Specific Inhibition of AQP1 Water Channels in Isolated Rat Intrahepatic Bile Duct Units by Small Interfering RNAs*  

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Patrick L. Splinter, Anatoliy I. Masyuk, and Nicholas F. LaRusso‡

From the Center for Basic Research in Digestive Diseases, Division of Gastroenterology and Hepatology, Mayo Medical School, and Foundation, Rochester, Minnesota 55905

Cholangiocytes express water channels (i.e. aquaporins (AQPs)), proteins that are increasingly recognized as important in water transport by biliary epithelia. However, direct functional studies demonstrating AQP-mediated water transport in cholangiocytes are limited, in part because of the lack of specific AQP inhibitors. To address this issue, we designed, synthesized, and utilized small interfering RNAs (siRNAs) selective for AQP1 and investigated their effectiveness in altering AQP1-mediated water transport in intrahepatic bile duct units (IBDUs) isolated from rat liver. Twenty-four hours after transfection of IBDUs with siRNAs targeting two different regions of the AQP1 transcript, both AQP1 mRNA and protein expression were inhibited by 76.6–92.0 and 57.9–79.4%, respectively. siRNAs containing the same base pairs of the AQP1-siRNAs but in random sequence (i.e. scrambled siRNAs) had no effect. Suppression of AQP1 expression in cholangiocytes resulted in a decrease in water transport by IBDUs in response to both an inward osmotic gradient (200 mosm) or a secretory agonist ( forskolin), the osmotic water permeability coefficient ($P_f$) decreasing up to 58.8% and net water secretion ($J_w$) decreasing up to 87%. A strong correlation between AQP1 protein expression and water transport in IBDUs transfected with AQP1-siRNAs was consistent with the decrease in water transport by IBDUs resulting from AQP1 gene silencing by AQP1-siRNAs. This study is the first to demonstrate the feasibility of utilizing siRNAs to specifically reduce the expression of AQPs in epithelial cells and provides direct evidence of the contribution of AQP1 to water transport by biliary epithelia.

Intrahepatic bile duct epithelial cells (i.e. cholangiocytes) play an essential role in bile formation, and by integrated absorptive and secretory processes, they contribute up to 40% of daily bile production in humans (1, 2). Because bile is a complex fluid composed of >98% water, cholangiocytes like other water-transporting epithelial cells are required to rapidly transport large amounts of water in response to osmotic gradients generated by transported ions and solutes, a situation in which specific water channel proteins (i.e. aquaporins (AQPs))

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† To whom correspondence should be addressed: Center for Basic Research in Digestive Diseases, Mayo Clinic, 200 First St., S. W., Rochester, MN 55905, Tel.: 507-284-1006; Fax: 507-284-0762; E-mail: larusso.nicholas@mayo.edu.

‡ To whom correspondence should be addressed: Center for Basic Research in Digestive Diseases, Mayo Clinic, 200 First St., S. W., Rochester, MN 55905. Tel.: 507-284-1006; Fax: 507-284-0762; E-mail: larusso.nicholas@mayo.edu.

The abbreviations used are: AQP, aquaporin; siRNA, small interfering RNA; PBS, phosphate-buffered saline; $P_f$, osmotic water permeability coefficient; $J_w$, net water secretion; IBDU, intrahepatic bile duct unit; ODIN, oligonucleotide.
A

