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Morphological changes in the rat exocrine pancreas after pancreatic duct ligation

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Summary. In the present study, morphological changes of the exocrine pancreas in rats after pancreatic duct ligation were examined with light microscopy (hematoxylin-eosin, TUNEL, and PCNA staining) and scanning electron microscopy in order to elucidate the effects of increased pancreatic duct pressure. On the fifth day after pancreatic duct ligation, ductular proliferation, periductal fibrosis, and disappearance of acini were obserbed. TUNEL and PCNA staining demonstrated many apoptotic acinar cells and proliferating ductal cells immediately after ligation, which reached a maximal number on the 2nd or 3rd day. Tortuous or helical interlobular pancreatic ducts with inner surfaces containing many crater-like depressions and long cilia were found after ligation. These changes were almost identical to those observed in the pancreatic tissue of model chronic pancreatitis rats, WBN/Kob rats, and stroke-prone spontaneously hypertensive (SHRSP) rats.

In summary, the morphological changes observed after pancreatic duct ligation were similar to those of chronic pancreatitis, therefore, the characteristic changes of pancreatic ducts observed in chronic pancreatitis may be caused by increased pancreatic duct pressure.

Key words: Pancreatic duct ligation, Scanning electron microscopy, Immunohistochemistory, Morphological change

Introduction

The morphological characteristics of chronic pancreatitis are an irregular tortuous dilatation of pancreatic ducts as well as the loss of acinar cells. Although these changes, as demonstrated by endoscopic retrograde pancreatography, are used as a diagnostic marker of chronic pancreatitis in humans, the pathogenetic mechanisms still remain unclear. Hydrostatic pressure in the main pancreatic duct is reported to be increased in patients with chronic pancreatitis (Okazaki et al., 1988). Therefore, morphological changes of the pancreatic ductal system and increased pressure in the pancreatic duct may have a causal relationship. This study is designed to investigate morphological changes of the exocrine pancreas after pancreatic duct ligation.

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Materials and methods

Animal preparation

Twelve-week-old male Wistar rats were used in this study. They were allowed to eat commercially available chow (CRF-1; Oriental Yeast Co., Tokyo, Japan) and drink water ad libitum, before and after surgery. The rats were divided into two groups.

A pancreatic duct ligation was performed on one group of rats (PDL group, n=8). They were laparotomized along the midline under anesthesia induced by intraperitoneal injection of sodium pentobarbital (4mg/100g body weight). The common hepatic duct was ligated proximal to its entry into the pancreas and the common bile-pancreatic duct was also ligated near its junction with the duodenum, after which the abdomen was closed. Another group of rats (n=4) were sham-operated (SH group). They were laparotomized using the same method as the PDL group, and the pancreas was exposed and then rubbed with fingertips for a few minutes. One, two, three, and five days after the operation, three groups of rats were killed under anesthesia induced by diethyl ether inhalation. After a laparotomy, the diameter of the common bile-pancreatic duct was measured.

Preparation of pancreatic tissue for histological and immunohistochemical studies

The whole pancreas was removed and weighed. Pancreatic tissue specimens to be used for light microscopic (LM) and immunohistochemical examinations were obtained from the splenic lobes close to the spleen and fixed in a 10% formaldehyde solution,

followed by embedding in paraffin. Paraffin sections (4 μm thick) were subjected to hematoxylin and eosin (H&E) staining, fluorescein-labeled terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method (TUNEL) staining, and proliferating cell nuclear antigen (PCNA) staining.

