

Enhanced aquaporin 8 expression after subtotal colectomy in rat

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ABSTRACT

Background: Aquaporins (AQPs), the family of water-selective channels, are localized in various organs and tissues, including the gastrointestinal (GI) tract. However, the roles of AQPs in the GI tract remain unclear. **Materials and Methods:** Male SD rats were subjected to subtotal colectomy (Group C, n = 22) or a sham operation (Group S, n = 16) and were sacrificed on postoperative days 7, 14, and 28. Total RNAs from the distal ileum and rectum were extracted. Quantitative RT-PCR was performed to measure AQP8 mRNA expression. For light-microscopy or immunohistochemistry, paraffin-embedded sections of 4 μm were prepared with H-E staining or anti-AQP8 antibody reaction. Mann-Whitney U-test was performed to compare the AQP8 distributions between the two groups, and the statistical significance was defined as $p < 0.05$. **Results:** AQP8 mRNA expression was enhanced in both the ileum and rectum in Group C at day 7. AQP8 protein expression was consistently observed in the ileum and rectum. The villus length in the ileum of Group C was significantly greater than that of Group S at days 7 and 14. **Conclusion:** Enhanced AQP8 mRNA expression in the subtotal colectomy model suggests that AQP8 plays an important role in maintaining the intestinal fluid balance.

Keywords: Aquaporin 8; Subtotal Colectomy; mRNA; Immunohistochemistry

1. INTRODUCTION

Water-selective channels (aquaporins; AQPs) have been identified as molecules located mainly on the plasma membrane of various cell types and increasing water permeability. The mammalian water channel, AQP1, was

first identified as a protein homologous to the major intrinsic protein of the bovine lens in erythrocytes [1,2], and was also shown to be present in red blood cells, renal proximal convoluted tubules and the thin descending limb of Henle [1,2]. Homology cloning techniques have been used to clone AQPs, which have been cloned from various organs: AQP2 [3] and AQP3 [4] from the kidney, AQP4 from the brain [5], AQP5 from the salivary gland [6], AQP6 from the kidney [7], AQP7 from the testis [8], AQP8 from the pancreas and liver [9], AQP9 from the liver [10], and AQP10 from the duodenum and jejunum [11].

Previous studies have demonstrated the expression of several AQP types in the gastrointestinal (GI) tract, suggesting their participation in water absorption or secretion [1,4,5,9]. We have demonstrated both mRNA and protein expression of AQP1, AQP3, AQP4, and AQP8 in the rat GI tract [12,13]. However, the functions of the AQPs in the GI tract have not been well elucidated. In the present study, we examined the mRNA expression and protein expression of AQP8 in rat subtotal colectomy models in order to investigate the possible functions of AQP8 in the GI tract during postoperative adaptation to subtotal colectomy.

2. MATERIALS & METHODS

2.1. Experimental Animals

All experiments and surgical procedures conformed to the guidelines for the proper care and use of laboratory animals issued by the Public Health Service, National Institutes of Health. Male Sprague-Dawley (SD) rats weighing approximately 200 g were purchased from Charles River Japan (Yokohama, Japan). Throughout the experimental period, except for 12 h prior to the surgical procedures, the animals were allowed free access to standard lab rat chow and tap water. Their condition and weight were

recorded daily.

2.2. Subtotal Colectomy Model

The rats were divided into two groups: Group C ($n = 22$) was subjected to subtotal colectomy with primary anastomosis and Group S ($n = 16$), a sham operation. They were denied food for 12 h and were anesthetized by intraperitoneal injection of pentobarbital. In Group C, a short segment of the ileum and the entire large intestine was resected from 1 cm proximal to the ileocecal valve to 2 cm proximal to the anal verge, and ileoproctal anastomosis was performed. In Group S, the ileum was divided at 1 cm proximal to the ileocecal valve, and the rectum was divided at 2 cm proximal to the anal verge. Subsequently, anastomosis was performed without removal of the small or large intestine. All bowel anastomoses were completed with eight interrupted 5-0 silk sutures. The abdominal wound was closed in two running layers, using 2-0 silk. Both groups were allowed free access to standard lab rat chow and tap water after the operation.

