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Dynamics of Aquaporin 1 and Aquaporin 8 in the Pancreas: An Experimental Study with a Caerulein-induced Pancreatitis Model

Chi-e Kitami[1], Yu Koyama[1], Hirokazu Nishino[2], Isao Kurosaki[1], Tsuneo Iiai[1] and Katsuyoshi Hatakeyama[1]

[1]Division of Digestive and General Surgery, Graduate School of Medical and Dental Sciences, Niigata University, Niigata; [2]Department of Gastroenterology and Hepatology, Jikei University School of Medicine, Tokyo, Japan

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Summary. Aquaporins (AQPs) are a family of water channel proteins comprising 13 members, of which five (AQP1, AQP 4, AQP 5, AQP 8 and AQP12) have been detected in the rat exocrine pancreas. However, the dynamics and role of AQPs in pancreatitis, which is characterized by interstitial edema and acinar cell injury, are unclear. This experimental study using a caerulein-induced pancreatitis model aimed to investigate the kinetics of AQPs in pancreatitis. Male Wistar rats received a caerulein infusion (10 μg/kg/h) for three h and then were sacrificed at three h and 72 h after the start of infusion. The expression of mRNA and protein for AQP1 and AQP8 were examined by quantitative reverse transcription polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry. In the control group, AQP1 was located in the pancreatic ductal cells and capillary endothelia, whereas AQP8 was positive only in the acinar cells. Macroscopically and microscopically, obvious pancreatitis was observed in the acute pancreatitis phase (three h group), while it disappeared in the recovery phase (72 h group). AQP1 expression in the pancreatic ductules decreased in both the three h and 72 h groups. AQP8 expression was depressed in the three h group when the acinar cell swelling was observed, while AQP8 expression was focally decreased in the 72 h group. These results suggest that the decrease in AQP1 and AQP8 show a latent injury in the ductal cells and acinar cells. They can be related to the mechanisms of edematous pancreatitis.

Key words— caerulein-induced pancreatitis model, aquaporin 1, aquaporin 8.

INTRODUCTION

In acute pancreatitis in humans, prominent edema of the pancreatic parenchyma and surrounding tissue is commonly observed from its onset. Experimentally, the caerulein, cholecystokinin analog induced acute pancreatitis which has been well established as a model of edematous pancreatitis.[1,2] Prominent edematous changes in the connective tissue, acinar cell swelling, and inflammatory cell infiltration are recognized as distinctive features in this model.[2,3] Recently, several reports documented that the pancreatitis induced by caerulein is characterized by acinar cell apoptosis.[4,5] However, the role of aquaporins (AQPs) in pancreatitis has not been elucidated.

AQPs are a family of small (~30 kDa) integral membrane proteins that function as water channels.[6,7,8] Currently, 13 varieties of AQPs, numbered from 0-12, have been confirmed to be widely distributed in various organs of mammals; the type of AQP or its numbered variety from the AQP family differ from organ to organ.[8-14] In the rat pancreas, AQP1, AQP4, AQP5, AQP8, and AQP12 have been identified. AQP4 and AQP5 expression were detected by reverse transcription polymerase chain reaction (RT-PCR) Southern blot analysis;[12] AQP12 was identified in 2005.[15] On the

Correspondence: Chi-e Kitami, Division of Digestive and General Surgery, Graduate School of Medical and Dental Sciences, Niigata University, Asahimachi-dori 1-757, Chuo-ku, Niigata 951-8510, Japan.

Abbreviations – AQP, aquaporin; GAPDH, glyceraldehydes3-phosphate dehydrogenase; HE, hematoxylin and eosin; RT-PCR, reverse transcription polymerase chain reaction.

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other hand, AQP1 and AQP8 have been detected by both RT-PCR and immunohistochemistry i.e.; AQP1 has been detected in the pancreatic ductal cells and microvasculature, and AQP8 in the acinar cells.[10-14] Therefore, we chose AQP1 and AQP8 to disclose the dynamics of mRNA and protein expression in the rat pancreas.

The aim of the study was to analyze the kinetics of AQP mRNA and protein expression in caerulein-induced pancreatitis, and to discuss the correlation between the AQP expression and the morphological changes in the pancreas.

MATERIALS AND METHODS

Caerulein-induced pancreatitis model

Male Wistar rats weighing 200-250 g were used. Under pentobarbital anesthesia, caerulein (Sigma Chemicals Co., St Louis, MO, USA) (10 μg/kg/h) was infused for three h. The rats were sacrificed at three h (n = 10) and 72 h (n = 10) after the start of infusion (three h group, 72 h group). Control group rats (n = 5) received an infusion of saline for three h and were sacrificed. All experiments and surgical procedures conformed to the Guidelines for the-Proper Care and Use of Laboratory Animals, prescribed by the Public Health Service, National Institutes of Health.[16]

Quantitative RT-PCR

Total RNA was isolated from the caudal site of the pancreas by using a modified acid guanidinium thiocyanate phenol-chloroform extraction method (TRIzol, GIBCO BRL, Life Technologies, Rockville, MD, USA). Total RNA (1 μg) of each samples was reverse-transcribed at 42°C for one h by using an oligo dT primer and Superscript II reverse transcriptase (GIBCO BRL) in a volume of 20 μl. Each reverse transcript was used as a template for real-time quantitative PCR to quantify AQP1, AQP8, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA expression was separated by means of an ABI PRISM 7700 Sequence Detection Instrument.

