THE KNOCKDOWN OF HEPATOCYTE AQUAPORIN-8 BY RNA INTERFERENCE INDUCES DEFECTIVE BILE CANALICULAR WATER TRANSPORT*

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ABSTRACT

Aquaporin-8 (AQP8) water channels, which are expressed in rat hepatocyte bile canalicular membranes, are involved in water transport during bile formation. Nevertheless, there is no conclusive evidence that AQP8 mediates water secretion into the bile canaliculus. In this study, we directly evaluated whether AQP8 gene silencing by RNA interference inhibits canalicular water secretion in the human hepatocyte-derived cell line, HepG2. By RT-PCR and immunoblotting we found that HepG2 cells express AQP8, and by confocal immunofluorescence microscopy that it is localized intracellularly and on the canalicular membrane, as described in rat hepatocytes. We also verified the expression of AQP8 in normal human liver. Forty-eight hours after transfection of HepG2 cells with RNA duplexes targeting two different regions of human AQP8 molecule, the levels of AQP8 protein specifically decreased by 60-70%. We found that AQP8-knockdown cells showed a significant decline in the canalicular volume of about 70% (p < 0.01) suggesting an impairment in the basal (nonstimulated) canalicular water movement. We also found that the decreased AQP8 expression inhibited the canalicular water transport in response either to an inward osmotic gradient (-65%, p < 0.05) or to the bile secretory agonist dibutyryl cAMP (-80%, p < 0.05). Our data suggest that AQP8 plays a major role in water transport across canalicular membrane of HepG2 cells, and support the notion that defective expression of AQP8 causes bile secretory dysfunction in human hepatocytes.

Key words: HepG2, human liver, bile secretion, dibutyryl cAMP
INTRODUCTION

Bile is composed of 98% water, which is mainly secreted by hepatocytes at bile canaliculi. Water transport at this level is driven by transient osmotic gradients generated across the hepatocyte membrane by active solute transport (1). Aquaporins (AQPs) are a family of integral membrane proteins that facilitate the osmotically-induced water transport through cell membranes (18). At least thirteen mammalian aquaporins have been identified in diverse human and animal cells. Rat hepatocytes express four AQPs, i.e., AQP8 (9, 13, 16), AQP9 (10, 16), AQP11 (15), and AQP0 (16). AQP8 is localized, as a glycosylated 34 kDa protein, in intracellular vesicles and at the canalicular plasma membrane (9, 13, 16). There is experimental evidence suggesting that AQP8 facilitates the canalicular water transport during hepatocyte bile formation (16) and that the defective expression of hepatocyte AQP8 may contribute to bile secretory dysfunction in cholestasis (4, 5, 20). Nevertheless, conclusive evidence for AQP8 involvement in bile canalicular water transport should come from studies performed in hepatocytes lacking AQP8 expression. Recently, it has been described that hepatocytes from AQP8 null mice show similar membrane water permeability to those from wild-type mice (31). Although this observation suggests that AQP8 does not play a major role in mouse canalicular water transport, it should be taken with caution because knockout animals often develop functional compensatory adaptations. In the present study, we directly evaluated whether AQP8 gene suppression by RNA interference is able to inhibit canalicular water secretion in hepatocytes, using HepG2 cells, a well differentiated polarized cell line derived from human hepatocytes (7, 33).

MATERIALS AND METHODS
Synthesis of Short Interfering RNA (siRNA) – The 21 nucleotides RNA duplexes were synthesized using the Silencer siRNA kit (Ambion, Austin, TX), and, as templates, oligonucleotides synthesized by Invitrogen. Four target sequences were chosen following the guidelines described by Elbashir et al. (8). Two of the RNA duplexes induced a significant decrease in AQP8 expression, as analyzed by immunoblotting: siRNA2 was specifically targeted to nucleotides 177-197 (AACGGTTTGTGCAGCCATGTC), and siRNA4 to nucleotides 117–137 (AATTTGGCAATGACAAGGCCA) of human AQP8. The control siRNA (siRNA C) was designed scrambling the nucleotides of one of these specific targets (AATGTGTCCGTGAGCACGTCT).