The animals in both Group C and Group S were sacrificed at postoperative days 7 ($n = 8$ and $n = 6$, respectively), 14 ($n = 7$ and $n = 5$, respectively), and 28 ($n = 7$ and $n = 5$, respectively). Total RNAs were isolated from the ileum and the remnant rectum by a modified acid guanidiniumthiocyanate phenol-chloroform extraction method using TRIzol (GIBCO BRL, Life Technologies, Rockville, MD, USA), as described previously [12].

2.3. PCR Cloning of Rat Aquaporin 8

Rat AQP8 (315 bp; +701 ~ +1015) cDNA fragments were obtained from the rat ileum and remnant rectum by the PCR-based cloning method using primers for AQP8 as reported previously [9,12]. The PCR products were subcloned into pGEM 11Z (Promega Japan Inc., Tokyo, Japan) and their sequences were verified using an automated DNA sequencer (Perkin Elmer, Foster City, CA, USA). A partial fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA of 123 bp was inserted in pGEM 3Z (Promega).

The plasmid with the rat AQP8 and GAPDH gene inserts was linearized with appropriate restriction enzymes and used as the template for quantitative RT-PCR.

2.4. Quantitative RT-PCR

The total RNA (1 μg) of each sample was reverse-transcribed at 42°C for 1 h by using an oligo (dT) primer and Superscript II reverse transcriptase (GIBCO BRL) in a volume of 20 μl . Each of these transcripts was used as a template for multiplex quantitative PCR to measure AQP8 mRNA and GAPDH expression in a single well by using an ABI PRISM 7700 sequence detection instrument (PE Biosystems Japan, Chiba, Japan).

The AQP8-specific primers were 5'-GGCAGGTGGTGGGATCTCT-3' and 5'-GCCTAATGAGCAGTCCCA CAA-3', and the fluorogenic probe was 5'-TGGATC TACTGGCTGGGCCCACTC-3'. A GAPDH TaqMan Rodent GAPDH Control Reagent VIC TM Probe (PE Biosystems Japan) was used to amplify rat GAPDH for use as an internal control.

The amplification reactions mixture (50 μl) contained a reverse-transcript (1 μl), 1 \times TaqMan Universal PCR Master Mix (PE Biosystems), 900 nM of each AQP8 primer, 250 nM of the AQP8 fluorogenic probe, 100 nM of rodent GAPDH primers, and 250 nM of the rodent GAPDH fluorogenic probe. All quantitative 2-step -PCR reactions were performed according to the manufacturer's instructions under the following thermocycler conditions: 50°C holding for 2 min, 95°C holding for 10 min followed by 40 cycles at 95°C for 15 seconds, and at 60°C for 1 min. Template-negative controls were run on each PCR plate. A calibrator reverse-transcript sample was amplified in parallel on all plates in order to allow a comparison of the samples run at different times. The data were analyzed using Sequence Detection Software (PE Biosystems Japan), and the values were represented as the mean (SEM) of ratios (AQP/GAPDH mRNA amplicon) \times 100%.

2.5. Immunohistochemistry

Since the results of mRNA quantification revealed enhanced AQP8 expression in the ileum and the remnant rectum of the Group C animals, immunohistochemistry was performed as described previously [13]. In brief, the rat ileum and rectum samples were cut and fixed with methyl-Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid) overnight, dehydrated with ethanol, embedded in paraffin, and sectioned at 4 μm . The slide-sectioned tissues were deparaffinated with xylene and ethanol, hydrated in distilled water, and then blocked with normal goat serum (1:20 dilution) for 1 h. After a three-time rinse with PBS, the slides were incubated with an anti-AQP8 antibody (2.0 $\mu\text{g}/\text{ml}$) (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) for 1 h at 37°C; this was followed by overnight incubation at 4°C, rinsing three times with PBS, and incubation with goat anti-rabbit immunoglobulins conjugated to a peroxidase-labeled polymer (En Vision, DAKO, Kyoto, Japan), and coloring by diaminobenzidine reaction. After rinsing with distilled water, the slides were counterstained with hematoxylin for observation.

2.6. Morphologic Measurements

To measure the villus length of the ileum and the crypt depth of the remnant rectum and the ileum, rat ileum and rectum samples were cut and fixed as described above.

The slide-sectioned tissues were deparaffinated with xylene and ethanol, hydrated in distilled water, and then immersed in a Hematoxylin-Eosin solution for 15 min. The measurements were performed 5 times in each slide section, and the values were described as the mean \pm SD.

