Functional impairment of lens aquaporin in two families with dominantly inherited cataracts

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Received 29 June 2000; Revised and Accepted 7 August 2000

Opacities in the crystalline lens of eye appear with high frequency in the general population. Dominantly inherited cataracts with differing clinical features were found in two families carrying different point mutations in the gene encoding lens water channel protein AQP0 (major intrinsic protein, MIP). Families with E134G have a uni-lamellar cataract which is stable after birth, whereas families with T138R have multi-focal opacities which increase throughout life. To establish pathophysiological relevance of cataract formation, the Xenopus laevis oocyte expression system was employed to evaluate functional defects in the mutant proteins, E134G and T138R. Both substitutions cause loss of membrane water channel activity due to impaired trafficking of the mutant proteins to the oocyte plasma membrane. Although missense mutations in AQP1 and AQP2 proteins are known to result in recessive traits in vivo and in vitro, when E134G or T138R are co-expressed with wild-type AQP0 protein, the mutant proteins exhibit dominant negative behaviour. To our knowledge, these studies represent the first in vitro demonstration of functionally defective AQP0 protein from humans with congenital cataracts. Moreover, these observations predict that less severe defects in the AQP0 protein may contribute to lens opacity in patients with common, less fulminant forms of cataracts.

INTRODUCTION

The lens is a unique tissue that increases in size throughout life by deposition of newly differentiated fiber cells in concentric lamellae resembling an onion skin. Part of this differentiation process involves the loss of both nucleus and organelles. Clarity to light in the visible range is maintained by highly ordered adherence of the fiber cells to each other and the packing of their intracellular contents, the crystallins. Lens transparency is known to depend on a critical balance between the concentration of these macromolecules and disruption of normal levels of hydration can lead to opacity (1).

Human cataract, a condition in which the crystalline lens of the eye becomes opacified, is the commonest cause of blindness world-wide (2,3) and can also result in irreversible amblyopia in children. Approximately half of all congenital cataracts are familial (4) and are a secondary feature of almost 200 genetic diseases (5). Significant advances towards understanding the genetics of inherited forms of cataract have recently been made. Most often a cataract is inherited non-syndromically as an isolated dominant abnormality. Independent chromosomal loci have been identified on several autosomes and mutations have been described in human genes encoding the transparent intracellular lens proteins (crystallins), membrane gap junction proteins (connexins) and a developmental transcription factor (PITX3) (6). As expected for a disorder with multiple genetic origins, congenital cataracts exhibit marked phenotypic heterogeneity (7).

The major intrinsic protein of the lens (AQP0) is a member of the aquaporins, a ubiquitous family of membrane water transport proteins that confer rapid movements of water across cell membranes (8). Aquaporins share a unique structure with six bilayer-spanning α-helices surrounding loops B and E which overlap between the leaflets of the lipid bilayer, forming the transmembrane ‘hourglass’ (Fig. 1) (9). In the native membrane, aquaporins exist as tetramers and most inherited defects in red cell and kidney aquaporins are autosomal recessive (10,11). AQP0 is only expressed in terminally differentiated fibres, the major cell type of the crystalline lens. Naturally occurring mutations in the orthologous gene Aqp0 from mice with cataracts (12) prompted a search for mutations in the human AQP0 gene from patients with cataracts. Two families were recently identified with cataracts linked to the aquaporin gene cluster at human chromosome 12q (13). Affected family members carry single amino acid substitutions, E134G or T138R (Fig. 1), producing congenital cataracts with distinctive clinical phenotypes (Fig. 2). Here we demonstrate molecular consequences of these mutations in AQP0 protein function in vitro.

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The *Xenopus laevis* oocyte swelling assay was employed to assess possible functional consequences of the E134G and T138R substitutions (14). The cDNA encoding the human AQP0 protein from a human lens library was cloned into the X. laevis expression vector. Coefficients of osmotic water permeability ($P_f$) were calculated from measurements of oocyte swelling after transfer from 200 to 70 mosM solution. AQP1 and most other mammalian aquaporins are known to exhibit a 10- to 20-fold increase in water permeability using this system (15); however, non-human AQP0 orthologues have previously been noted by multiple laboratories to exhibit much smaller increases in $P_f$ (16–18).

