocol governed the acquiring of the human samples, and informed consent was obtained from all patients. AH was obtained by paracentesis of the anterior chamber at the beginning of the operation. Two samples from patients with primary open-angle glaucoma and three samples from patients with cataract, but no history of glaucoma were obtained. The AH was frozen at −70°C until use. To determine whether the AH was contaminated with intracellular proteins as a result of cells that were damaged during the paracentesis, a commercially available assay for lactic acid dehydrogenase (LDH; Sigma, St. Louis, MO) was performed on each of the AH samples. The assay followed the manufacturer's protocol, but the volumes were reduced to allow the assay to be conducted in a 96-well plate. For gel electrophoresis, after the human AH (0.75 μg) was thawed, it was dried (SpeedVac system; Savant, Farmingdale, NY). The AH was then reconstituted in sample buffer (62.5 mM Tris [pH 6.8]), 2% sodium dodecyl sulfide [SDS], and 5% β-mercaptoethanol) and placed in boiling water. Samples were run on 12.5% gels (PhastGels; Pharmacia Biotech, Piscataway, NJ) and blotted onto nitrocellulose (BioRad, Hercules, CA) as directed by the manufacturer's protocol. Protein standards (BenchMark Protein Ladders) were obtained from Life Technologies (Grand Island, NY). The western blot analyses were developed with a kit (Chemiluminescence Reagent Plus; NEN Life Sciences, Boston, MA) after incubation with primary antibody to myocilin[12] overnight at 4°C and incubation with secondary antibody (Kirkegaard and Perry, Gaithersburg, MD) for 1 hour. Chemiluminescence was detected using film (XAR; Kodak, Rochester, NY) or with an image analysis system (Image Station 400; NEN Life Sciences).

Bovine eyes were transported from the abattoir, and the AH was removed within 5 hours of death. For column chromatography, the AH was concentrated 10-fold (Centricon-10 concentrator; Amicon, Danvers, MA), and sample was diluted 10-fold with water and reconcentrated. Samples of up to 200 μl were applied to a gel filtration column (Zorbax GF250; DuPont, Wilmington, DE) with a running buffer of 50

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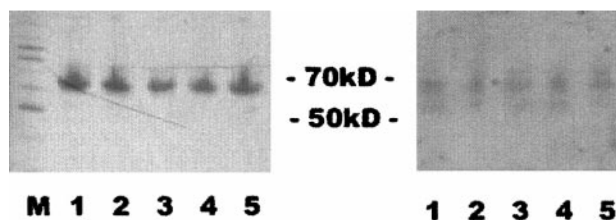

Figure 1. SDS-PAGE (*left*) and western blot (*right*) of human AH samples from three patients who underwent cataract removal (*lanes 1, 2* and *3*) from two patients who underwent glaucoma surgery (*lanes 4* and *5*). The protein ladder (M) is the manufacturer's standard. The western blot analysis using antibody to myocilin showed two immunoreactive bands at approximately 65 and 55 kDa in each of the AH samples.

mM HEPES (pH 7.5), 0.2M NaCl, and 0.02% NaAzide. The flow rate through the column was 330 μl/min. Fractions were collected each minute. Fractions were concentrated on the concentrators, gel sample buffer was added, and the samples were placed in boiling water. Samples were run on 12.5% gels and western blot analysis developed according to the manufacturer's protocol (Pharmacia Biotech). The blotted gel was silver stained according to the protocol.

Monkey AH was obtained at the time of death. The monkey AH was treated in a manner similar to the bovine AH, with the exception that it was not diluted with water after concentration.

To study the obstruction of microporous filters, the bovine AH was first centrifuged at 100,000*g* for 45 minutes. Between 1 and 1.25 ml of the supernatant was passed through a polycarbonate 0.2-μm filter that had not been treated with polyvinylpyrrolidone (PVP; Nucleopore, Pleasanton, CA). These PVP-free filters are hydrophobic.[15] The filters were washed with 1 ml of Dulbecco's phosphate-buffered saline (PBS) after the aqueous had been perfused through and then rinsed briefly in this saline. Ten filters were placed in 1 ml of 0.1% Nonidet P40 (American Bioanalytical, Natick, MA) overnight in the refrigerator. Samples of all the fractions at each step of the process (uncentrifuged, centrifuged supernatant, material perfused through the filter, and proteins eluted from the filters) were concentrated (Centricon-10; Amicon) and then used in the biochemical analysis.