Detection of apoptotic cells

After rehydration, the sections were treated with 20 mg/ml proteinase K (Code 9033; TakaRa, Kyoto, Japan) for 1 hour at room temperature. They were then incubated with 3% hydrogen peroxide diluted in phosphate buffer saline (PBS) to neutralize endogenous peroxidase activity, followed by treatment with terminal deoxynucleotidyl transferase (TdT enzyme; TakaRa Code MK502) and biotinylated digoxigeninuridine triphosphate (dUTP) for 60 minutes at 37 °C in a moist chamber. To visualize the nick end-labeling with a light microscope, the sections were treated with 70 μ/ml Anti-FITC HRP Conjugate (Code MK 503; TakaRa, Kyoto, Japan) for 30 minutes and then incubated with diaminobenzidine-tetrahydrochrloride (DAKO, Denmark) as the substrate after rinsing in PBS three times.

Detection of proliferating cells

Mouse anti-rat PCNA monoclonal antibodies (clone 5A10; MBL Co., Nagoya, Japan) were used to detect proliferating nuclei. After rehydration, the sections were incubated with 1% hydrogen peroxide diluted in PBS to neutralize endogenous peroxidase activity, before an overnight incubation at 4 °C with anti-PCNA antibody diluted 1:100 in PBS. Bound antibody was detected with biotinylated goat anti-mouse immunoglobulin G second antibody (Dakopatts, Denmark) and streptavidin-peroxidase complex, followed by incubation with diaminobenzidine-tetrahydrochloride (0.03%) as the substrate.

Preparation of pancreatic tissue for scanning electron microscopic studies

Rats were anesthetized with sodium pentobarbital and perfused trans-aortically with a physiological saline solution, and then with 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4. The pancreatic tissue specimens were then obtained from splenic lobes close to the spleen. Conventionally fixed pancreatic tissue specimens to be used for scanning electron microscopic (SEM) examination were prepared as previously described (Ashizawa et al., 1991).

Scanning electron microscopic examination of pancreatic ducts

After drawing pancreatic juice from the common bile-pancreatic ducts, corrosion casts of the pancreatic ducts were prepared as previously described (Ashizawa et al., 1991). Using a SEM (Hitachi S-800, Hitachi: Co. Ltd., Tokyo Japan), the corrosion casts and pancreatic ductal inner surfaces in conventionally fixed specimens were examined, with acceleration voltages of 5kV and 20kV, respectively.

Quantitation of labeling

In each tissue section, PCNA indices and Apoptotic indices (detected by TUNEL) were determined in the interlobular duct cells and intralobular ductular cells and in the acinar cells. Each PCNA index was calculated by counting the number of positive cells in epithelial cells of the interlobular and intralobular pancreatic duct in at least 10 randomly selected fields (magnification: x400) and expressed as a percentage. Apoptotic index was calculated by counting the number of positive cells among 1,000 cells distributed in at least 10 randomly selected fields (magnification: x400).

Statistical analysis

The results obtained were processed statistically and differences observed were compared using analysis of variance, followed by Mann-Whitney U test. $P<0.05$ was considered to be statistically significant difference.

Results

General appearance of pancreatic tissue

Marked swelling of pancreatic tissue was found on the 1st and 2nd experimental days after ligation in the PDL. In the SH group, on the other hand, there was no such pathological change found. Swelling began to decrease from the 3rd day and those with markedly dilated pancreatic ducts became atrophic on the 5th day (Fig. 1). In the PDL, marked dilated common bile-pancreatic ducts were found and their diameter increased in a time-dependent manner after ligation (Fig. 2).

Light microscopic examination of pancreatic tissue

In the PDL group, infiltration by inflammatory cells, interstitial edema, and dilatation of the pancreatic ducts were found on the 1st day after ligation. On the 2nd day, dilated interlobular ducts with a bead-like deformity were observed (Fig. 3A). On the 5th day, a tubular complex resulting from the deletion of pancreatic acini and a proliferation of pancreatic ductules was found (Fig. 3B). No massive necrosis or hemorrhaging was seen at any time point after the ligation. Rats in the SH group showed no pathological change in pancreatic tissue (data not shown).

Immunohistochemical detection of apoptotic cells

Many apoptotic cells (detected by TUNEL) were

Table 1. Time course changes of TUNEL and PCNA positive cells after pancreatic duct ligation.