**AQP1-siRNA(a)**

- 5′-AUC AAG AAG AAG CUC UUC UU
- UUA UAG UUC UUC UUC GAG AAG A - 5′

**AQP1-siRNA(b)**

- 5′-CUU CUC AAA CCA CUG GAU UU
- UUU GAA GAG UUU GGU GAC CUA A - 5′

**Scrambled AQP1-siRNA(a)**

- 5′-UGU UCA GGC AAA UAU AAC CUU
- UUU ACA AGR GCC UUU AUA UGC G - 5′

**Scrambled AQP1-siRNA(b)**

- 5′-ACU UUC CAU ACA UGA CUC UUU
- UUU UGA ACG GUA UGU ACU GAG A - 5′

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**Fig. 1.** Sequences and expected duplexes for siRNAs (A) and sites of targeting (B) within AQP1 mRNA. A, the sense (top) and antisense (bottom) strands of siRNAs targeting AQP1 message and the scrambled AQP1-siRNAs are shown. B, the partial mRNA sequence of rat AQP1 (GenBank™ accession number NM_012778). Two potential sites of targeting by siRNAs are underlined. The start codon is in a boldface.

**B**

1. gaggcctccgg agggggctag cccagggctc tcgaaggtc gctggccgca
gagcgggtgga
gagcgggtgga
gagcgggtgga
gagcgggtgga
gagcgggtgga
gagcgggtgga
gagcgggtgga
gagcgggtgga
gagcgggtgga

2. 5′-ACU UUC CAU ACA UGA CUC UUU
- UUU UGA ACG GUA UGU ACU GAG A - 5′

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**Fig. 2.** Uptake of AQP1-siRNAs by IBDUs. A, visualizing of AQP1-siRNA in IBDUs. IBDUs were incubated 24 h in normal rat cholangioocyte medium containing naked Cy3-labeled AQP1-siRNA(b) from 0 to 20 nm. IBDUs analyzed by fluorescent microscopy showed a significant increase in Cy3-AQP1-siRNA(b) fluorescence (red). B, IBDUs take up AQP1-siRNAs in a dose-dependent manner. 24 h after incubation with Cy3 labeled AQP1-siRNA(b) in the absence (white bars) or presence (black bars) of the lipid carrier, the fluorescence intensity of IBDUs was measured and normalized to 100 μg of protein. The uptake of Cy3-AQP1-siRNA(b) increased with increasing concentrations of siRNA(b) independent of the presence of the lipid carrier. Values are the mean ± S.E. of three separate experiments with 4–6 IBDUs in each group.
AQP1 Suppression by siRNAs

IBDUs were transfected with 10 nM AQP1-siRNA(a), AQP1-siRNA(b), and corresponding scrambled AQP1-siRNAs for 24 h. A significant suppression of AQP1 mRNA level occurred in IBDUs transfected with AQP1-siRNA(a) and AQP1-siRNA(b) but not with scrambled (scr) AQP1-siRNAs. Representative immunoblots (B) and quantitative analysis (C) show a significant suppression of AQP1 protein levels by both AQP1-siRNAs. AQP1 Suppression by siRNAs

AQP1 mRNA and protein expression levels was seen (\( p < 0.05 \)). All of the values are expressed as the mean ± S.E. Statistical analysis was performed by the Student's \( t \) test, and results were considered statistically different at \( p < 0.05 \).

RESULTS

Design of AQP1-siRNAs—We selected two target regions of rat AQP1 mRNA (i.e. 71–91 and 673–693 sequences) by scanning the length of the AQP1 gene for AA-dinucleotide sequences and downstream 19 nucleotides without significant homology to other genes by using an appropriate genome database. The antisense strands of synthesized AQP1-siRNAs are the reverse complement of the target sequences (Fig. 1). The sense strands of the AQP1-siRNAs have the same sequences as the target mRNA sequences with the exception that they lack the 5'-AA sequence (Fig. 1). A uridine dimer was incorporated at the 3' end of the sense strands siRNAs (Fig. 1). Thus, the end products are two double-stranded 21-mer siRNAs (i.e. AQP1-siRNA(a) and AQP1-siRNA(b)) that theoretically should reduce the expression of AQP1 mRNA and protein and two siRNAs (i.e. scrambled AQP1-siRNA(a) and AQP1-siRNA(b)) that theoretically should not be effective in AQP1 gene silencing.