The AQP1 specific primers were 5'-CTGGGTGGGACCATTCATTG-3' and 5'-TGCGGTCTGTAAAGTCGCTG-3', and the fluorogenic probe was 5'-ACTTCATCCTGGCCCCACGCAG-3. The AQP8 specific primers were 5'-GGCAGGTGGTGGGAT CTCT-3' and 5'-GCCTAATGAGCAGTCCCACAA-3', and the fluorogenic probe was 5'-TGGATCTACTGGC TGGGCCCAGCTC-3'. The Taq Man® GAPDH Control Reagent was used for amplifying the rat GAPDH as an internal control.

For AQP1 or AQP8 mRNA quantification, the amplification reaction solutions (50 μl) contained a reverse transcript (1 μl), 1 × Taq Man Universal PCR Master Mix (PE Biosystems), 900 nM of each AQP primer, and 250 nM of each corresponding AQP fluorogenic probe. For the GAPDH amplifications, the amplification reaction solutions (50 μl) contained a reverse-transcript (1 μl), 1 × Taq Man Universal PCR Master Mix, 100 nM of rodent GAPDH primers, and 250 nM of 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 for two min, followed by 95°C for 10 min, followed by 40 cycles of 95°C for 15s, and then 60°C for one 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 compare with the sample run at different times. These data were analyzed using Sequence Detection Software (PE Biosystems).

Western blotting

For protein preparation, tissues from the tail of the pancreas were frozen in liquid nitrogen, and then stored at -80°C until they are analyzed. The tissue was homogenized in an ice-cold isolation solution containing 0.3 M sucrose, 25 mM Tris, and 1mM EDTA 2Na. The homogenates were centrifuged at 3500 rpm for 15 min at 4°C. The supernatants were obtained and supercentrifuged at 13000 rpm for one h at 4°C. The pellet (plasma membrane) was diluted by 1-ml lysis solution, and the protein concentrations were measured by a modified Lowry Method.[17] The total proteins (30 μg/sample) were solubilized in a lysis solution and a sample buffer, and then boiled for five min. SDS-PAGE was carried out on 12.5% gradient gels. The proteins were transferred electrophoretically to the polyvinylidene difluoride (PVDF) membranes, blocked for one h at room temperature in PBS-T (phosphate-buffered saline containing 0.1% Tween 20), and incubated two nights at 37°C with an anti-AQP1 antibody (diluted 1:500) and an anti-AQP8 antibody (1:50) in PBS-T containing 2 % non-fat dry milk. After washing in PBS-T, the membranes were incubated with goat anti-rabbit immunoglobulin conjugated to peroxidase labeled polymer (EnVision, DAKO, Kyoto) for 90 min at room temperature. The blots were developed with enhanced chemiluminescence agents (ECL Plus; Amersham) before exposure to X-ray film. For quantification, images of the Western blots were scanned and the labeling density was quantified using Scion Image Software.

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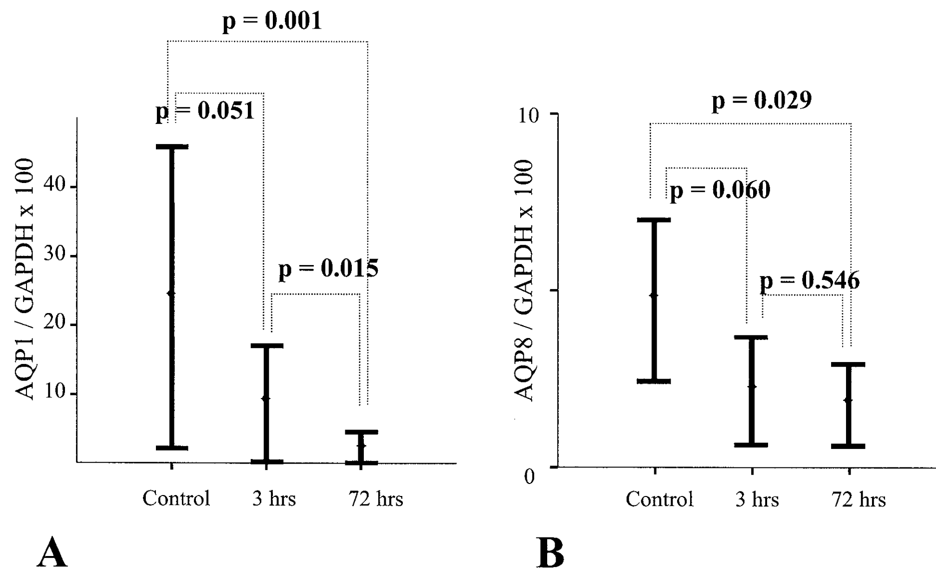

Fig. 1. Reverse transcription polymerase chain reaction (RT-PCR) quantification of aquaporin (AQP) mRNA expression in the pancreas. **A.** AQP1 mRNA expressions: the level of AQP1 mRNA in the 72 h group is significantly lower than that in both the control and three h groups. **B.** AQP8 mRNA expressions: the level of AQP8 mRNA in the 72 h group is significantly lower than that in the control group. The values are means ± SD of ratios (AQP/GAPDH mRNA amplicon) × 100

