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Intact AQP0 performs cell-to-cell adhesion

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Abstract

Aquaporins (AQPs) constitute a major conduit for movement of water across plasma membranes. AQP0 is expressed in the fiber cells and is critical for lens transparency and homeostasis as mutations and knockout have resulted in dominant lens cataract. Several functions have been attributed for AQP0. *In vitro* and *ex vivo* experiments from several laboratories have confirmed the water permeability function of AQP0. However, this function seems paradoxical when the lens switches protein expression from AQP1 in the equatorial epithelial cells to 40 times less efficient AQP0 in the differentiating fiber cells. A possible explanation is AQP0 may perform unique function/s besides being a water pore. Indirect evidences including those from structural studies indicate a cell-to-cell adhesion role for AQP0. However, there is a lack of experimental evidence directly demonstrating the cell-to-cell adhesion capability of AQP0. We studied the adhesion property of human intact AQP0 by expressing it in adhesion-deficient mouse fibroblast L-cells using a newly devised method as well as a traditional assay. Our results reveal that AQP0 indeed can perform cell-to-cell adhesion. AQP1, two alternate splice variants of AQP4 (AQP4-M1 and AQP4-M23) and E-cadherin were also tested to validate the results. Cell-to-cell adhesion and cell aggregation properties of AQP0 expressing L-cells were less than those of the positive control L-cells expressing mouse E-cadherin and greater than those of AQP4-M23. AQP1 or AQP4-M1 expressing cells did not show cell-to-cell adhesion or cell aggregation. To our knowledge, this is the first report validating the possible structural role of intact AQP0 as a cell-to-cell adhesion protein, using an *in vitro* expression system.

Keywords

AQP0; AQP1; AQP4-M1; AQP4-M23; Cell-to-cell adhesion; E-cadherin; Mouse L-cells; Adhesion deficient cell; Water channel; Lens; Lens fiber cells; Cataract; Transparency; Eye; Human; Aquaporin; MIP26

Introduction

The superfamily of aquaporins (AQPs) consists of integral plasma membrane proteins that allow passive movement of water (aquaporins) or water and some small neutral solutes like glycerol and urea (aquaglyceroporins) across the plasma membrane. Aquaporins play an important role in cellular water homeostasis and alterations or lack of expression in mammals leads to pathophysiological conditions. Among the six AQPs expressed in the eye, two are in the lens. AQP1 is expressed in the lens epithelial cells and AQP0 in the fiber cells; the latter contributes over 50% of the total membrane protein. Mutation and knockout of a single copy or both copies of AQP0 have resulted in reduced lens size and bilateral lens cataract in mice

[1,2] highlighting the inevitability of this protein for normal lens growth and transparency. Functional studies on AQP0 mutant and knockout mouse models showed considerable reduction in lens fiber cell membrane water permeability [2–4]. AQP0 is synthesized as a 28 kDa protein; the C- and N-terminal ends get progressively cleaved off resulting in 24 and 22 kDa forms in the mature fibers of the inner cortical and nuclear regions [1]. Expression of AQP1 is down-regulated and replaced by AQP0 during fiber cell differentiation from equatorial epithelial cells.

Since the identification of AQP0 ~ 30 years ago, several functions have been proposed and water permeability of AQP0 in the fiber cell membranes has been tested and confirmed [1]. However, this function does not rationalize why lens switches protein expression from AQP1 in the equatorial epithelial cells to 40 times less efficient AQP0 in the differentiating fiber cells. We have shown previously that AQP0 and AQP1 are expressed temporally and spatially in the lens [5]. AQP0 began to express in the primary fiber cells at E11.25 and the onset of AQP1 expression in the anterior epithelial cells was at E17.5. The spatial and temporal nature of aquaporin expression in the lens and other indirect evidences [6–9] suggest that AQP0 may perform more than one function. We hypothesized that apart from being a water channel, AQP0 has unique function/s and tested it by developing a transgenic mouse model expressing human AQP1 in the fiber cells of AQP0 knockout mice, using α A-crystallin promoter. The transgenic AQP1 expression reduced the severity of lens cataract but did not restore complete lens transparency. Lack of AQP0 in the fiber cells caused disorganization of the fiber cells leading to the loss of the ordered compact arrangement [10] indicating a possible cell-to-cell adhesion role for AQP0.