Cell Culture and Treatments – HepG2 cells (kindly donated by Dr. Pablo Schwarzbaum, Universidad Nacional de Buenos Aires/CONICET, Argentina) were grown on plastic dishes in DMEM with 4.5 g of glucose per liter, supplemented with 10% fetal calf serum and antibiotics. Media was changed every other day. Cells were trypsinized after reaching confluency. For immunofluorescence microscopy analysis, the cells were grown on glass coverslips and, at the end of the experiments, washed three times with phosphate buffered saline (PBS), and fixed in 4% paraformaldehyde. For AQP8 expression analysis by immunoblotting, the cells where washed twice with cold PBS, scraped and pelleted at 200 x g, for 3 min at 4 ºC.

Reduction of AQP8 Expression by siRNA: 5 x 10^5 HepG2 cells were seeded in 35-mm plastic Petri dishes. After 18 h of culture, the cells were transfected with AQP8 siRNAs using the Dharmafect I transfection reagent (Dharmacon, Lafayette, CO), following the manufacturer instructions. The cells were checked for AQP8 protein expression 24 and 48 h afterwards. As mentioned above, there was a significant decrease in AQP8 expression with two of the RNA duplexes (siRNA2 and siRNA4) at
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48 h, but not at 24 h after transfection. AQP8 knockdown efficiency was assessed in each experiment by immunoblotting. Cells transfected with the siRNA2 for 48 h consistently showed the lowest AQP8 levels, so we chose to use them for the functional experiments. In these cells, the percentage of decrease of AQP8 related to β-actin levels, in three different experiments, was 64% ± 10% (p < 0.05). All the functional studies where performed 3 days after plating the cells, time of maximal number of canalicular structures in HepG2 cells (7).

Canalicular Osmotic Water Transport Studies: HepG2 cells transfected with siRNA C or siRNA2 for 48 h were exposed to a hypotonic Hepes-buffered medium (100 mosM, pH 7.4) to stimulate water influx to the canalicular space. A first set of experiments was performed at 37 °C, and the canalicular changes were recorded using time lapse microscopy with phase contrast optics. Alternatively, cells were incubated at 20 °C, and canalicular changes were assessed by analysis of the intensity of fluorescence in canaliculi loaded with the impermeant fluorophore glutathion-methylfluorescein (GS-MF).

cAMP-induced Canalicular Secretion Studies: siRNA C or siRNA2 transfected cells were cultured in the presence of 200 µM dibutyryl cAMP (DBcAMP) for 5 min at 37 °C to stimulate secretion into the canalicular space. Canalicular morphology was followed by time lapse microscopy with phase contrast optics.

Morphometric Analysis of the Canaliculus – Phase contrast images were obtained using an inverted microscope Zeiss Axiovert 25CFL, and captured with a Nikon camera at 40X. Images were processed using Adobe Photoshop to optimize the visualization of the canaliculi. For the functional studies, images were captured before changing osmolarity of the medium or adding DBcAMP, and every 30 s or 60 s after initiation of the choleretic challenge. All images were taken using the same settings.
Aquaporin-8 mediates canalicular water transport for each experiment. Canalicular diameters were determined using a measure tool of the Image Pro program. The relative canalicular volume (RCV) was calculated as the ratio between each canalicular volume after the generation of the osmotic gradient or the addition of DBcAMP related to its volume at time 0. The number of canaliculi every 100 cells was determined using the same program.