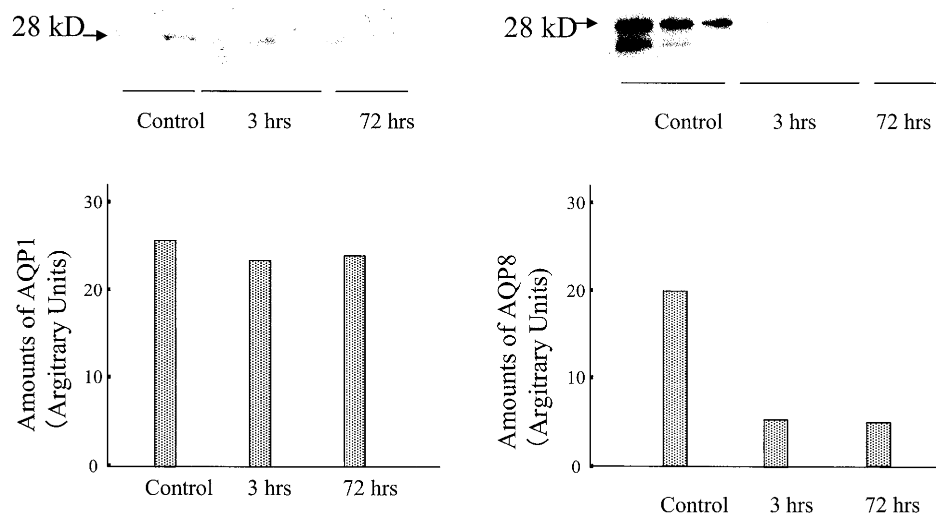

Fig. 2. Representative of the immunoblot for AQPs of the pancreas. **A.** AQP1 (30 μg/*lane*): AQP1 protein levels are not altered in either the three h or 72 h group. **B.** AQP8 (30 μg /*lane*): AQP8 protein levels are significantly lower in both the three or 72 h groups than that in the control group. Densitometric analysis is shown under the immunoblots. The data are expressed in arbitrary units as the means of amounts AQP for four-six animals in each experimental group.

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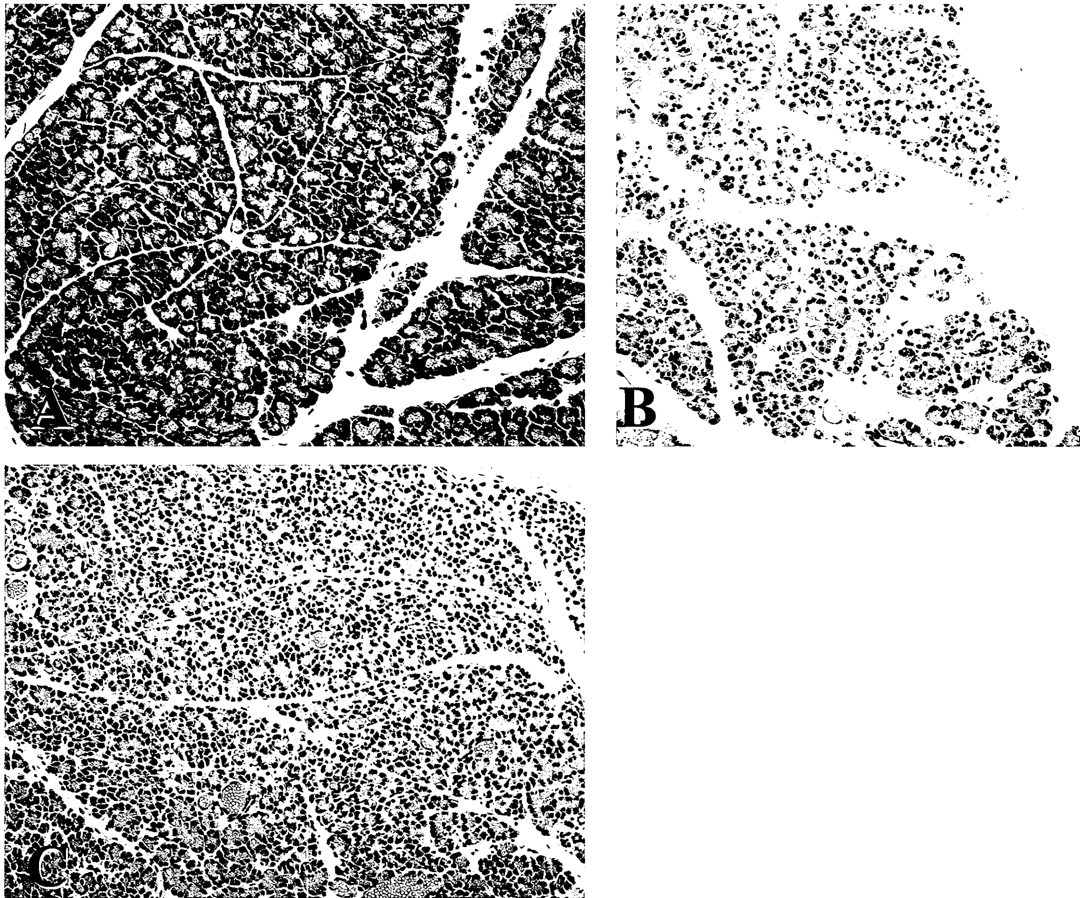

Fig. 3. Hematoxylin and eosin staining (*low magnification*). **A.** Control rat pancreas. × 100 **B.**The pancreas of the three h group showed intestinal edama, lobular mesenchymal rarefaction, and inflammatory cell infiltration. **C.** Evidence of pancreatitis is not detected in the pancreas of the 72 h group.