Results

All the human samples had lactic acid dehydrogenase levels below 22 U/l. This value has previously been reported to be the upper level of normal AH.[16] This result suggests that the paracentesis at the time of surgery did not cause rupture of cells in the eye and was not contaminated with secondary aqueous. AH obtained from a donor eye that arrived in the laboratory more than 24 hours after death had a value of 55 U/l showing that the LDH assay of AH could detect cellular damage. Using SDS-polyacrylamide gel electrophoresis (PAGE) of the human samples and western blot analysis with an antibody developed to a peptide sequence of human myocilin, two immunoreactive bands were seen in the human AH samples (Fig. 1). The antibody has been used to detect myocilin in various tissues in the eye.[12] The topmost band in the AH from both the patients with glaucoma and the ones undergoing cataract extraction was located at approximately 65 kDa. The lower one was present at approximately 55kDa. In a separate experiment, the proteins from human serum were also run on SDS-PAGE, but western blot analysis did not reveal any immunoreactive bands with this antibody, which suggests that the level of myocilin was below the level of detectability (data not shown).

Myocilin was also present in bovine and monkey AH. Both monkey and bovine AH were used for the column chromatography experiments. Initial attempts with the bovine AH to determine the apparent molecular size of the myocilin were not successful. Gel exclusion columns that were based on agarose beads appeared to interact with the myocilin in the aqueous. Positive immunoreactivity on western blot analysis could be detected from the void volume to the column volume fraction. This finding suggests that the myocilin may interact hydrophobically with the beads. Gel filtration was undertaken using a silica-based column (Zorbax GF 250; Dupont) to minimize the hydrophobic interactions. The pattern of the bovine AH eluting from the column indicated that there was a small peak of absorbance at 280 nm at the void volume (Fig. 2). Blue dextran (2000 kDa) eluted from the column in this fraction, but under the conditions of flow, proteins greater than approximately 250,000 Da could have been present in the first collected tube. A considerably larger peak was observed at fraction 26 that corresponded to the fraction in which the albumin standard eluted in another run. There was a shoulder on the major peak and a couple of other peaks of low molecular size that trailed the major peak.

SDS-PAGE and western blot analysis of the fractions were performed, and immunoreactive myocilin was observed principally in the first fraction that eluted from the column. Myocilin was not the only protein present in this fraction. In addition to the bands that resolved on the gel, stained material was also present at the stacking gel interface suggesting that there might be some additional large proteins present in the complex. A very small amount of immunoreactivity to myocilin was observed in other fractions, particularly in fraction 24, that corresponded to the fraction in which the standard aldolase eluted, which suggests an apparent size of this myocilin of approximately 150,000 Da and may correspond to a dimeric form of this protein. The silver-stained gel did not reveal many

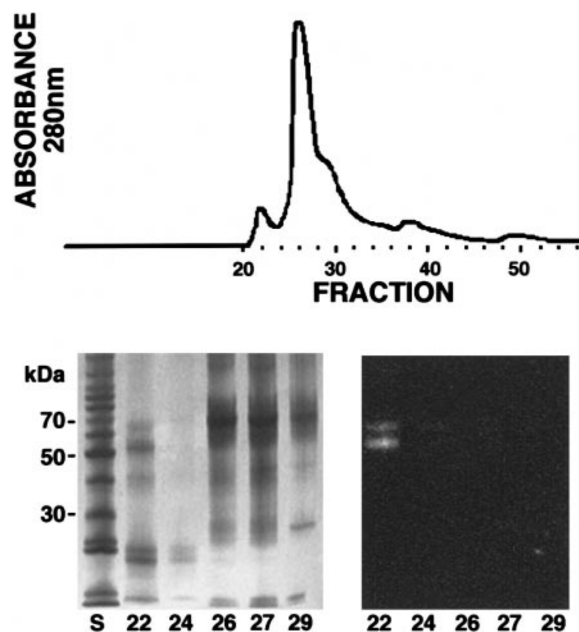

Figure 2. The elution pattern (*top*) of bovine AH from a gel filtration column. Standards eluted from the column in the following fractions: blue dextran, fraction 22; aldolase, fraction 24; albumin, fraction 26; and carbonic anhydrase, fraction 31. SDS-PAGE (*bottom left*) and western blot analysis (*bottom right*) of the proteins eluting in the different column fractions indicated that immunoreactive myocilin was present in the void volume fraction. A small amount of immunoreactive myocilin was present in fraction 24, although the protein content of this fraction was small.