A normal ocular lens cross section reveals the spectacular, compact architecture of the fiber cell arrangement. How do these cells accomplish this tight adherence to each other? Plasma membranes of differentiating and differentiated lens fiber cells develop very sophisticated contact with the adjacent cell membranes and form fiber junctions throughout the length of the cell. Fiber cells in the outer cortex have ball-and-socket interdigitations and those in the inner cortex have tongue-and-groove interdigitations which interlock to hold the cells in close proximity. Ultra-structural studies revealed two types of fiber membrane junctions (1) 11–13 nm thick, present very frequently towards the center of the lens and (2) 18–20 nm wide, abundant in the lens cortex fiber cells. These junctions also differ in protein composition and structure. Immunocytochemical and freeze-fracture studies have shown that AQP0 is abundant in the 11–13 nm junctions; the intercellular space is much narrower (0.5–0.7 nm) than that in gap junctions (3–5 nm) [11–13]. The remarkable narrowness of the space in the thin junction could be due to the interaction between the positively charged amino acids in the extracellular loops of AQP0 and the negatively charged lipid molecules of the adjacent cell membrane [6, 7,12,13]. Another school of thought is, the narrowness could be the result of the interactions between the amino acids of two opposing AQP0 molecules [8]. In the reconstituted unila-mellar liposomes, purified intact 28 kDa as well as cleaved 22 kDa form of AQP0 promoted cell-to-cell adhesion [6,7]. According to a cell-to-cell adhesion model based on electron crystallography [8] only the cleaved form of AQP0 can promote cell-to-cell adhesion. However, two other structural studies [9,14] suggest the possible involvement of intact AQP0 in cell-to-cell adhesion. Due to the natural discrepancies in the methods followed, investigators were unable to conclude with confidence whether both intact and cleaved or only one form of AQP0 promotes adhesion. So far, no direct experimental evidence exists to demonstrate whether AQP0 is capable providing cell-to-cell adhesion.

We investigated the possible unique role of intact human AQP0 as a cell-to-cell adhesion structural protein. L-cells devoid of endogenous adhesion molecules were used to transfect AQP0 and other constructs to test for the cell-to-cell adhesion capability. A newly developed

assay and a customary technique were followed for cell-to-cell adhesion studies. The experiments clearly demonstrated the cell-to-cell adhesion property of intact AQP0.

Materials and methods

Constructs, expression and localization

cDNAs of human intact AQP0, AQP1, AQP4-M1, AQP4-M23, and mouse E-cadherin were PCR amplified [15] and cloned individually into pcDNA 3.1 *myc*-His vector (Invitrogen, CA) carrying a CMV promoter as described by Varadaraj et al. [4,16].

Cell culture and transfection were performed as reported [16]. In brief, mouse fibroblast L-cells (CCL-1.3, ATCC, VA) were grown in Minimum Essential Medium (MEM) supplemented with 10% FBS. Cells were cultured at 37 °C and 5% CO₂ in a humidified incubator. Transfections were carried out using Lipofectamine™ LTX and Plus Reagent (Invitrogen, CA). Stable cell lines were selected with 1 mg/ml G418. To investigate whether the expressed protein localizes in the plasma membrane, colocalization studies were conducted using wheat germ agglutinin (WGA) and protein-specific antibodies [16]. Cells transfected separately with the desired construct were grown on sterile coverslips, fixed in 4% paraformaldehyde, washed in PBS and incubated with WGA (Molecular probes, CA) conjugated to Texas Red-X, in a humidified chamber. WGA binds to glycosylated proteins in the membrane. Cells were washed with PBS, fixed, washed again and immunostained with protein-specific polyclonal antibodies against human AQP0, AQP1 (Abcam Inc., MA), AQP4 (Sigma-Aldrich, MO) or mouse E-cadherin (Santa Cruz, CA). After washing, the cells were incubated with FITC-conjugated anti-rabbit or anti-goat IgG, as appropriate (Santa Cruz, CA) and processed as described [16].