Immunohistochemistry

For immunohistochemistry, the tissues from the caudal site of the pancreas were isolated and fixed with Methyl-Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid), dehydrated with ethanol, embedded in paraffin, and sectioned at 4 μm. The sections were deparaffinated with xylene and ethanol, hydrated in distilled water, and then blocked with normal goat serum (1:20 dilution) for one h. After rinsing with PBS three times, the slides were incubated with an anti-AQP1 antibody (Chemicon, Temecula, CA, USA) (0.4 μg/ml) and an anti-AQP8 antibody (Alpha Diagnostic, San Antonio, TX) (2.0 μg/ml) for one h at 37°C followed by two nights at 4°C, and then rinsed with PBS three times, incubated with goat anti-rabbit immunoglobulins conjugated to peroxidase labeled polymer (EnVision, DAKO), and colored by diaminobenzidine reaction. After rinsing with distilled water, the slides were counterstained with hematoxylin for observation.

Detection of apoptosis

Apoptotic cells were visualized using the ApopTag® In Situ Oligo Ligation (ISOL) technique (Chemicon, Temecula, CA, USA). The staining procedures were modified based on the manufacturer's instructions. Briefly, after routine deparaffinizaton and rehydration and washing in PBS, slides were put into 3% H_2O_2 for five min and washed with PBS or were then digested with proteinase K for 15 min at room temperature and washed. After adding the equilibration buffer for 10 sec, working strength DNA ligase ezyme was pipetted onto the sections, which were incubated at room temperature for 16 h. After washing in PBS, streptavidin-peroxidase

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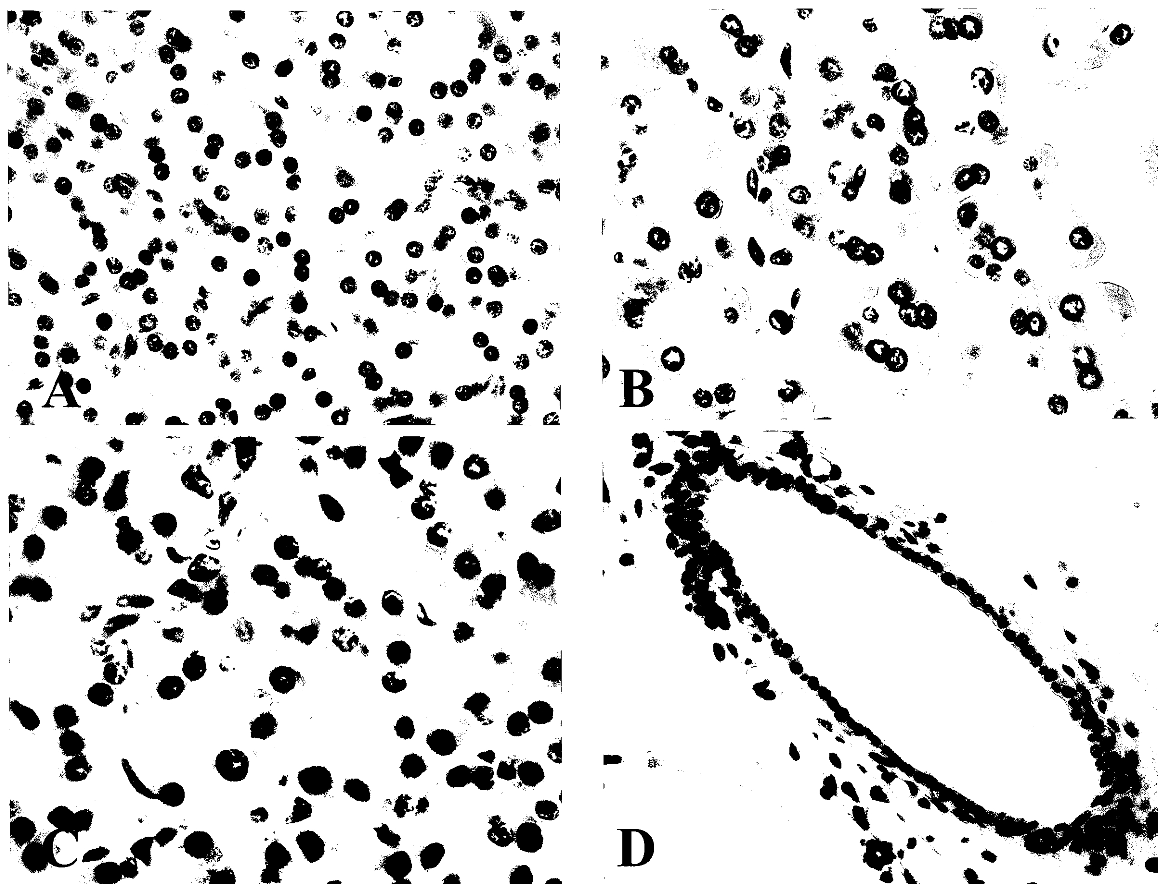