In six separate experiments, oocytes injected with 5 ng of human AQP0 cRNA exhibited ∼3-fold increase in osmotic water permeability (Fig. 3a). In each of four separate experiments, oocytes injected with 5 ng of E134G or T138R mutant cRNA had water permeabilities similar to that of control oocytes, whereas oocytes co-injected with 5 ng of wild-type AQP0 cRNA plus 5 ng of E134G or T138R mutant cRNA showed water permeabilities which were less than half of the wild-type level (Fig. 3a). The abilities of the E134G and T138R mutant proteins to inhibit the wild-type subunit were compared with each other in the four experiments. Although oocytes injected with wild-type AQP0 cRNA plus T138R mutant cRNA exhibited slightly lower water permeability than did oocytes injected with wild-type AQP0 cRNA plus E134G cRNA, no statistically significant differences were noted (data not shown). Whereas E134G or T138R proteins do not increase the oocyte membrane water permeability, both inhibit the function of co-expressed wild-type subunits.

Oocyte membranes were analysed on immunoblots incubated with rabbit antiserum specific for the C-terminus of AQP0. Since methods for purification of oocyte plasma membranes do not eliminate the presence of substantial amounts of intracellular membranes (16), our samples included total plasma membrane and intracellular membrane pools. After 3 days of

**RESULTS**

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incubation, membranes from control oocytes exhibited no immunoreactivity on immunoblots, whereas membranes from oocytes injected with wild-type AQP0 cRNA exhibited a strong band at ∼30 kDa (Fig. 3b). In each of the four separate experiments, immunoblots of membranes from oocytes injected with E134G or T138R mutant cRNA exhibited ∼30 kDa bands with lesser intensity than the wild-type signal (Fig. 3b) and the E134G samples yielded a slightly stronger signal than the T138R samples. In each of the four experiments, immunoblots of oocytes co-injected with wild-type AQP0 cRNA plus the T138R mutant cRNA exhibited an ∼30 kDa band of lesser intensity (Fig. 3b). Therefore, total membranes from oocytes injected with the mutant cRNAs express ∼30 kDa polypeptides which appear reduced by semi-quantitative immunoblot analysis.

The likelihood that the mutant polypeptides may fail to traffic to the oocyte plasma membrane was studied by confocal immunofluorescence microscopy. Control oocytes exhibited negligible immunofluorescence, whereas oocytes injected with wild-type AQP0 cRNA had a sharp rim of immunofluorescence at the plasma membrane. Oocytes injected with E134G or T138R mutant cRNA exhibit immunofluorescence at both the plasma membrane and over the cytoplasmic space. Panels shown are representative of confocal studies performed on three separate occasions with three different sets of oocytes.
the bovine and frog AQP0 confirmed that the protein functions as a membrane water channel (16–18); however, the inherent permeability is much lower than that of other aquaporins. Similar to other aquaporins, AQP0 is a homo-tetramer and all known naturally occurring mutations in human AQP0 are expressed as recessive traits (10). Recessive mutations in human AQP2 are known to result from improper membrane protein trafficking (11,19). A secondary structural function has been proposed for AQP0 as a cell-to-cell adhesion protein (20), and mutations in structural proteins are known to result often in dominantly inherited traits. Of note, two naturally occurring mouse Aqp0 mutants (11) and both of the human families reported here suffer from cataracts of dominant inheritance. Thus, the pathological mutations in the gene encoding AQP0 may perturb cell fibre water content and cell-to-cell adherence.

The three-dimensional structures of several aquaporins, including AQP0 (21), are being solved by cryoelectron microscopy. In the human red cell AQP1 is presently understood at 4.5 Å and is a right-handed helical bundle with six tilted bilayer-spanning domains surrounding an intracellular ‘hourglass’ formed by loops B and E (Fig. 1) (22). The importance of transmembrane α-helices is becoming appreciated. The first and fourth transmembrane domains each contain a highly conserved motif (ExxxTxx/F/L) which was recently proposed to line the aqueous channel within each subunit (23). The E and T residues are predicted to reside on the internal face of the fourth transmembrane α-helix.