	1 DAY (after ligation)	2 DAYS (after ligation)	3 DAYS (after ligation)	5 DAYS (after ligation)
TUNEL positive acina cells (%)				
SH group	1.3±0.5	1.2±0.5	1.1±0.4	1.2±0.5
PDL group	3.2±0.5*	8.0±1.6*	11.0±0.8*	3.2±0.9*
PCNA positive interlobular duct cells (%)				
SH group	6.0±0.4	7.2±1.5	4.4±0.8*	3.1±0.4
PDL group	55.5±5.4*	77.1±8.2*	79.6±9.0*	4.5±1.7
PCNA positive intralobular ductular cells (%(				
SH group	7.3±1.1	6.7±0.8	5.3±0.6	5.6±0.6
PDL group	36.1±6.4*	41.6±5.2*	49.0±8.2*	31.6±9.9*

found in acini as early as the 1st day after ligation, and their number increased and reached a maximum two to three days after the start of the experiment (Fig. 4). On the 5th experimental day, there were scarcely any apoptotic cells detected in the pancreatic lobules. In ductal and ductular cells, no TUNEL-positive apoptotic cells were found. In the SH group, scarcely any apoptotic cells were detected at any time, not only among ductal and ductular cells but also acinar cells (Table 1).

Immunohistochemical detection of proliferating cells

After ligation, many PCNA-positive proliferating cells were detected in the interlobular pancreatic ducts as well as in the intralobular ductules, though the time-course changes in the appearance of proliferating cells in these two areas were different. In the interlobular ducts, many PCNA-positive nuclei appeared one day after ligation and more nuclei became positive by the 3rd day (Fig. 5A). On the 5th day, proliferating PCNA-positive nuclei were decreased and only a few nuclei showed positive staining (Fig. 5B).

In intralobular ductules, the number of PCNA-positive cells began to increase as early as the 1st day and many proliferating cells were still found five days after ligation of the pancreatic ducts. No proliferating acinar cells were found throughout the duration of the experiment.

In the SH group, only a few PCNA-positive cells were found in interlobular ducts and intralobular

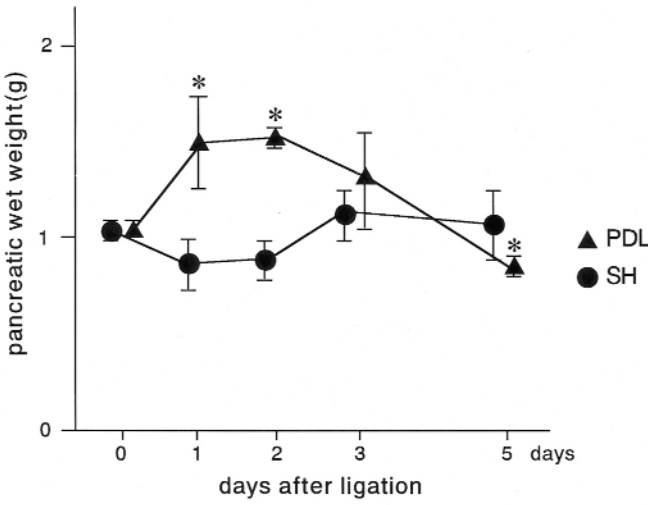


Fig. 1. In the PDL group, swelling of pancreatic tissue is revealed by the increased weight of the pancreas. Vertical lines represent means±standard deviation (SD). *p<0.05, significantly different from SH group; triangles: PDL; Circles: SH.