**Fluorometric analysis of water transport to the canaliculi** – Hepatoma cells were plated on 25 mm-diameter glass coverslips (Fischer Scientific, Pittsburg, PA) that had been previously coated with 0.1% w/v poly-L-lysine. Each coverslip with attached cells was mounted in a chamber filled with isotonic medium and placed on the stage of a Nikon TE-200 epifluorescence inverted microscope. Cells were then loaded with 5-Chloromethylfluorescein diacetate (CMFDA) (kindly donated by Dr. Marcelo G. Roma, Universidad Nacional de Rosario/CONICET, Argentina) (2.5 µmol/L) for 3 min. CMFDA is taken up by hepatocytes and, after being metabolized to GS-MF, is actively secreted by the organic anion transporter Mrp2 to the canaliculus, where it concentrates (29). Control and AQP8 knockdown cells showed similar efficiency for accumulating GS-MF. The loading solution was then washed out with isotonic medium. Changes in canalicular volume were inferred from readings of the fluorescence intensity recorded by exciting GS-MF through a 470 CWL excitation filter (Nikon Inc., Melville) and were imaged with a 500 nm LP dichroic mirror (Nikon Inc.) and a 515 LP barrier filter (Nikon Inc.). Temporal changes in fluorescence intensity recorded from a small region of dye-loaded canaliculi reflect changes in intracanalicular fluorophore concentration, and therefore alterations of canalicular water volume (11). Fluorescence images were acquired by use of a charge-coupled device camera (Hamamatsu C4742-95) connected to a computer and the Metafluor acquisition program (Universal Imaging). RCV changes during these
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experiments were calculated as the inverse of the ratio between the fluorescence 60 s after initiated the exposure to the hypotonic media, and the fluorescence at time 0. It is worth mentioning that although this methodology works as a useful tool for determining the canalicular water transport rate driven by an osmotic gradient, it is not suitable for determining cAMP-induced canalicular water flow. This is because cAMP stimulates the canalicular membrane insertion of Mrp2 and therefore the amount of GS-MF transported (27).

Human liver tissue – Samples of normal human liver tissue were taken from adult patients undergoing partial hepatectomy. All patients had previously given informed consent for the study, and the experimental protocol was approved by the Bioethics Committee of the Universidad Nacional de Rosario in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction – Total RNA was extracted from HepG2 cells using the TRIZol reagent (Invitrogen) following the manufacturer’s instructions. 5 µg of total RNA was reverse transcribed with random priming, using an avian myeloblastosis virus reverse transcriptase system (Promega, Madison, WI). The cDNA was amplified using PCR with primers (forward GCCTGTCGGTCTTGAGAAT and reverse CCAGATGCATTCCAGAACCT) designed to amplify a specific sequence of human AQP8 (nucleotides 241-507), synthesized by Invitrogen. cDNAs prepared from HeLa cells (kindly donated by Dr. Fabiana Garcia, Universidad Nacional de Rosario/CONICET) where used as negative controls. The PCR products were electrophoresed in 1% agarose gels, and the bands were visualized by ethidium bromide staining.

Preparation of total cellular membranes – Cells were sonicated or livers homogenized in 0.3 M sucrose containing 0.1 mM phenyl-methylsulfonyl fluoride and 0.1 mM
leupeptin (Sigma Chemical Co., St. Louis, MO) and were subjected to low-speed centrifugation to obtain post-nuclear supernatants. These supernatants were centrifuged at 200,000 x g for 60 min, yielding the total HepG2 (liver or HeLa) membrane fractions (13). Membrane proteins were determined according to Lowry et al. (22).

**Immunoblotting** – Solubilized membranes were heated 10 min at 65 °C in sample buffer (20 mM TRIS, pH 8.5, 1% SDS, 400 μM DTT, 10 % glycerol), subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (Perkin Elmer Life Sciences). After blocking and washing, blots were incubated overnight at 4°C with rabbit affinity-purified antibodies against rat AQP8 (1 μg/ml; Alpha Diagnostics International), goat antibodies against human AQP8 (1 μg/ml; Santa Cruz Biotechnology) or rabbit antibodies against human AQP0 (2 μg/ml; Alpha Diagnostics International), or incubated for 1 h at room temperature with a monoclonal antibody against β-actin (1 μg/ml, Sigma). The blots were then washed and incubated with the horseradish peroxidase-conjugated corresponding secondary antibodies, and bands were detected by enhanced chemiluminescence detection system (ECL) (Amersham Pharmacia Biotech). Auto-radiographs were obtained by exposing polyvinyl difluoride membranes to Kodak XAR film. The bands were quantitated by densitometry using the NIH Image J program. Adjustments of brightness and contrast were applied to the entire images for the sake of clarity.