Fig. 4. Hematoxylin and eosin staining (*high magnification*). **A.** Control rat pancreas. × 800 **B.** The pancreas of the three h group shows interstitial edema, inflammatory cell infiltration, prominent swelling of the acinar cells with pyknotic nuclei, and acinar cell vacuolation. × 800 **C.** The pancreas of the 72 h group. × 800 **D.** The pancreatic duct of the three h group. There were no changes observed in the pancreatic ductal cells. × 800

was added to the slides and incubated for 30 min. The slides were washed, then stained with working strength peroxidase substrate, and finally counterstained with methyl green.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U-test. The data were expressed as means ± standard deviation. The statistical significance was defined as $p < 0.05$.

RESULTS

Quantitative RT-PCR

The levels of expression of AQP1 mRNA were 23.9± 21.8 in the control group, 8.6 ± 8.4 in the three h group, and 2.3 ± 2.2 in the 72 h group. The level of AQP1 mRNA in the 72 h group was significantly lower than that in either the control or three h groups ($p = 0.001$, $p = 0.015$, respectively) (Fig.1A).

The levels of expression of AQP8 mRNA were 2.1± 1.5 in the three h group and 1.7±1.1 in the 72 h group. The levels of AQP8 mRNA in the 72 h group were significantly lower than that in the control group (4.7± 3.2 in the control group, $p = 0.029$) (Fig.1B). There was no significant difference between the three h and 72 h groups ($p = 0.546$).

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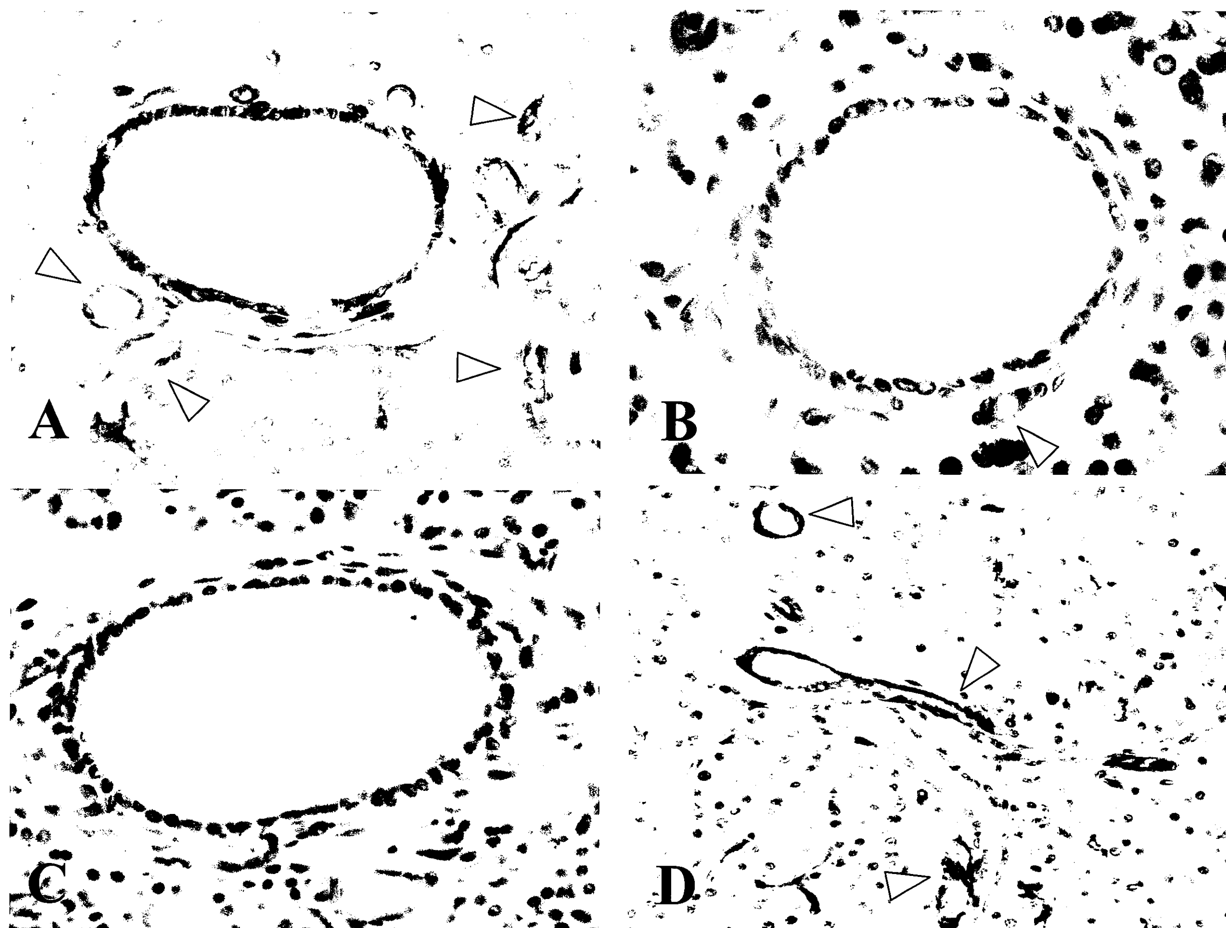

Fig. 5. Immunohistochemistry of AQP1 in the rat pancreas. **A.** AQP1 on the pancreatic ducts is clearly positive in the control rat pancreas, × 400 but negative in three h (**B:** × 400) and 72 h (**C:** × 400) groups. AQP1 staining on the capillary endothelia was positive in all the three groups. *Arrowheads* in **Fig.3A** reveal AQP1 on the capillary endothelia in the control rat. **D.** AQP1 on the capillary endothelia in the 72 h group. × 400 (*arrowheads*, capillary endothelia).