2.7. Statistical Analysis

Mann-Whitney U-test was performed to compare the distributions between the two groups. A two-tailed p value of <0.05 was considered to indicate statistical significance.

3. RESULTS

3.1. Overall Animal Health

During the current experimental series, the perioperative lethality was 40%. Twenty-five rats were sacrificed due to bleeding from the mesenterium, ileus or peritonitis. The weight of the Group C rats decreased and diarrhea was observed until day 7. The weight of the Group S rats decreased until day 3; however, it gradually increased after day 4. Moreover, no diarrhea was observed. The rats in Group C showed a temporary decrease in weight from 210.5 ± 14.9 g to 165.8 ± 20.0 g, 7 days after the operation. The other group showed a temporary decrease from 200.2 ± 8.2 g to 192.7 ± 8.4 g, 3 days after the operation. The weight loss after surgery was more severe in Group C as compared to Group S (Group C: 210.5 ± 14.9 to 165.8 ± 20.0 , Group S: 200.2 ± 8.2 to 192.7 ± 8.4). However, it recovered gradually in both groups, although the postoperative weight on days 7, 14, and 28 was significantly lesser in Group C than in Group S (**Figure 1**).

Watery stool was observed for 7 days post operation in Group C, but not in Group S.

3.2. Quantitative RT-PCR

Significant quantitative RT-PCR analyses enhanced

AQP8 mRNA expression in the ileum and remnant rectum of Group C rats at days 7 ($p < 0.01$); however, this was not observed at days 14 and 28 (**Figure 2**). In the ileum at days 7, the AQP8 mRNA expression level of Group C was almost 4 times that of Group S. In the remnant rectum, it was almost 3 times that of Group S. The level, however, returned to normal at days 14 and 28.

3.3. Immunohistochemistry

Immunohistochemistry was used to detect the AQP8 protein expression in the apical membrane of the ileum in Group C and the apical membrane of the remnant rectum in both groups (**Figure 3**). In the ileum of Group S, no protein expression was detected. AQP8 mRNA expression in the ileum was low at days 14 and 28, but AQP8 protein in the ileum was maintained through the experimental period.

3.4. Morphological Measurements

The villus length of the ileum and crypt depth of the ileum and rectum were measured. The results are shown in **Table 1**. The villus length of the ileum on days 7 and 14 of Group C was significantly greater than that of Group S ($p < 0.002$ and 0.02 , respectively). The crypt depth remained unchanged (**Table 2**).

4. DISCUSSION

Since the discovery of AQP1, 12 mammalian AQPs have been recognized, and the clinical or physiological importance of AQPs has been shown or suggested; AQP0 mutations have been identified in some forms of congenital cataracts [14]. AQP1-null individuals have shown limited ability to maximally concentrate urine under water-deprived conditions [15] and have shown decreased pulmonary vascular permeability [16]. AQP2 mutations

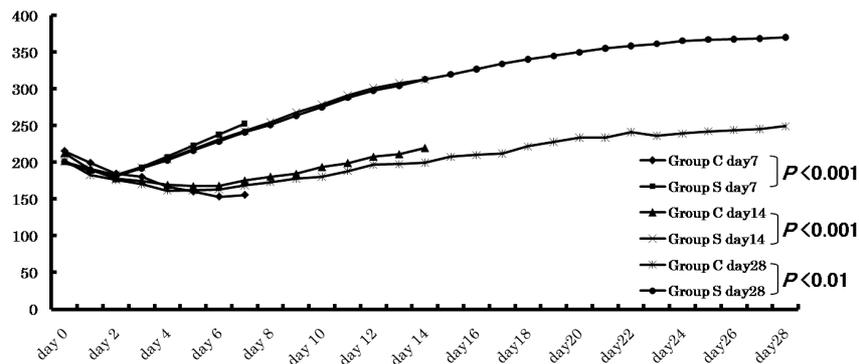


Figure 1. Weight of experimental animals. The rats in Group C showed a temporary decrease in weight from 210.5 ± 14.9 g to 165.8 ± 20.0 g at 7 days post operation. Group S showed a temporary decrease from 200.2 ± 8.2 g to 192.7 ± 8.4 g at 3 days post operation. The weight on postoperative days 7, 14, and 28 were significantly lesser in Group C than in Group S ($p < 0.001$ on days 7 and 14, and $p < 0.01$ on day 28).