**Immunofluorescence and Confocal Microscopy** – Fixed cells were permeabilized and blocked with 0.2% Triton X-100/Bovine Serum Albumin 3% for 10 min, and incubated overnight at 4 °C with rabbit affinity-purified AQP8 antibodies (10 μg/ml). After washing, coverslips were incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody and Alexa 560-conjugated Phalloidin (Molecular Probes) for 1 h.
Coverslips were washed, incubated with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) (50 µM) for 10 min, and mounted with ProLong (Molecular Probes). Fluorescence localization was detected by confocal microscopy (Nikon C1SiR with inverted microscope Nikon TE200). Images were collected with the same confocal settings in each set of experiments. With these settings no autofluorescence was detected. Controls omitting primary or secondary antibodies revealed no labeling. For the sake of clarity, contrast and brightness adjustments were equally applied to the entire images using Adobe Photoshop software.

Statistical Analysis—Data are expressed as mean ± S.E. Significance was determined using Student’s t-tests; p < 0.05 was considered statistically significant.

RESULTS

AQP8 expression in HepG2 cells. Although AQP8 expression in mammals has been described in different tissues from rat and mouse (9, 12, 13, 16), its protein expression in cells derived from human tissues has not been characterized so far. Thus, we first investigated if AQP8 was expressed in HepG2 cells. We designed primers targeting human AQP8 sequence (see Materials and Methods) and performed PCR studies using cDNAs from human derived cell lines. Figure 1A shows that a band with the expected size for human AQP8 sequence was amplified from HepG2 cDNA, but not from HeLa cDNA. We and others have previously validated the anti-AQP8 antibody from Alpha Diagnostics International to assess rat hepatic AQP8 protein expression (9, 13). We compared the peptide towards which this antibody was raised in rat, with its homologue in the human sequence, and found that there was an 81 % of aminoacid-identity (Figure 1B, top). Thus, we used this antibody to assess protein expression in membrane fractions from HepG2 and HeLa cells. Figure 1B shows that this antibody
recognized two main specific bands of about 36 kDa and 56 kDa, which were consistently present in total cellular membranes from HepG2 but not from HeLa cells. The same results were obtained by using an affinity-purified antibody raised against human AQP8 (Santa Cruz Biotechnology, Santa Cruz, CA) (not shown). Furthermore, the specificity of the AQP8 immunoblot was verified by the decrease of the intensity of both immunodetected bands in samples from AQP8 knock-down cells (Fig. 2A). The predicted MW for each AQP8 monomer based on human AQP8 aminoacidic sequence is 27.4 kDa. The 36 kDa apparent molecular weight of the low band may be due to protein glycosylation. In fact, human AQP8 contains three potential sites for N-glycosylation and the N-glycosylated rat hepatocyte AQP8, which predicted MW is 27.9 kDa, has a similar apparent molecular weight in poliacrylamide/SDS gels (13). The presence of the higher band of 56 kDa may also be due to protein glycosylation, although incomplete depolymerization of the AQP8 tetramer, as suggested for mouse colon AQP8 (31), can not be ruled out. Since the patterns of protein expression may differ between cell lines and normal tissue, we analyzed AQP8 expression in samples derived from normal human liver. Fig 1C shows the presence of identical anti-AQP8 immunoreactive bands in total cellular membranes from HepG2 and human liver samples. Overall, these data indicate that AQP8 is expressed in normal human liver and that its expression is retained in HepG2 cells. To characterize AQP8 subcellular localization, we performed confocal immunofluorescence microscopy. Phalloidin was used to identify canalicular structures (34) and DAPI for nuclear staining. Figure 1D shows that AQP8 is localized to vesicular structures throughout the cytoplasm as well as to the canalicular membranes in HepG2 cells. The same distribution has been previously described for AQP8 in rat hepatocytes (13).
Decrease of AQP8 expression by RNA interference. In order to induce a decrease in AQP8 expression, we designed and synthesized siRNAs containing sequences specific for human AQP8 (see Materials and Methods). HepG2 cells were transfected with the siRNAs, and AQP8 expression checked 24 and 48 h afterwards. Figure 2A shows that 24 h after transfection AQP8 expression was unaffected. Nevertheless, 48 h after transfection, the siRNAs 2 and 4 induced an important decrease in AQP8 expression. The densitometric analysis relating AQP8 to β-actin levels indicated a decrease in AQP8 expression at 48 h of 79 % and 77 % in siRNA2 and 4 treated cells, respectively (Fig 2A). The analysis of AQP8 expression by immunofluorescence confocal microscopy confirmed these results, as shown in Figure 2B. Specifically, the canalicular staining in AQP8 knockdown cells was significantly decreased by the treatment with both AQP8 specific siRNAs.