Uptake of AQP1-siRNA by IBDUs—IBDUs incubated for 24 h in normal rat cholangiocyte culture medium containing AQP1-siRNAs but not scrambled siRNAs. D, a correlation between AQP1 mRNA and protein expression levels was seen (\( r = -0.298.34 + 32.079 \times x; r = 0.8821 \)). Values are the mean ± S.E. of three independent experiments with 4–6 IBDUs in each group (*, \( p < 0.05 \)).
various amounts (i.e. 0–20 nM) of Cy3-labeled AQP1-siRNA(b) in the absence or presence of the lipid carrier took up AQP1-siRNA, an increase in Cy3 immunofluorescence (white bars) and decrease in AQP1 immunofluorescence (black bars) occurred during 12–24 h, reflecting accumulation of AQP1-siRNA in cholangiocytes and rapid and effective suppression of AQP1 expression in these cells, respectively. Values are the mean ± S.E. of three separate experiments with 3–4 IBDUs in each group. (*, p < 0.05 for AQP1 fluorescence compared with point 0 min).

**Fig. 4. Visualizing of AQP1 suppression in IBDUs by AQP1-siRNA.**

A, AQP1-siRNA(b) was labeled with Cy3 and transfected into cholangiocytes of IBDUs. At the times indicated, IBDUs were analyzed using confocal fluorescent microscopy for fluorescence intensity of Cy3-labeled AQP1-siRNA(b) (red) and AQP1 (green). Top left patterns show higher magnification of region in inset. B, in IBDUs treated with AQP1-siRNA, an increase in Cy3 immunofluorescence (white bars) and decrease in AQP1 immunofluorescence (black bars) occurred during 12–24 h, reflecting accumulation of AQP1-siRNA in cholangiocytes and rapid and effective suppression of AQP1 expression in these cells, respectively. Values are the mean ± S.E. of three separate experiments with 3–4 IBDUs in each group. (*, p < 0.05 for AQP1 fluorescence compared with point 0 min).

**AQP1 Gene Suppression in IBDUs by siRNAs**—The levels of AQP1 mRNA and protein in IBDUs transfected with 10 nM each of four different naked siRNAs (i.e. two siRNAs to different regions of the AQP1 and two corresponding scrambled AQP1-siRNAs) are shown in Fig. 3. Both siRNAs to different sequences within the AQP1 gene effectively inhibited AQP1 mRNA (Fig. 3A) and protein expression (Fig. 3B). AQP1 mRNA and protein levels were inhibited by AQP1-siRNA(a) by 76.6 and 57.9%, respectively. AQP1-siRNA(b) was more effective, suppressing AQP1 mRNA and protein levels by 92.0 and 79.4%, respectively. In contrast, two scrambled AQP1-siRNAs had no effect on AQP1 gene expression. Moreover, AQP1-siRNAs had no effect on mRNA expression for AQP4 (data not shown), an AQP, which is also expressed in rat cholangiocytes (11), providing evidence that both AQP1-siRNA(a) and AQP1-siRNA(b) were specific for AQP1. Because there is a strong correlation between AQP1 mRNA and protein suppression by siRNAs (Fig. 3C), these data suggest that AQP1 silencing in cholangiocytes results from a reduction in the amount of AQP1 mRNA available for translation. These data also suggest that AQP1-siRNAs were highly specific and efficient in AQP1 gene silencing in rat IBDUs.