Cell-to-cell adhesion assays

Cell aggregation assay using rotary gyrotory shaker: Following a published protocol [17] AQP1, AQP4-M1 (negative controls), AQP4-M23, E-cadherin (positive controls) and AQP0 were examined for cell aggregation property using mouse fibroblasts (L-cells) which lack endogenous adhesive elements. Single cell suspensions of stably transfected L-cells expressing empty vector, AQP1, AQP4-M1, AQP4-M23, AQP0 or E-cadherin were plated separately in 6-well plates precoated with 2% agarose (Sigma) or 2% bovine serum albumin (Sigma), and rotated on a gyrotory shaker (80 rpm) for 30, 60, 90 and 120 min, at 37 °C. At the end of each time point, equal volume of 2% glutaraldehyde was added to prevent further aggregation or dissociation. Particle number (aggregates + individual cells) was determined by counting representative aliquots at different intervals using a hemocytometer. The extent of aggregation at various intervals was calculated from the ratio of total particle numbers at time *t* of incubation (*N_t*) to the initial particle numbers (*N₀*) and expressed as percentage. The results were averaged for four experiments.

In another experiment, L-cells expressing empty vector (negative control) or intact AQP0 were each divided into two groups having fairly equal number of cells. One group of control and a group of AQP0 expressing cells were loaded with 5 µM/ml of CellTraker Red CMPTX; the other two matching groups were loaded with CellTraker Blue CMAC dye (Invitrogen, CA). CellTraker Red CMPTX loaded control cells were mixed with CellTraker Blue CMAC loaded control cells in 1:1 ratio. Similarly, AQP0 expressing dye-loaded cells were mixed and cell aggregation assay was performed as above for 1 h. At the end of 1 h, equal volume of 2% glutaraldehyde was added and aggregates were scanned and imaged under an epifluorescent microscope.

Novel adhesion assay using microplate reader: Fig. 1 is a schematic diagram showing the Novel Adhesion Assay protocol. L-cells stably expressing empty vector, AQP1, (negative

controls), E-cadherin, AQP4-M23 (positive controls) or AQP0 were grown to 80% confluency, washed with $1 \times$ PBS and incubated with $5 \mu\text{M/ml}$ CellTracker™ Red CMPTX (Invitrogen, CA) in Opti-MEM I without serum for 1 h. After washing with $1 \times$ PBS, cells were incubated for 30 min in MEM with 10% serum. The cells were trypsinized and suspended in Opti-MEM I without serum. A monolayer of L-cells stably expressing vector, AQP1, E-cadherin, AQP4-M23 or AQP0 was maintained separately in 24-well microplates and background fluorescence was measured from the bottom of the plates using a POLARstar OPTIMA microplate reader at excitation/emission wavelengths of 584/612 nm. A $200 \mu\text{l}$ aliquot of the respective CellTracker-Red-loaded cell suspension was added to the microplate well containing the monolayer of L-cells stably expressing the corresponding protein and incubated in Opti-MEM I without serum at 37°C for 1 h. At the end of the incubation period fluorescence was measured using a microplate reader as above. Cells were washed with PBS to remove non-adherent cells and fluorescence was read again. Background fluorescence was subtracted from the values obtained post-incubation and after washing. Percent of adhered cells was estimated as a ratio between the fluorescence units of CellTracker-Red loaded cells that remained adhered after washing to those of CellTracker-Red loaded cells post-incubation. A minimum of 10 wells were used to estimate background fluorescence, total cell load, or fluorescence due to adhered cells. The experiment was repeated five times and data were analyzed using Sigma Plot 2000 software.