Effect of AQP8 knockdown on canalicular structures. The morphometric analysis of three separate experiments indicated that there is not a cell phenotype associated with transfection of siRNA2 or siRNA4 at 24 h; nevertheless, at 48 h, when AQP8 silencing is evident, there is a clear decrease in the number and size of canalicular structures (Fig. 3). The quantitative assessment of the number of canaliculi by phase contrast microscopy (Fig. 3B) and by the fluorometric analysis of GS-CF accumulation (data not shown) confirmed that AQP8 knockdown cells showed a lower number of canaliculi, what could be due to the generation of very small-not detectable canaliculi. Control cells demonstrated an increase in canalicular volume between 24 and 48 h which is expected to be caused by continuous fluid secretion, although a contribution of canalicular membrane biogenesis cannot be disregarded (7, 34). AQP8 knockdown prevented this canalicular volume increase which suggests a defective
Aquaporin-8 mediates canalicular water transport. Together these data suggest that AQP8 plays an important role in basal (nonstimulated) canalicular water movement.

Effect of AQP8 knockdown on Canicular Osmotic Water Transport. We evaluated the role of AQP8 on osmotic water secretion into the canaliculus in live HepG2 cells by exposing control or AQP8 knockdown cells to a 200 mosM inwardly directed osmotic gradient. We assessed the osmotically induced canalicular water influx by using two different methodological approaches: phase contrast microscopy and image analysis (Fig. 4A and B) and fluorometric assessment of fluorescent probe-loaded canaliculi (Fig. 4C and D). The time course of canalicular volume (Fig. 4A) and relative fluorescence intensity (Fig. 4C) were markedly reduced in AQP8 knockdown cells. In accordance, the corresponding relative canalicular volumes (Fig. 4B and D) were significantly reduced in AQP8 knockdown cells by 60-70% (p<0.05). Together these results suggest that AQP8 mediates the osmotic transport of water towards the bile canaliculus.

Effect of AQP8 knockdown on the DBcAMP-induced Canicular Secretion. To test the relevance of AQP8 on water transport to the canaliculus under more physiological conditions, we made use of DBcAMP. DBcAMP is a choleretic agent, i.e. stimulates bile formation via the secretion of osmotically active solutes (16). The time course of canalicular volume after the exposure of live HepG2 cells to 200 μM DBcAMP is shown in Figure 5A. DBcAMP induced an increase in the canalicular volume of control cells but not in AQP8 knockdown cells. As shown in Fig. 5B, the reduced AQP8 expression significantly inhibited the DBcAMP-induced increase of the relative canalicular volume by about 80% (p<0.05). These data suggest that AQP8 facilitates the cAMP-induced bile canalicular water secretion.
DISCUSSION

Our major aim was to study directly the role of the water channel AQP8 in the molecular mechanisms of hepatocyte canalicular water transport utilizing RNA interference. Using a polarized human hepatocyte cell line, HepG2, we found for the first time that: (i) human hepatocyte AQP8 protein is expressed and localized to canalicular plasma membranes and cytoplasmic vesicular structures and (ii) the knockdown of AQP8 expression caused a decrease in the basal canalicular volume as well as an impairment in the osmotically and agonist-induced canalicular water transport. Our data suggest that AQP8 plays a major role in water transport during hepatocyte bile formation and also support the notion that defective AQP8 expression causes bile secretory dysfunction.

This is the first study to our knowledge that reports the expression of AQP8 protein in human hepatocyte-cells. By using an antibody which efficacy for detecting AQP8 in rat hepatocytes was previously validated (9, 13), we found that AQP8 is present on HepG2 canalicular membranes (i.e., the bile secretory pole of hepatocytes) and cytoplasmic vesicular structures. This is the same subcellular localization found for AQP8 in rat and mouse hepatocytes (12, 13, 16). Furthermore, we verified that the 36 and 56 kDa AQP8 bands in HepG2 cells are also present in normal human liver. The fact that HepG2 cells treated with siRNAs specifically designed for diminishing AQP8 expression distinctly decreased the immunolabeling using the same antibody, confirms the specificity of these results.