**Inhibition of AQP1-mediated Water Transport by siRNAs**—Water transport characteristics (i.e. $P_f$ and $J_v$) in AQP1-siRNAs-nontransfected IBDUs and in IBDUs transfected with 10 nM AQP1-siRNAs were determined from the volume of water transported into the lumen of IBDUs either in response to a 200 mOs\text{m} transepithelial osmotic gradient (lumen osmolality > bath osmolality) (Fig. 5) or in response to 100 $\mu$M forskolin (Fig. 6), an agent known to stimulate biliary bicarbonate and water secretion (2–4). The $P_f$ in AQP1-siRNA-nontransfected IBDUs and IBDUs transfected with scrambled AQP1-siRNAs...
ranged from $50 \times 10^{-3}$ to $72 \times 10^{-3}$ cm/sec (Figs. 5 and 6). When a transepithelial osmotic gradient was established in IBDUs transfected with AQ1P1-siRNA(a) and AQ1P1-siRNA(b), $P_f$ was decreased by 29.4% and 58.8%, respectively (Fig. 5A). In IBDUs transfected with AQ1P1-siRNA(b), $P_f$ was also decreased by 53.1% in response to forskolin (Fig. 6A). In IBDUs transfected with AQ1P1-siRNA(b), $J_{\nu}$ across biliary epithelia in response to an osmotic gradient or forskolin was decreased by 42.8% and 87.6%, respectively (Figs. 5B and 6B). A strong correlation between AQ1P1 protein expression and water transport in IBDUs transfected with AQ1P1-siRNAs was shown (Fig. 5C), suggesting that a decrease of cholangiocyte osmotic water permeability is a result of specific AQ1P1 gene silencing by AQ1P1-siRNAs.

Fig. 5. Water transport in response to transepithelial osmotic gradient in IBDUs transfected with AQ1P1-siRNAs. A, osmotic water permeability coefficients ($P_f$) reflect rapid water transport across biliary epithelia in response to an inward osmotic gradient (200 mos M) in nontransfected IBDUs and in IBDUs transfected with scrambled (scr) AQ1P1-siRNAs (white bar) and in IBDUs transfected with AQ1P1-siRNA(b) (gray bar) stimulated with forskolin (100 µmol) showed rapid water transport across biliary epithelia. In IBDUs transfected with AQ1P1-siRNA(b) (black bar) for 24 h, osmotic water permeability in response to forskolin decreased by 53.1%. B, net water secretion ($J_{\nu}$) in IBDUs transfected with AQ1P1-siRNA(b) (black bar) in response to forskolin decreased by 87% compared with nontransfected IBDUs (white bar) and IBDUs transfected with scrambled AQ1P1-siRNA(b) (gray bar). Values are the mean ± S.E. of three microperfused IBDUs in each group (*, $p < 0.05$).

Fig. 6. Water transport in response to forskolin in IBDUs transfected with AQ1P1-siRNA. A, osmotic water permeability coefficient ($P_f$) in AQ1P1-siRNA-nontransfected IBDUs (white bar) and in IBDUs transfected with scrambled (scr) AQ1P1-siRNA(b) (gray bar) stimulated with forskolin (100 µmol) showed rapid water transport across biliary epithelia. In IBDUs transfected with AQ1P1-siRNA(b) (black bar) for 24 h, osmotic water permeability in response to forskolin decreased by 53.1%. B, net water secretion ($J_{\nu}$) in IBDUs transfected with AQ1P1-siRNA(b) (black bar) in response to forskolin decreased by 87% compared with nontransfected IBDUs (white bar) and IBDUs transfected with scrambled AQ1P1-siRNA(b) (gray bar). Values are the mean ± S.E. of three microperfused IBDUs in each group (*, $p < 0.05$).
AQP1 Suppression by siRNAs

DISCUSSION

The major objectives of this study were to develop and utilize siRNAs to directly investigate the importance of AQP1 in water transport by biliary epithelia. Although several lines of evidence indicate that AQPs are involved in water transport by cholangiocytes (4–11), the absence of specific AQP inhibitors has impaired direct testing of hypotheses related to this issue. Indeed, until this study, no specific pharmacological inhibitors of AQPs had been reported.