To clearly demonstrate cell-to-cell adhesion, a bi-color fluorescence assay was developed. A monolayer of confluent L-cells expressing empty vector (negative control) and AQP0 separately was loaded with CellTracker Blue CMAC. Two comparable groups were loaded with CellTracker Red CMPTX and plated over the monolayer of matching cells (e.g., control to control) loaded with CellTracker Blue CMAC and incubated for 1 h at 37°C . Cells were washed with $1 \times$ PBS, fixed in 2% glutaraldehyde to prevent cell dissociation and adherent cells were imaged under an epifluorescent microscope.

Results and discussion

Until now, documentation of the cell-to-cell adhesion potential of AQP0 existed only as indirect evidences in publications [5–9,11–14,18,19]. To the best of our knowledge this is the first report revealing the cell-to-cell adhesion property of intact human AQP0 using an *in vitro* model. Parallel experiments were performed on AQP1, two alternate splice variants of AQP4 (AQP4-M1, AQP4-M23) as well as on E-cadherin which is a *bona fide* adhesion protein, to compare and verify the results. A method from literature, and a novel method devised were put into practice to ensure reproducibility and consistency of the findings.

All the constructs were first tested to ensure proper trafficking and membrane localization. Mouse L-fibroblast cells (L-cells) which do not express cell-to-cell adhesion molecules [17] endogenously were stably transfected with AQP1, AQP4-M1 (negative controls), E-cadherin, AQP4-M23 (positive controls), or intact human AQP0 and individual clones were selected. Cells expressing each protein were stained separately with a membrane-specific probe WGA conjugated to Texas Red-X to reveal membrane localization (Fig. 2a,d,g,j,m; red fluorescence).¹ Next, these cells were immunostained with protein-specific antibodies (anti-AQP1, anti-E-cadherin, anti-AQP4 or anti-AQP0) and exposed to secondary antibody conjugated to FITC for detecting membrane localization (Fig. 2b,e,h,k,n; green fluorescence). Merging of corresponding images of WGA and antibody binding assured membrane localization of the proteins (Fig. 2c,f,i,l,o; yellow fluorescence). The level of functional protein in the membrane was not quantified due to the usage of different protein-specific antibodies and the difference

¹For interpretation of the references to color in this figure text, the reader is referred to the web version of this paper.

in the affinity of binding. However, clones which showed very high level of expression of the test proteins were selected for further studies.

After confirming appropriate trafficking, cell-to-cell adhesive property was tested using cell aggregation measurements, following a published protocol using a rotary gyratory shaker [17] as described in Materials and methods section. As opposed to L-cells transfected with empty vector (control), AQP1 or AQP4-M1, cells expressing E-cadherin, AQP4-M23 and AQP0 displayed considerable cell aggregation (Fig. 3A); aggregation of cells as a function of time (Fig. 3B) revealed that with passage of time more and more E-cadherin, AQP4-M23 or AQP0 transfected cells formed cell clusters consequently reducing the number of non-aggregating individual L-cells. Extent of aggregation exhibited by AQP0 was more than that of AQP4-M23 and less than that of E-cadherin. Cells transfected with AQP1, AQP4-M1 or empty vector did not show considerable aggregation with passage of time, thus served as negative controls. Lack of cell cluster formation by AQP1 had also been reported by Hiroaki et al. [20]. AQP4-M23 [20] and mouse E-cadherin [21] served as positive controls; however, the latter was used as standard to calculate relative aggregation by other proteins. In order to clearly show cell aggregation, Cell-tracker Blue or Red loaded AQP0 transfected cells were mixed together and tested on a gyratory shaker. Similarly fluorophore-labeled vector-transfected cells served as control (Fig. 3C, a,b). AQP0 transfected cells exhibited effective cell aggregation (Fig. 3C, c,d).