Bile secretion by hepatocytes is an osmotic secretory process resulting from the inflow of water into the biliary space in response to osmotic gradients created by active solute excretion (1). Osmotic water transport across hepatocyte sinusoidal (basolateral) and canalicular plasma membranes is thought to occur, at least in part,
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Aquaporin-8 mediates canalicular water transport through AQPs (23). Thus, hepatocyte sinusoidal AQP9 would contribute to water uptake, while AQP8 to the canalicul, rate-limiting water flow (16, 23). Water transport studies in isolated rat hepatocytes (13, 16) and canalicular plasma membrane vesicles (23) suggest the involvement of AQP8 in canalicular water movement by hepatocytes. Nevertheless, the lack of specific AQP8 inhibitors has precluded conclusive experimental evidence on this issue.

AQPs gene silencing in hepatocytes has previously been accomplished by developing transgenic AQP-null mice (28, 31). The phenotype analysis of transgenic mice deficient in AQP8 has been useful in providing insight into its role in water transport in several cells. Surprisingly, the AQP8-knockout does not seem to affect membrane water permeability of isolated mice hepatocytes (31). This suggests, in disagreement with the above mentioned findings in rat hepatocytes (13, 16, 23), that AQP8 does not play an important role in mouse canalicular water transport. Nevertheless, direct water transport studies in canalicular membrane vesicles or canalicular bile secretion studies were not performed. On the other hand, deletion of a single AQP might not significantly affect cell water transport because other known or still unidentified AQPs could undergo compensatory up-regulation. The advantages of siRNA technology for specific and effective inhibition of endogenous genes expression are becoming apparent (24), and it has been used for assessing the role of aquaporins in water transport in cholangiocytes (6, 30), colonocytes (19), astrocytes (26), and vascular endothelial cells (17). Using this technology, we provide direct experimental evidence supporting the notion that AQP8 mediates the water transport necessary for canalicular bile secretion, i.e., AQP8 knockdown caused a decrease in basal canalicular volume (Fig. 3) as well as a marked impairment in the osmotically (Fig. 4) and the bile secretory agonist-induced (Fig. 5) canalicular fluid flow. This
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strongly suggests that AQP8 is required for the efficient coupling of osmotically active solutes and water transport during canalicular bile formation in human hepatocytes.

Our findings may also have pathophysiological implications for human cholestatic disorders. Cholestasis is a clinical condition defined as an impairment of normal bile formation or flow with the risk to produce severe liver injury and systemic disease. The contribution of alterations in membrane solute transporters expression to this condition has been widely documented (1). Nevertheless, this is the first study that provides direct experimental evidence supporting that defective AQP8 membrane expression can impair normal bile physiology, and thus would contribute to the development of cholestasis. Therefore, cholestasis may result from a mutual occurrence of an impaired solute transport and an AQP8-mediated decrease in canalicular water permeability. Our previous in vivo studies are in line with this notion. Thus, different established models of cholestasis, such as extrahepatic obstructive cholestasis (4), estrogen-induced cholestasis (5), and sepsis-associated cholestasis (20, 21) have shown a severely impaired functional expression of hepatocyte canalicular AQP8.

AQP0, the major intrinsic protein of lens, is another member of the AQP family of channel proteins with the particularity of having low water permeability (25, 32). AQP0 has been found to be expressed in rat hepatocytes (16) and in the rat hepatoma/human fibroblast hybrid cell line WIF-B (14). In these cells, AQP0 is located mainly in intracellular compartments, although in rat hepatocytes it also shows canalicular expression. Therefore, we decided to assess whether AQP0 is also present on the canalicular membranes of HepG2 cells. Nevertheless, we found by immunoblotting analysis that HepG2 cells do not express any significant amount of AQP0 protein (Supplementary Figure 1). In accordance, AQP0 was not detected in
normal human liver samples either (Supplementary Figure 1). Our data suggest that AQP0 plays no role in human canalicular bile formation.