Western blotting

A band at 28 kDa was observed in the AQP1 immunoblot. Densitometry of the bands revealed that AQP1 protein levels were not altered by caerulein infusion (Fig.2A).

In the AQP8 immunoblot, a band at 28 kDa with the glycosylation was observed. Densitometry of the bands revealed that AQP8 protein levels were significantly lower in both the three h and 72 h groups than in the control group (Fig.2B).

Hematoxylin and eosin (HE) staining

In the control group, morphology of the pancreas was normal, the lobule structure existed, and mesenchyma was clear (Fig.3A and 4A). In the pancreas of the three h group, lobular mesenchymal rarefaction, edema, and inflammatory cell infiltration were observed at low magnification (Fig.3B). At high magnification, prominent swelling of the acinar cells with pyknotic nuclei and acinar cell vacuolation were detected (Fig.4B). These microscopic features of acute pancreatitis had been disappeared in the 72 h group (Fig.3C and 4C). There were no changes observed in the pancreatic ductal cells resulting from caerulein infusion (Fig.4D).

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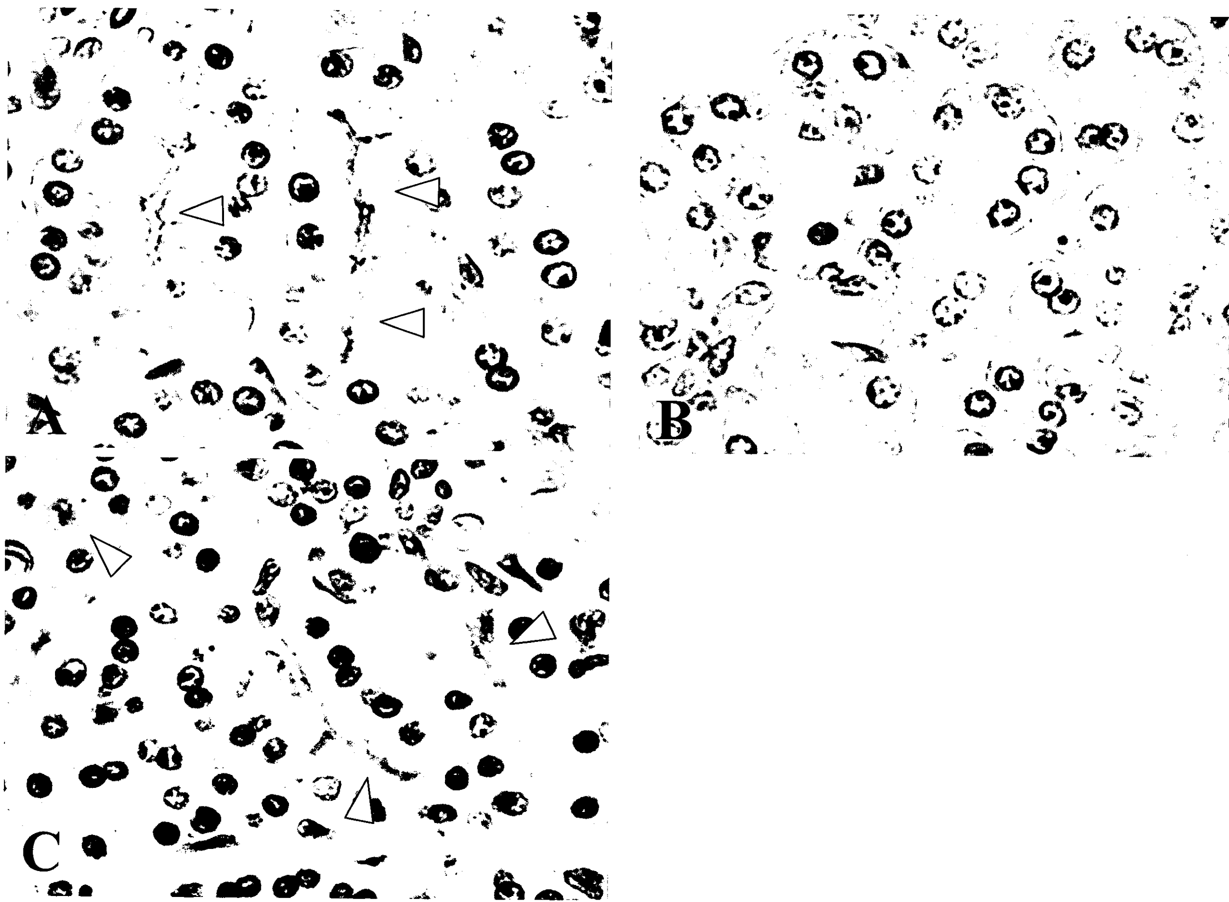

Fig. 6. Immunohistochemistry of APQ8 in the pancreas. **A.** AQP8 was positive on the apical membrane of acinar cells in the normal pancreas. (control rat pancreas, × 800) **B.** AQP8 staining has disappeared in the three h group. × 800 **C.** AQP8 staining is partly observed in the 72 h group. × 800 (*arrowheads*, membrane of the acinar cells).