A novel method was also developed (Fig. 1) to test the cell-to-cell adhesion capability of AQP0. The new method can be employed for small and large scale screenings of samples. Adhesion-deficient L-cells transfected separately with empty vector, E-cadherin, AQP1, AQP4-M23 or AQP0 and grown to 80% confluency were loaded with membrane permeable CellTracker™ Red CMPTX dye, dissociated by trypsinization, neutralized, washed and added to a monolayer of L-cells in 24-well microplates stably expressing the respective protein but were not labeled with Cell-Tracker™ Red CMPTX. Intensity of fluorescence was measured after 1 h of incubation as well as after washing using a microplate reader. Relative adhesion induced by each protein was estimated using E-cadherin as a positive control. Cell-to-cell adhesion induced by AQP0 and AQP4-M23 expressing cells were statistically significant (**P* value: <0.001; Fig. 4A). As in the cell aggregation measurements, results of the newly developed method followed the same trend (compare data from Figs. 3B and 4A). In both methods, E-cadherin, AQP0 and AQP4-M23 revealed significant cell-to-cell adhesion whereas AQP1 did not (Figs. 3B and 4A). Cell-to-cell adhesion exhibited by AQP0 was less than that of E-cadherin and more than that of AQP4-M23 (Fig. 3B). A previous study reported weak adhesion capability for AQP4-M23 compared to an adhesion protein nectin-3 [20]. AQP4-M23, however, is a highly efficient water channel whereas the water permeability of AQP0 is much less than that of AQP4-M23 and AQP1. Fig. 4B provides visible evidence for the cell-to-cell adhesion property of AQP0. To a monolayer of vector or AQP0 transfected cells loaded with CellTracker™ Blue corresponding cells loaded with CellTracker™ Red CMPTX were added. After an hour of incubation fluorescent microscopic analysis showed that in contrast to empty vector expressing L-cells (Fig. 4B, a) AQP0 expressing cells clearly demonstrated cell-to-cell adhesion (Fig. 4B, b).

In spite of being the most abundant lens membrane protein, and the first cloned member [22] among vertebrate AQPs, the role of AQP0 remained vague for a considerable length of time. Cell-cell coupling, ionic conductance, water permeability and cell-to-cell adhesion were speculated as possible functions of AQP0 [1]. While the first two functions have been ruled out [23,24], water permeability has been authentically proven [2,3,24]; cell-to-cell adhesion suffered lack of direct experimental proof. We developed a transgenic mouse model expressing AQP1 in the fiber cells of AQP0 knockout mouse lens [10] to test whether AQP0 performs unique functions. Even though water permeability was more than compensated and cataract

reduced, complete lens transparency was not restored. Ultrastructural analysis showed that fiber cells could not regain their compact architecture indicating that presence of AQP0 is essential for cell-to-cell adhesion and AQP1 cannot replace AQP0 completely.

Adhesion-promoting function of intact (28 kDa) and cleaved (22 kDa) forms of AQP0 extracted from bovine [6] and human [7] lens was characterized using artificial neutral phosphatidylcholine (PC) lipid vesicles. Interaction of the lipid vesicles carrying intact or cleaved forms of AQP0 with negatively charged phosphatidylserine (PS) vesicles and neutral PC vesicles was investigated by resonance energy transfer [6,7]. Both forms of AQP0 promoted vesicle adhesion with the negatively charged PS lipid vesicles and not with neutral PC vesicles indicating the involvement of charge interaction. Structural studies by Gonen et al. [8] suggested that only the cleaved form participates in the adhesion function and the channel pore is closed; intact AQP0 does not participate in adhesion function and the channel is in open configuration. According to the structural analyses by Harries et al. [9] and Palanivelu et al. [14], intact AQP0 could function as an adhesion protein and the positive charges in the extracellular domain might play a significant role in cell-to-cell adhesion. Our investigation supports the hypothesis of Michea et al. [6,7], Harries et al. [9], and Palanivelu et al. [14] with experimental proof that intact AQP0 can function as an adhesion protein.