Conceptually, water can be transported into the biliary space by either of two pathways: a paracellular pathway between the tight junctions of adjacent cells or a transcellular pathway across the cells. While the quantitative contribution of these two pathways of water transport is still unclear, previous experimental evidence (2, 16, 23), together with the fact that AQP8 knockdown markedly reduced canalicular water transport, clearly implicates the significance of the transcellular pathway.

In summary, we designed and utilized siRNAs that specifically and effectively diminished the endogenous expression of AQP8 in the human hepatocyte cell line HepG2. As a result, osmotically-driven and bile agonist-induced canalicular water secretion were significantly reduced, suggesting that AQP8 plays a major role in water transport into the canalicular space during bile formation. These data also support the notion that defective AQP8 expression might lead to bile secretory dysfunction.
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REFERENCES


   Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured

   S. Immunolocalization of aquaporin-8 in rat kidney, gastrointestinal tract, testis,

10. Elkjaer M, Vajda, Z, Nejsum, LN, Kwon, TH, Jensen, UB, Amiry-
    Moghaddam, M, Frokiaer, J, Nielsen S. Immunolocalization of AQP9 in liver,
    epididymis, testis, spleen, and brain. Biochem Biophys Res Commun 276: 1118-

    Volumetric and ionic responses of goldfish hepatocytes to anisotonic exposure and

    Ontogeny, distribution, and possible functional implications of an unusual

    Marinelli RA. The water channel aquaporin-8 is mainly intracellular in rat
    hepatocytes, and its plasma membrane insertion is stimulated by cyclic AMP. J

14. Gradilone SA, Tietz PS, Splinter PL, Marinelli RA, LaRusso N. Expression
    and subcellular localization of aquaporin water channels in the polarized
    hepatocyte cell line, WIF-B. BMC Physiol 5:13, 2005.


FIGURE LEGENDS

Figure 1. AQP8 expression in HepG2 cells. HepG2 cells were grown for 3 days, and then processed to analyze AQP8 mRNA and protein expression, and subcellular localization as described in Materials and Methods. A, cDNAs were synthesized using total RNA prepared from HepG2 or HeLa cells. We performed PCR reactions using human AQP8 specific primers. DNA with the expected size for AQP8 sequence was amplified from HepG2, but not from HeLa cDNA. B, the figure shows the high homology between the sequences of human AQP8 and rat AQP8 C-terminal peptide, against which the antibody used for immunoblotting and immunofluorescence was raised. Total cellular membranes (TCM) from HepG2 or HeLa cells were prepared and analyzed for AQP8 expression. The western blot detected two main immunoreactive bands of about 36 kDa and 56 kDa, which were present in TCM from HepG2, but not from HeLa cells. C, TCM from samples of normal livers of two different donors were analyzed for AQP8 expression. The western blot detected two immunoreactive bands with identical molecular mass to those observed for HepG2 cells. D, the immunofluorescence images obtained by confocal microscopy showed AQP8 localization to intracellular vesicular structures and to canaliculi (arrowheads) labeled with phalloidin. Nuclear DAPI staining is in blue. Bar, 5 µm.

Figure 2. Decrease in AQP8 expression by RNA interference. HepG2 cells were cultured over night and then transfected with oligo RNA duplexes targeting specific sequences of human AQP8 mRNA (siRNA2 and 4) or a scrambled siRNA2 sequence (siRNA C). AQP8 expression and subcellular distribution in these cells were analyzed as described in Materials and Methods. A, AQP8 expression was analyzed 24 and 48 h after transfection by western blot, using β-actin as loading controls. The figure shows
that siRNA2 and siRNA4 induced a specific decrease in AQP8 expression 48 h after transfection. The bars graphic indicate the densitometry of AQP8 related to β-actin, and expressed as percentage of the control at 48 h. The densitometric analysis of either the 36 kDa or the 56 kDa bands raised similar results. B, the immunofluorescence images obtained by confocal microscopy showed that the fluorescence intensity of both the canalicular (arrowheads) and the cytosolic AQP8 stainings were decreased in the siRNA2 and siRNA4 48 h-transfected cells. The third row shows the overlapped images of AQP8 (green), canalicular (red) and nuclear (blue) stainings. Bar, 5 µm.