Immunohistochemistry

Immunohistochemistry for AQP1: In the control group, the capillary endothelia and the pancreatic ductal cells were positive for AQP1 immunoreactivity. AQP1 in the ductal cells disappeared in the three h and 72 h groups although HE staining did not show any changes in the ductal cells (Fig.5B and C). AQP1 expression in the ductal cells was reduced by caerulein infusion, and the damage was not restored — even in the recovery phase. In contrast, the AQP1 that was strongly expressed in the capillary endothelia was not impaired in the caerulein treated groups (Fig.5D). There was no immunoreactivity in the islet cells or the acinar cells for the AQP1 antibody (Fig.5A).

Immunohistochemistry for AQP8: AQP8 was observed at the apical membrane of pancreatic acinar cells in the control group (Fig.6A). Almost all the acinar cell membranes were stained in the control group. In the three h group, the positivity for AQP8 in the acinar cell membranes disappeared (Fig.6B). In the 72 h group, AQP8 staining was focally observed, and the percentage of AQP8 labeled acinar cells was approximately 40% (Fig.6C). The decrease in AQP8 in the recovery phase continued although swelling of the acinar cells was restored by histology.

Staining for detection of apoptosis

A number of apoptotic acinar cells were observed in the three h group (Fig.7B), while such was not seen in either

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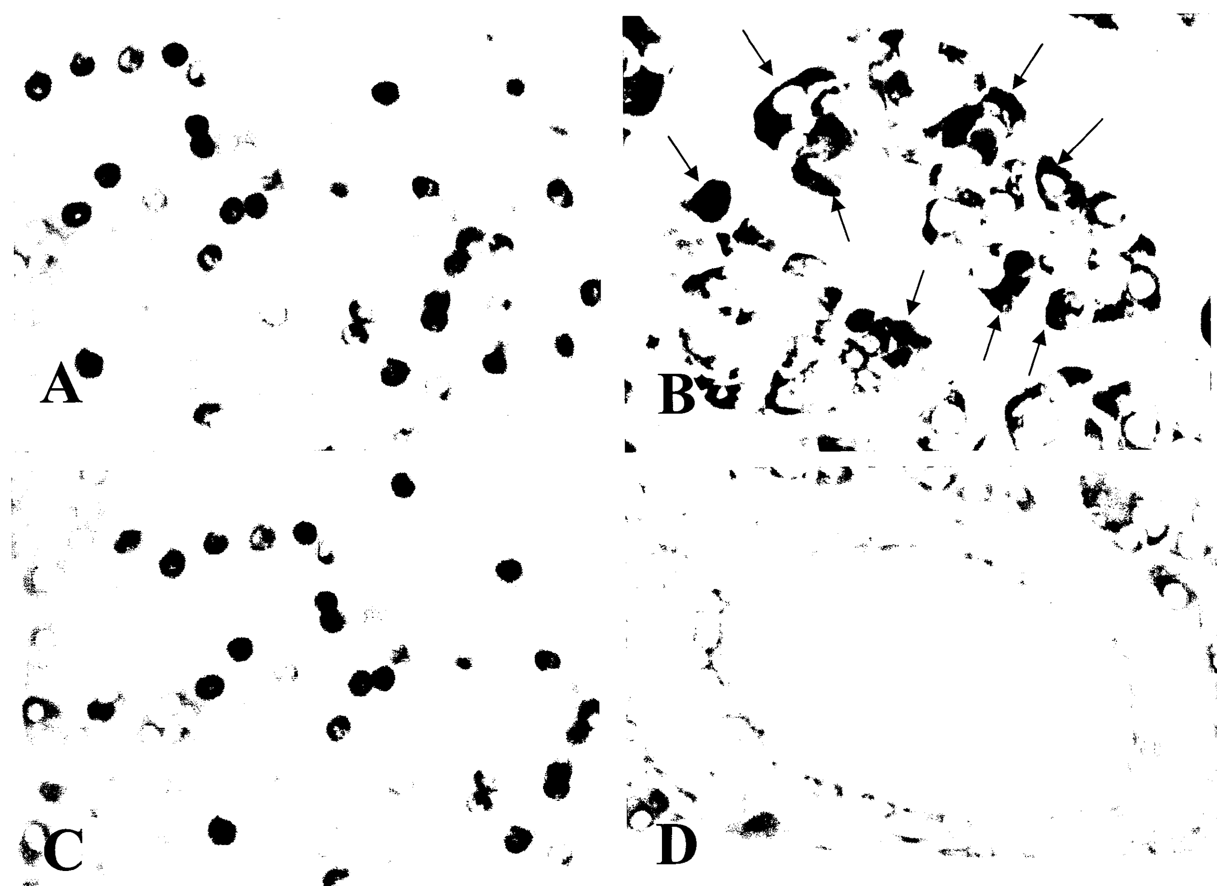

Fig. 7. Detection of apoptotic cells in the rat pancreas using ApopTag® ISOL Kit. **A.** Apoptotic cells were not detected in the acinar cells in the normal pancreas. (control rat pancreas, × 400) **B.** Apoptosis was diffusely observed in the acinar cells in the three group (three h group rat pancreas, × 800) (*arrows*, apoptotic cells). **C.** Apoptotic cells were not detected in the acinar cells in the 72 h group (72 h group rat pancreas, × 400). **D.** The pancreatic duct of the three h group: There are no apoptotic cells observed in the pancreatic ductal cells. × 800

the control or 72 h groups (Fig.7A and C). There were no apoptotic cells observed in the ductal cells (Fig.7D).