The temporal nature of AQP expression, AQP0 at an earlier stage E11.25 than AQP1 (E17.5), in the lens [5] indicates that AQP0 may have a special role; AQP0 is expressed as the primary fiber cells began to develop in the embryonic lens. Our data indicate that intact AQP0 is a highly efficient cell-to-cell adhesion structural protein. AQP0 which shares the function of water permeability in the lens, however, is a less efficient water channel [3,5,25]. AQP1 functions as a highly efficient water channel and our results show that it lacks the cell-to-cell adhesion property. Presence of AQP0 is necessary to establish the orderly arrangement of the fiber cells and this could possibly be the reason for the switching of protein expression from AQP1 in the equatorial epithelial cells to AQP0 during secondary fiber cell differentiation in the normal lens.

Natural mutations in human AQP1 and AQP2 which do not have structural roles resulted in recessive phenotypes [26,27] whereas most of the mutations in structural proteins caused dominant inherited traits [28]. All of the thus far documented mutations in AQP0, six in human and three in mice, have led to dominant lens cataract which further strengthens the putative structural role for AQP0 in the lens.

In conclusion, the present investigation demonstrates *in vitro* that intact human AQP0 which expresses predominantly in lens fiber cells is capable of providing cell-to-cell adhesion in L-cells which lack cell adhesion molecules. It is reasonable to assume that AQP0 could be performing an equivalent role *in vivo* in the lens, given the ordered and compact cellular arrangement of the fiber cells in the normal lens, disorganization of the cells in the AQP0 knockout lens and the failure in regaining compactness of the cells in AQP0 knockout lens even with the abundant transgenic expression of AQP1. It becomes apparent that the cell-to-cell adhesion property of AQP0 could play a major role in accomplishing the highly ordered and compact fiber cell architecture that is necessary to minimize extracellular space and light scattering at the cell boundaries in order to aid in the focusing of objects on the retina. Our current focus is to find out whether N- and C-terminal cleaved forms perform cell-to-cell adhesion and to create animal models to investigate the structural role of AQP0 *in vivo*.

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Abbreviations

AQP0	aquaporin 0
AQP1	aquaporin 1
AQP4	aquaporin 4

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Fig. 1.
Schematic diagram of the novel cell-to-cell adhesion assay using CellTracker Red CMPTX and microplate reader.

Fig. 2.

Expression and trafficking of intact human AQP0 in L-cells. Epifluorescent images of L-cells transfected with AQP1 (a–c), AQP4-M1 (d–f), E-cadherin (g–i), AQP4-M23 (j–l) and AQP0 (m–o). Cells were stained first with membrane specific wheat germ agglutinin (WGA) conjugated to Texas Red-X and then immunostained with protein-specific antibody. a,d,g,j,m – Cells viewed under Texas Red fluorescent filter for plasma membrane staining by WGA; b,e,h,k,n – the same cells viewed under FITC fluorescent filter for protein-specific antibody binding; c,f,i,l,o – overlaid images.

Fig. 3.

Cell aggregation assay using rotary gyratory shaker. (A) Cell aggregation exhibited by L-cells expressing empty vector, AQP1, AQP4-M1, E-cadherin, AQP4-M23 or AQP0. (B) Graph showing cell aggregation induced by intact human AQP0 and other proteins as marked, in relation to incubation time. (C) Bi-color fluorescence aggregation assay (see Methods section) using L-cells expressing empty vector (a,b) or AQP0 (c,d) labeled with CellTraker Blue and CellTraker Red. Micrographs: a,c – phase-contrast; b,d – fluorescence.

Fig. 4.

Novel cell-to-cell adhesion assay using microplate reader. (A) Histogram showing cell-to-cell adhesion induced by different proteins using the novel assay. (B) Bi-color fluorescence adhesion novel assay. Over a monolayer of CellTracker Blue CMAC loaded L-cells (a) empty vector (negative control) and (b) AQP0, corresponding cells loaded with CellTracker Red were plated. a,b – Representative fluorescence micrographs of adhered cells after washing.