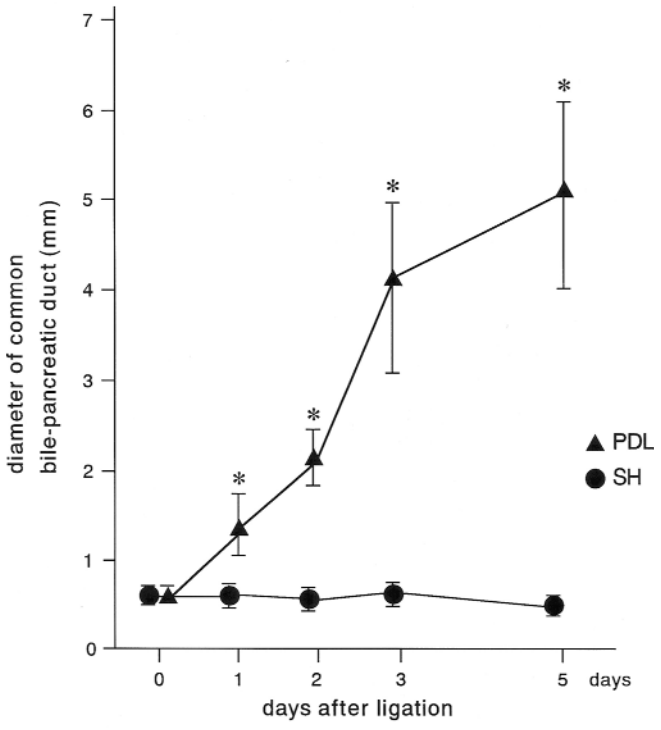


Fig. 2. Dilated common bile-pancreatic ducts are found and their diameter increases in a time-dependent manner following pancreatic duct ligation. Vertical lines represent means±standard deviation (SD). *p<0.05, significantly different from SH group; triangles: PDL; Circles: SH.

ductules at any time of the experimental period (Table 1).

Scanning electron microscopic examination of pancreatic duct corrosion casts

In all the SH group rats, the pancreatic duct system had a tree-like structure and all of the ducts were cylindrical in shape (Fig. 6).

In the PDL group, resin injected into the dilated pancreatic ducts easily leaked out because of the vulnerability of the ducts. Several portions of the corrosion casts of interlobular ducts obtained from the pancreas two days after ligation showed tortuous ductal channels with helical deformity and many luminal blebs, especially in the small interlobular ducts (Fig. 7A,B).

Scanning electron microscopic examination of the interlobular duct inner surfaces

In the SH group, the interlobular ducts were cylindrical in shape and their smooth inner surfaces had microvilli and short cilia (Fig. 8A,B).

In the PDL group, the inner surfaces of the interlobular ducts showed dynamic morphological changes after pancreatic duct ligation. In most, small