Our results show that AQP1-siRNAs effectively (i.e. by 57.9–79.4%), specifically (i.e. AQP1-siRNAs but not scrambled AQP1-siRNAs), and rapidly (i.e. during 12 h after transfection of IBDUs) down-regulate endogenously expressed AQP1 but not AQP4 in cholangiocytes. Moreover, direct quantitation of water transport in IBDUs transfected with siRNAs to AQP1 demonstrates that P_{f} and J_{w} are significantly decreased compared with IBDUs transfected with scrambled siRNAs. The observed decrease of net water secretion in IBDUs transfected with AQP1-siRNA(b) under different experimental conditions (i.e. an inward osmotic gradient or a secretory agonist) suggests high affinity AQPs. Taken together, these findings demonstrate for the first time that inhibition of AQP1 expression in mammalian cells by siRNAs results in inhibition of water transport.

We found that cholangiocytes effectively take up siRNA duplexes if IBDUs are incubated with AQP1-siRNAs either in the absence or presence of transfection reagent. This observation is in contrast to published data (29) that siRNAs are taken up by cultured HeLa, COS-1, and 293 cells only in the presence of transfection agent. However, our data are consistent with recently published results that hepatocytes in vivo can be effectively transfected with naked siRNAs (30, 31). Although a precise explanation for these differences is not apparent, they emphasize the importance of assessing both forms of siRNAs when employing this gene-silencing approach.

In our study, we utilized two different siRNAs constructed against different regions of AQP1 mRNA. Whereas both constructs inhibited AQP1 expression and function in cholangiocytes, siRNA-designated AQP1-siRNA(b) was more effective. We have no explanation as to why these two siRNAs differed in their inhibitory activity, but this phenomenon has been observed for other genes. For example, several siRNAs synthesized against different sites on the same target mRNA demonstrated striking differences in silencing efficiency (29).

To our knowledge, this study is the first to successfully utilize siRNA gene-silencing technology to achieve AQP gene suppression in epithelial cells. AQP gene silencing has previously been achieved primarily by developing transgenic null mice lacking AQPs and by using dominant negative mutants or antisense oligonucleotides (ODNs). The phenotype analysis of transgenic mice deficient in AQP1, AQP3, AQP4, and AQP5 has provided new insights into their critical role in water transport in Xenopus oocytes (41–43), LLC-PK1 cells (44), and mouse cholangiocytes (45). This experimental approach also has its limitations being applicable principally to studies utilizing cultured cell systems. Antisense ODNs have been used to a limited degree to suppress AQP function. For example, the P_{f} of Xenopus laevis oocytes injected with poly(A)^{+}RNA from cultured bovine corneal epithelial cells was inhibited by coinjection with AQP5 ODNs (46). Also, the expression of AQP1 in human trabecular meshwork cells was suppressed by corresponding ODNs (47). Although useful, the specificity of this approach is somewhat limited because non-specific hybridization of ODNs with intracellular protein rather than mRNAs has been reported previously (48). Moreover, the efficiency of gene silencing by ODNs is relatively low (e.g. only one of eight antisense oligonucleotides is expected to provide a specific suppression of a targeted gene) (49). Our own experience has been that AQP1 ODNs are not effective in inhibiting AQP1 expression and function in isolated rat IBDUs (data not shown).

The advantages of siRNA technology for effective and specific suppression of selected genes are becoming increasingly apparent. Recent studies (19–26) indicate that siRNAs directed against endogenous genes can inhibit the expression of virtually any protein-coding gene in any mammalian cells. Importantly, RNA and protein analysis indicate that only the targeted gene is affected by the corresponding siRNA (15–26).

In summary, we designed and utilized siRNAs to AQP1 that markedly diminished the expression of this protein in IBDUs. As a result, cholangiocyte osmotic water permeability and net water secretion were significantly reduced, suggesting that at least 65% of water transported across biliary epithelia is AQP1-mediated. These data are the first to demonstrate the feasibility of utilizing siRNAs to specifically reduce the expression of AQPs in epithelial cells. They also provide further evidence of the importance of AQP1 in water transport by biliary epithelia.

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