Interestingly, these residues correspond exactly to the sites of the two mutations in our two families, E134G and T138R. Each of these substitutions would be expected to be disruptive. Replacement of negatively charged residue glutamate (E134) with glycine (G), a smaller but uncharged residue, eliminates a fixed charge lining the aqueous pathway. Replacement of the polar residue threonine (T138) with the large residue arginine (R) introduces a strong positive charge in the aqueous pathway. Our studies in oocytes indicate that the human mutant E134G and T138R polypeptides fail to traffic to the plasma membrane (Fig. 4). Moreover, when co-expressed, both E134G and T138R mutants interfere with the water permeability of wild-type AQP0 protein (Fig. 3a).

Our studies do not yet provide firm biochemical explanations for the differences in the clinical phenotypes of the two families. Lens opacities in the E134G family (Fig. 2) are stationary and limited to specific central lamellae, corresponding to those fibres that differentiated in the late fetal period (1). We speculate that the lens may be resistant to the consequences of the E134G mutation except during this developmental stage when the tissue may be particularly stressed. In contrast, individuals carrying the T138R mutation have multiple punctate opacities that can be located in any region of the lens and that progress during life (Fig. 2). We speculate that such a random pattern of opacification indicates that localized precipitations of lens crystallins or disruption of fibre cell architecture may occur in areas where the T138R mutant polypeptides interfere with water permeability and cell-to-cell adherence.

Together our studies demonstrate that the recently described AQP0 mutations from human patients with dominantly inherited congenital cataracts (13) result in dominant negative loss of AQP0 function in vitro. It remains unclear whether the molecular basis for reduced water permeability of oocytes expressing wild-type AQP0 plus the E134G polypeptide and oocytes expressing wild-type AQP0 plus T138R mutant polypeptide are identical. The T138R defect could result from aberrant membrane-trafficking and degradation of tetramers containing a mix of wild-type and mutant subunits, whereas the E134G defect could represent proper trafficking tetramers containing a mix of both subunits but resulting in a reduction in the unit water permeability. Moreover, it is yet unknown whether trafficking of these mutants is retarded at the endoplasmic reticulum or at the Golgi. Nevertheless, based on these studies we predict that other inherited or acquired defects in the AQP0 protein may contribute to some of the common forms of human cataracts. Thus, it may also be informative to determine the deduced amino acid sequence encoded by genomic AQP0 from cataract patients with predisposing conditions, such as diabetes mellitus, as well as from patients with sporadic forms of cataract which are frequently found in older populations (1–3).

**MATERIALS AND METHODS**

**AQP0 cDNA**

A lens cDNA library was prepared from RNA isolated from lenses of a 40-year-old human (Life Technologies, Gaithersburg, MD) cloned into the pCMVSPORT6 vector. Expressed sequence tag (EST) analysis showed the presence of AQP0 in the library. To obtain a complete coding sequence, specific primers were designed from published genomic sequence (GenBank accession no. U36308) and from EST data: HMP1, 5′-agatctgtgacatcccccctgccatgtgg-3′; HMP3, 5′-agatctCTA-CAGGGCTTGTTGTCAGTT-3′. Template DNA was produced from 1.5 × 106 c.f.u. of the non-normalized lens library in luria broth/ampicillin followed by plasmid isolation using Turbo Prep (Qiagen, Valencia, CA). Amplification by polymerase chain reaction was performed using AmpliTaq (PE Biosystems, Foster City, CA) and a dilution series of the template DNA. The product of expected size (821 bp) was subcloned using the pCR2.1 TOPO cloning system (Invitrogen, Carlsbad, CA) and verified by restriction digestion and sequencing.

**Xenopus laevis oocyte expression**

The human AQP0 cDNA with EcoRI and BstEII linkers was cloned into the pXBG X.laevis expression vector (14). Mutations were introduced into AQP0 using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene, La Jolla, CA) with specific 5′-phosphorylated primers (E134G, 5′-CGTCAG-GAGATCCCCACTGTGGTGTC-3′; T138R, 5′-CGAACT-GAGCCCTCAGGAAGATCTCC-3′) and were confirmed by sequencing. Defolliculated stage V–VI X.laevis oocytes were isolated. Control oocytes were either not injected or injected with 50 nl of water. Test oocytes were injected with 50 nl of water containing 5 ng of cRNA prepared from the indicated human AQP0 construct (14).