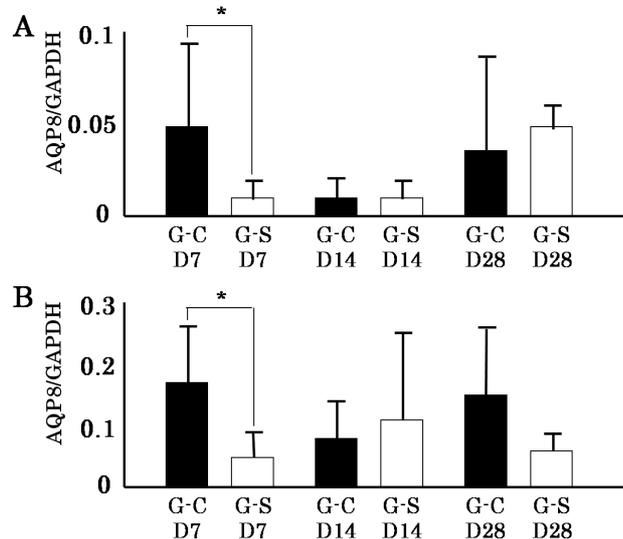


Figure 2. AQP8 mRNA expression. AQP8 mRNA expression in the ileum (A). B, AQP8 mRNA expression in the remnant rectum (B). AQP8 mRNA expression was enhanced in the ileum and the remnant rectum of Group C rats at day 7 (* $p < 0.01$).

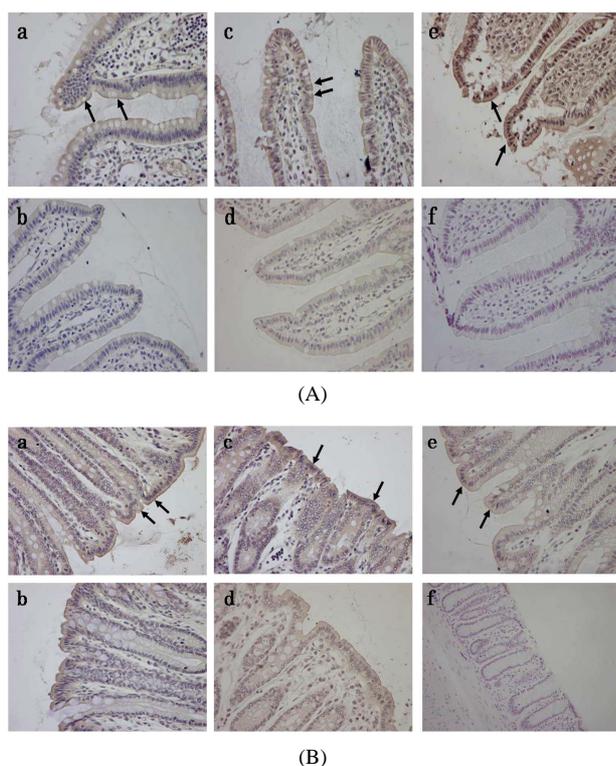


Figure 3. AQP8 protein expression. A, AQP8 protein expression in the ileum (A). AQP8 protein expression in the remnant rectum (B). AQP8 protein expression (arrow) was detected in the apical membrane of the ileum of Group C and the apical membrane of the remnant rectum of both groups. The top line (a, c, e) is a specimen from Group C and the bottom line (b, d, f), from Group S. The right column (a, b) is day 7; the middle column (c, d), day 14; and the left column (e, f), day 28.

cause clinical congenital diabetes insipidus [17], and AQP3 and AQP4 seem to play a role in urinary concentration in knockout mice studies [18,19]. Abnormalities in AQP5 distribution have been shown to be related to Sjogren's syndrome [20]. Although several experimental models using transgenic mice lacking AQPs have been examined previously [21], the functions of AQPs in digestive organs remain unclear.

Previously, we have shown the expression, distribution, and localization of AQPs in rat digestive organs, including the GI tract: Generalized expression of AQP1 and AQP3 mRNA is observed widely along the gastrointestinal tract; AQP4 mRNA is expressed selectively in the lower portion of the stomach and small intestine; and AQP8 mRNA is expressed more selectively in the jejunum and colon [12,13]. Recently we cloned AQP10 from the human jejunum. AQP10 was selectively expressed in the upper sites of the small intestine, such as the duodenum and jejunum in humans [11]; however, AQP10 has not been identified yet in rats.