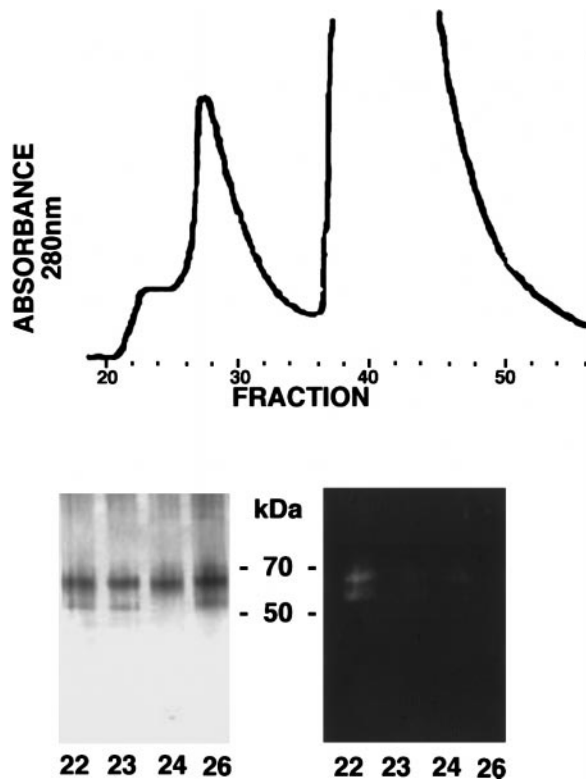

Figure 3. The elution pattern (*top*) of monkey AH from the gel filtration column. SDS-PAGE (*bottom left*) and western blot analysis (*bottom right*) of the proteins eluting in the different fractions indicated that most of the immunoreactive myocilin was present in the void volume fraction.

proteins in this fraction. In fraction 26 at the expected elution of albumin, the most prominent band on the silver-stained SDS-PAGE gel had an apparent molecular mass of 67 kDa.

Monkey AH was collected at death and had LDH values less than 17 U/l. Column chromatography was also performed with the monkey AH. With the monkey AH, a distinct peak at the void volume was not seen, rather the absorbance appeared to plateau until the peak at fraction 26. Because the monkey sample was not washed to reduce ascorbate, a peak of very low molecular weight material was present. Ascorbate, which has a very high concentration in AH,[2] readily absorbs light at 280 nm (Fig. 3). The majority of the immunoreactive myocilin was present in the first fraction eluting from the column. A very small amount of immunoreactive material was present in other fractions from the column, as was true in bovine AH.

Because the agarose column results suggested that myocilin might be a very hydrophobic protein, the possibility was examined that myocilin might be one of the proteins responsible for the reported capability of AH to obstruct flow through hydrophobic filters (Nucleopore). Previous work had shown that bovine AH obtained immediately after death or obtained within a few hours after death caused obstruction of the filters.[14] The proteins present in the bovine AH, the supernatant from the 100,000*g* centrifugation of the AH, the AH proteins that passed through the filter, and the AH proteins that were tightly bound to the filter were examined by SDS-PAGE (Fig. 4). Only approximately 2.2% of the proteins were retained on the filter. Several of the proteins in the AH were present in the fraction that was bound tightly to the filters; however, certain of the proteins were not as prominent on the filter as in the material that went through the filter, such as the protein migrating at approximately 180,000 Da.

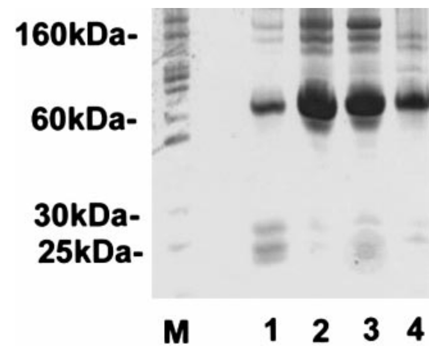

Figure 4. Silver-stained SDS-polyacrylamide gel of proteins present in the bovine AH. *Lane 1*: The proteins present in the AH when it was first obtained; *lane 2*: the proteins present in the supernatant of the 100,000*g* centrifugation of bovine AH; *lane 3*: the proteins that were perfused through the filter; *lane 4*: proteins that were tightly bound to the filter. The protein standard (M) is the manufacturer's protein ladder.

By western blot analysis, the myocilin could be detected in all these fractions (Fig. 5). Other immunoreactive bands were also detected with lower molecular masses. These bands, such as the one at approximately 25 kDa, may represent some degraded fragments of the myocilin. Two of these bands were prominent in the material that was closely associated with the filter. One of the bands had a molecular mass of approximately 32 kDa and the other of approximately 15 kDa.