DISCUSSION

It has been reported that AQP plays a major role in water permeability and transport in the cell membranes in several abnormal conditions [6,9,18-21] or diseases.[22,23,24] The caerulein-induced pancreatitis model, in which acute edematous changes occur in the acinar cells and the interstitial tissues, has been well established. In this model, acute pancreatitis is characterized by an imbalance of digestive enzyme segregation, transport, exocytosis, and activation. In general, supramaximal caerulein stimulation causes interstitial edema and cytoplasmic vacuoles in the acinar cells, but these histological changes are reversible within 48 h.[2,5] However, the dynamics of AQPs in the caerulein induced pancreatitis model has not been elucidated. From a viewpoint of edema, several reports have mentioned that a relationship exists between AQP and edema, for both pulmonary edema and brain edema.[25-28] Especially in the brain, AQP1 is mainly expressed in the chorioid plexus and AQP4 in the astrocyte endfeet and the ependymal cells. In rats, AQP4 is highly expressed in the astrocyte endfeet that face blood vessels, and AQP4 expression is decreased in perivascular astrocytic processes in the contused brain with acute global ischemia.[28, 29] Kobayashi et al. noticed that the decrease in AQP4 expression on the perivascular membrane might be the cause of the edema formation, by reducing the water transport across the

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perivascular membrane into the vascular lumen.[28] In our study, AQP8 expression in the acinar cell membrane was apparently decreased in the acute pancreatitis phase in which prominent swelling of acinar cells was observed. Similar to the AQP4 in the brain, the decrease in AQP8 seemed to reduce the water transport from acinar cells into the vascular lumen. In addition, the decrease in the expression of AQP8 continued even when the acinar cell swelling was restored in HE staining in the recovery phase. Therefore, the recovery of AQP8 in the acinar cell membrane is slower than that from the morphological change.

In this study, there was a discrepancy between AQP mRNA and protein levels. AQP1 mRNA expressions decreased during the pancreatitis phase; the protein levels were stable in all three phases in Western blotting. AQP8 m RNA expressions also decreased during the pancreatitis phase, but the immunohistochemistry showed the expression of AQP8 partially recovered 72h after the induction of pancreatitis. Sato et al. also reported a discrepancy between the expression of mRNA and protein of AQP in pulmonary edema in rat lungs.[25] They considered that it could be related to the difference in the stability and turnover between AQP mRNA and proteins. Several studies investigated the stability and degradation of AQP.[30,31,32,33] Leight et al. revealed that the half-life of the AQP1 protein was markedly increased by the exposure of cells to a hypertonic medium.[30] Although the turnover and half-life of AQP in the pancreas are not yet known, AQP mRNA might be rapidly degraded by caerulein infusion compared with AQP protein. In the present study, the discrepancy between AQP1 mRNA expression and protein levels in Western blotting might also be related to the differences in turnover and half-life.

The features of acute pancreatitis for HE staining of the pancreas restored after 72 h caerulein infusion and apoptosis was detected only in the acinar cells in the three h group. However, the decrease in AQP1 and AQP8 mRNA and protein expression continued 72 h after caerulein injection. Several studies have documented that the caerulein-induced pancreatitis model was characterized by acinar cell injury.[3,34,35,36] Acinar cell apoptosis in the models is recognized as a common event in pancreatic pathology.[36,37] In our study, AQP8 expression never recovered completely in the recovery phase even though there was morphological evidences. This result indicate that the latent damage of the acinar cell membrane. On the other hand, immunohistochemistry showed that AQP1 on pancreatic ducts was not stained, in both acute pancreatitis and the recovery phase. However, apoptosis was not detected in the ductal cells in either phase in our study. Kimura et al. also reported that the damage induced by caerulein was observed selectively in acinar cells but was not shown in ductal epithelial cells or islet cells in the model.[38] Contrary to this, our study revealed that caerulein impaired AQP expressions not only in the acinar cells but also in the pancreatic ductal epithelial cells. The decrease in the AQP1 on the pancreatic duct was prolonged although the damage did not involve the duct cell death. This suggests that a latent injury of the duct cells can persist even though the features of acute pancreatitis for the HE staining are restored. The decrease in AQP1 on the pancreatic duct indicate that the ductular network is destroyed with pancreatitis, contributing partialy to exocrine insufficiency.

We have shown the dynamics of AQP1 and AQP8 in caerulein-induced pancreatitis. AQP8 was damaged by caerulein, which is likely to be related to the swelling of the acinar cells. The decrease in both AQP1 in the pancreatic duct cells and AQP8 in the acinar cells can play a part in the mechanisms of edematous pancreatitis.

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