Purdy *et al.* [22] reported a change in AQP3 expression in the ileostomy model. We have previously shown enhanced expression of AQP8 mRNA after colectomy in rats. Here, we decided to construct a rat colectomy model to elucidate the function of AQP8 and intestinal adaptation.

We performed a quantitative investigation using Real-Time PCR to elucidate the changes in AQP family gene expression of mRNA in the rat gastrointestinal tract after bowel removal. Our result revealing a prominent enhancement in AQP8 mRNA expression in the ileum and remnant rectum of the subtotal colectomy rats suggest that AQP8 in the ileum and remnant rectum is useful for water absorption.

We also used immunohistochemistry to confirm the enhancement of the AQP8 protein in the rectum of the subtotal colectomy rats being in accordance with the enhancement of AQP8 mRNA expression. Our results showed prominent AQP8 protein on the columnar epithelial cells of the ileum and the remnant rectum of the subtotal colectomy rats; in the sham-operated rats, the staining was negligible. However, AQP8 mRNA expression was normalized on days 14 and 28, suggesting that AQP8 protein expression in Group C was enhanced even on days 28. This suggests that the half-life of the AQP8 protein would continue after a decrease in AQP8 mRNA expression, and the AQP8 protein would function for water absorption.

Morphologic measurements showed an elongated villus length and crypt depth of the ileum on days 7 and 14 in Group C. On days 28, there was no significant difference in the villus length between the groups. No significant difference was observed in the crypt depth of the rectum. Willis *et al.* [23] reported an increased villus

Table 1. Villus length and crypt depth of ileum.

| | Day 7 | | Day 14 | | Day 28 | |
|--------------------------|--------------------|--------------------|---------------------|---------------------|------------------|------------------|
| | Group C | Group S | Group C | Group S | Group C | Group S |
| Villus (μm) | 345.1 \pm 36.7** | 265.4 \pm 36.3** | 357.2 \pm 112.8** | 240.9 \pm 143.3** | 346.9 \pm 53.9 | 307.4 \pm 37.7 |
| Crypt (μm) | 193.6 \pm 41.7* | 131.7 \pm 21.8* | 208.3 \pm 50.2* | 154.5 \pm 34.1* | 203.4 \pm 42.9 | 157.6 \pm 29.8 |

Group C, subtotal colectomized rat; Group S, sham operated rat; **p < 0.005; *p < 0.05.

Table 2. Crypt depth of rectum.

| | Day 7 | | Day 14 | | Day 28 | |
|-------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | Group C | Group S | Group C | Group S | Group C | Group S |
| Crypt (μm) | 315.7 \pm 47.8 | 309.1 \pm 53.5 | 305.6 \pm 73.1 | 290.0 \pm 29.8 | 303.9 \pm 47.4 | 283.5 \pm 34.6 |

Group C, subtotal colectomized rat; Group S, sham operated rat.

length and density after colectomy in rats. They concluded that the increased ileal mucosal surface is probably responsible for the elevation in electrolyte and glucose absorption. Our results suggested that the elongated villi would assist AQP8 in water absorption in the subtotal colectomy model. We considered that most part of the adaptation of the ileum would be completed after 14 days.

Continuous diarrhea and weight loss were observed in Group C. The diarrhea continued and the bodyweight continued to decrease until 7 days after the operation, possibly suggesting that bodyweight loss and/or diarrhea would accelerate the adaptation of the intestine after colectomy in rats.

In summary, in the ileum and rectum in Group C, AQP8 mRNA expression was enhanced on days 7 and AQP8 protein expression was enhanced on days 7, 14 and 28. The villus length of the ileum was increased on days 7 and 14 in Group C. These results suggested that intestinal adaptation after subtotal colectomy occurred after 7 postoperative days.

5. CONCLUSIONS

The physiological roles of water channels in water transportation through the epithelial cell layer in the GI tract remain unclear. However, our results suggest an important role of AQP8 in the maintenance of intestinal fluid balance, and for the adaptation to postoperative conditions.

AQP8 may facilitate water movement through the epithelium of the ileum and the remnant rectum after subtotal colectomy.

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