Discussion

Myocilin was detected in AH. This protein was present in human, monkey, and bovine AH at molecular masses of approximately 65 and 55 kDa according to results of SDS-PAGE. This result suggests that both the glycosylated form and an unglycosylated form are present in the AH. Both forms have been previously reported to be present in media from human trabecular meshwork cells treated with glucocorticoids.[17] The ratios of the glycosylated and unglycosylated forms appeared similar in all the human samples. There is perhaps more glycosylated form present, but additional studies with multiple human samples will have to be undertaken to accurately quantitate any differences. The cells responsible for the release of

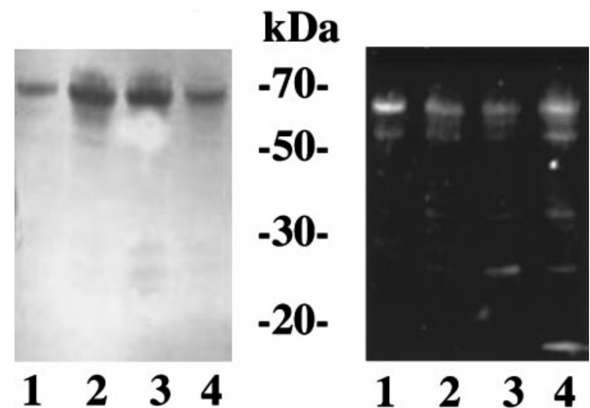

Figure 5. The blotted and silver-stained SDS- polyacrylamide gel of proteins identical with those shown in Figure 3 (*left*) and the western blot of this gel (*right*) showing the immunoreactive myocilin present at 65 and 55 kDa in the fractions. In the lane showing the fraction of the proteins tightly bound to the filter, additional bands were seen at approximately 32 and 15 kDa.

the myocilin into the AH are not known at this time. The absence of immunoreactivity of the antibody with human serum suggests that the amount of this protein is low or perhaps not normally present in the blood. Because of the high turnover rate of the AH,[1] the possibility that myocilin is present in the AH as a result of the diffusion of serum proteins at the iris root[5] appears remote. Myocilin has been reported to be in a number of tissues in the eye,[10–12, 17] and therefore several tissues could release myocilin into the AH, not just the trabecular meshwork. Candidates may be cells of the corneal endothelium, nonpigmented ciliary epithelium, or anterior iris stroma, in that positive immunolabeling with the antibody against myocilin was shown in a previous study.[12]

The apparent molecular mass of the myocilin in the bovine and monkey AH appears to be greater than expected of a dimeric protein. The gel filtration studies suggest that this protein could be oligomeric or could be associated with other proteins in the AH. An aggregate of proteins might also be suggested by the apparent hydrophobicity of the protein itself. This possibility suggests future experiments to determine the proteins that may be interacting with the myocilin in the AH and whether the composition of these aggregated may be changed during glaucoma. A small amount of myocilin appears to exist in the dimeric state, because there was some immunoreactivity in fractions that eluted after the void volume fraction.

The apparent hydrophobic interaction of the protein with the agarose beads led us to re-examine the earlier findings of Johnson et al.[14] that AH contains proteins that can obstruct hydrophobic, microporous membranes. They concluded that a protein with molecular mass of approximately 28 kDa was involved with the filter obstruction process. However, the possibility that the very prominent spot seen at 67 kDa might contain not only albumin but also another protein was not considered.

The tight association of the myocilin with the polycarbonate filters is consistent with the apparent hydrophobic nature of this protein. Although it is currently not possible to say whether the myocilin is the component in the AH that is responsible for obstructing the filters, it certainly is a candidate. Future experiments with purified myocilin that is glycosylated or unglycosylated may answer this question. It is conceivable that certain proteins selectively bind to either one form or the other. The finding of small molecular weight immunoreactive bands in the group of proteins tightly adhering to the filter may indicate that some proteolysis of the myocilin is occurring in the AH. This is particularly interesting in the case of the 32-kDa band, which is close to the 28-kDa one reported by Pavao et al.[13] as being related to the filter-obstruction process. It may be that these fragments of myocilin are more influential in obstructing the filters than the full-length protein.

In summary, myocilin was present in AH. Both the glycosylated and unglycosylated forms were found. The majority of the myocilin was found in a fraction that included proteins with apparent molecular masses greater than 250,000 Da. This result suggests that myocilin may exist as an oligomer or may be in association with other proteins in the AH. Myocilin appears to be a hydrophobic protein and is one of the proteins that is tightly bound to polycarbonate filters that become obstructed when AH is perfused through them.

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