**Oocyte water permeability**

After incubation for up to 3 days, the oocytes were analysed. The coefficient of osmotic water permeability (Pf) of oocytes was calculated from measured changes in volume after transfer from 200 to 70 mosM modified Barth’s solution at 22°C while monitoring the cell by videomicroscopy as reported (15). The
co-efficient of osmotic water permeability ($P_f$) was calculated from the changes in volume between 15 and 30 s, the initial volume ($V_o = 9 \times 10^{-4}$ cm$^3$); the initial oocyte surface area ($S = 0.045$ cm$^2$) and the molar volume of water ($V_w = 18$ cm$^3$/mol) using the equation: 

$$P_f = \frac{V_o \times \Delta V \times \Delta t}{S \times V_w \times (osm_{in} - osm_{out})}.$$ 

Immunoblot analyses

Whole cell membranes were prepared as described (15). Groups of 5–10 oocytes were lysed by vortexing in 0.5 ml of ice cold buffer (7.5 mM sodium phosphate, 1 mM NaEDTA, pH 7.4 with 20 mg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin). Celluar debris was removed by pelleting for 5 min at 750 g at 4°C and whole oocyte membranes were then pelleted for 30 min at 16 000 g at 4°C and solubilized in 10 μl of 1.25% (w/v) SDS at 37°C. SDS–PAGE electrophoresis was performed using 12% acrylamide (24), blot-transferred to nitrocellulose membranes and incubated with 1:10 000 dilution of polyclonal antiserum specific to the C-terminus of bovine AQP0 (14 of 15 residues identical to the human C-terminus) and visualized using ECL chemiluminescence (Amersham Life Sciences, Amersham, UK) (16).

Confocal immunofluorescence microscopy

Oocytes from three different experiments were analysed as described (9). Oocytes were fixed in 80 mM PIPES pH 6.8, 5 mM NaEDTA, 1 mM MgCl$_2$, 3.7% (v/v) formaldehyde, and 0.2% (v/v) Triton X-100 for 4 h at room temperature, followed by overnight post-fixation in absolute methanol at −20°C. The oocytes were rehydrated in phosphate-buffered saline (PBS) (8 mM sodium phosphate, 2 mM potassium phosphate pH 7.2, 128 mM NaCl, 2 mM KCl), incubated for 16 h at room temperature in PBS and bisected prior to processing for immunofluorescence. All antibody incubations and washes were at 4°C. The oocytes were incubated with polyclonal antiserum specific to the C-terminal of bovine AQPO (14 of 15 residues identical to the human C-terminus) and visualized using ECL chemiluminescence (Amersham Life Sciences, Amersham, UK) (16).

Clinical summaries

Ten patients carrying the T138R mutation were identified from four generations of a family living in southern England. The disease was completely penetrant, and each patient had bilateral cataracts that were present at birth and progressed throughout life. The lens opacities were typically pinhead shaped and were found in all regions of the lens except the centre of the nucleus (Fig. 2). Some patients had additional opacities at the anterior and posterior lens poles. Their loss of vision varied from moderate to severe and the patients underwent cataract removal between the ages of 6 weeks and 65 years.

Six patients with the E134G mutation were identified from three generations of a family living in south-eastern England. All patients had congenital cataracts that were stationary and specifically limited to a uni-lamellar region of the lens probably formed in the late fetal period (Fig. 2). This pattern of opacification was consistently noted in both eyes of each patient. Their loss of vision was moderate and the patients underwent cataract removal between 20 and 30 years of age. Except for cataracts, neither family suffered any other ocular or systemic abnormality.

ACKNOWLEDGEMENTS

The authors wish to thank W.B. Guggino for use of the core oocyte injection and videomicroscopy facility, Eric Beitz for assistance with topology software program and Shalesh Kaushal for helpful discussions. This work was supported by grants from the National Institutes of Health (EY11239, HL33991 and HL48268), the Human Frontier Science Program and the Wellcome Trust 053416/Z/98.

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