**Figure 3.** Decrease in number and volume of canalicular structures in AQP8 knockdown cells. AQP8 knockdown was induced by transfecting cultured HepG2 cells with two different AQP8 specific siRNA (siRNA2 and siRNA4). The number and volume of the canaliculi were analyzed in control cells (i.e. transfected with a scrambled siRNA2, siRNA C) and AQP8 knockdown cells in three separate experiments. A, phase contrast images taken 24 and 48 h after transfection. Arrows indicate canalicular structures. B, number of canaliculi every 100 cells. C, canalicular diameters were measured, and canalicular volumes calculated for each group of cells assuming spherical shape. Bars are means ± S.E. of at least 300 cells in each experimental group. * p < 0.05. Bar, 10 µm.

**Figure 4.** Impaired canalicular osmotic water transport in AQP8 knockdown cells.
Canalicular size changes in response to a 200 mosM hypotonic gradient were analyzed in live cells transfected with siRNA2 (AQP8-) or its scrambled control siRNA (control) 48 h after transfection, using two different methodologies. A,B) Phase contrast images of the same field were captured before and every 30 s after the change
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in medium osmolarity. Canalicular volumes were estimated from canalicular diameter measurements assuming spherical shape. The figure shows the canalicular volumes for typical control or AQP8- canaliculus (A). After 60 s of exposure to the hypotonic medium, the increase in control relative canalicular volume (RCV) was significantly higher than the corresponding to AQP8- RCV (B). C,D) Previous to the exposure to the osmotic gradient, the canaliculi were loaded for 3 min with a cholephilic fluorophore (see Materials and Methods). Fluorescence intensity in the canaliculi was recorded every second and the rate of water flow to the canaliculus in the presence of the osmotic gradient was estimated from the rate of relative fluorescence intensity decrease. The figure illustrates the relative intensity of fluorescence recorded for typical control and AQP8- canaliculi (C), and average RCV changes (D). Values are means ± S.E. of 23 and 17 canaliculi (B) or 10 and 7 canaliculi (D) in control and AQP8- cells, respectively. Figures are representative of three (A,B) or two (C,D) separate experiments. * p < 0.05, # p < 0.01.

**Figure 5.** Defective cAMP-induced canalicular water secretion in AQP8 knockdown cells. Control and 48 h-AQP8 knockdown (AQP8-) live cells were incubated with the bile secretory agonist DBcAMP (200 µM), and changes in canalicular size analyzed by time lapse microscopy. Phase contrast images of the same field were captured every 60 s. A, Canalicular volume changes in typical control and AQP8- cells. DBcAMP induced an increase in canalicular volume in control cells as soon as 2 min after initiated the incubation, which was delayed in AQP8- cells. B, RCV after 5 min incubation with DBcAMP. Values are means ± S.E. of 23 canaliculi in each group. Figures are representative of three independent experiments. * p < 0.05.
Figure 1

A) cDNA

HepG2

HeLa

markers

250 bp

B) Trans-membrane domain

40

250

rAQP8 RLFIGDEKTRLILKSR 263

hAQP8 RCFIGDEKTRLILKAR 261

56 kDa

36 kDa

C) Human

HepG2

Liver 1

Liver 2

56 kDa

36 kDa

D) AQP8

Actin

Overlap

Figure 1
Figure 2

A)

siRNA 24 h

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<th>4</th>
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siRNA 48 h

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B)

siRNA C  siRNA2  siRNA4

AQP8

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<th>siRNA4</th>
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Actin

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Overlap

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Figure 3

A)  

B) 

Canaliculi/100 cells

C) 

Canalicular Volume (μm³)

<table>
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<tr>
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<tbody>
<tr>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>siRNA 2</td>
<td>2</td>
<td>4</td>
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<tr>
<td>siRNA 4</td>
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* *
Figure 4

A) Canalicular Volume (µm³)

B) RCV

C) Relative Fluorescence

D) RCV
Figure 5

A) Canalicular Volume (µm³) over time with DBcAMP and control conditions.

B) RCV comparison between Control and AQP8 conditions, indicating a significant difference (*).