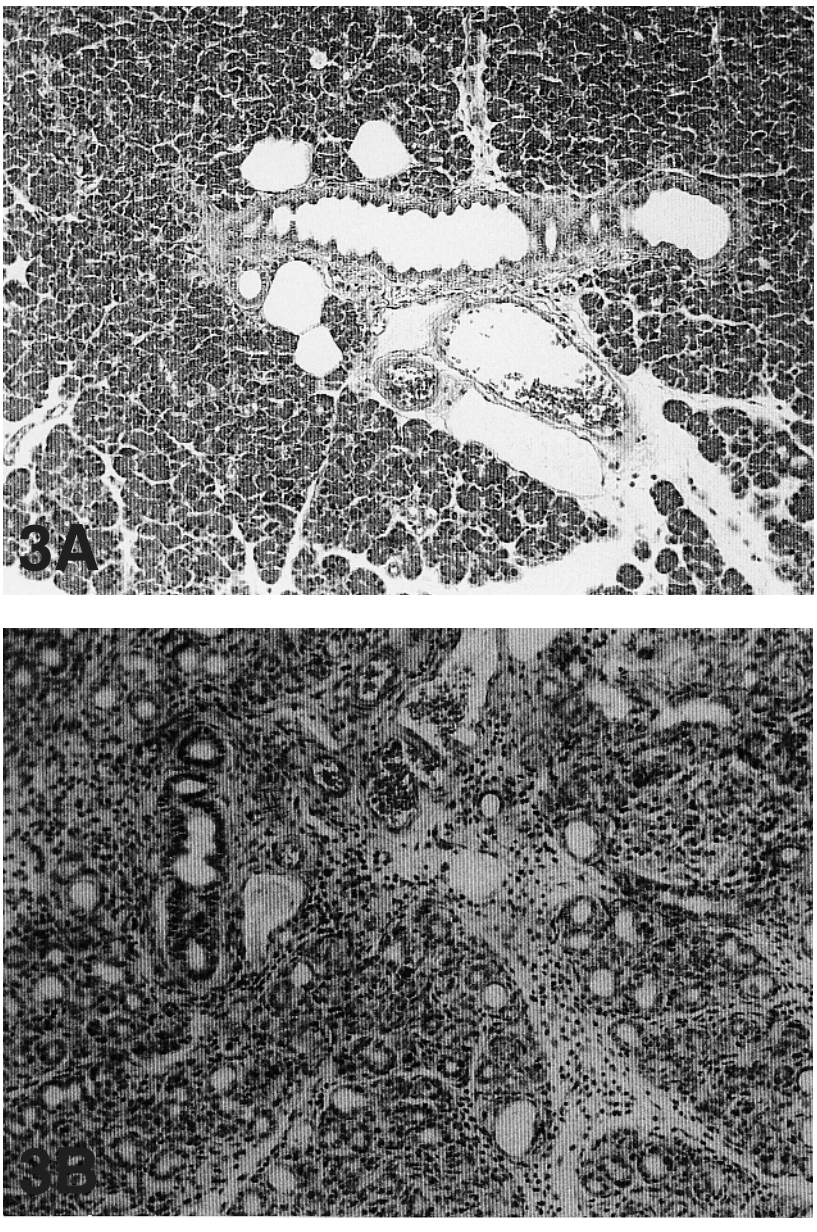


Fig. 3. Light microscopic view of pancreatic tissue after ligation, stained with hematoxylin and eosin. **A.** Two days after ligation, dilatation of central lumina and bead-like deformity of a dilated interlobular duct with periductal fibrosis are shown. **B.** Five days after ligation, note the deletion of acini and ductular proliferation. x 100

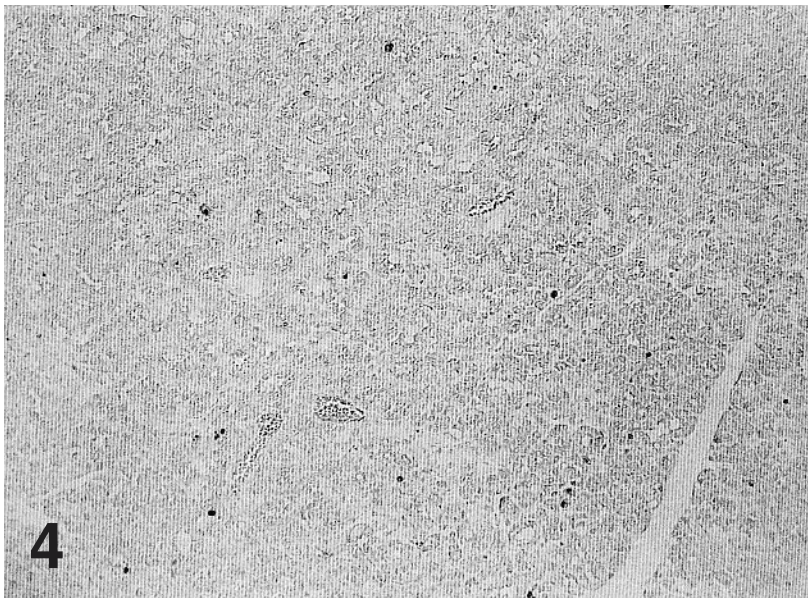
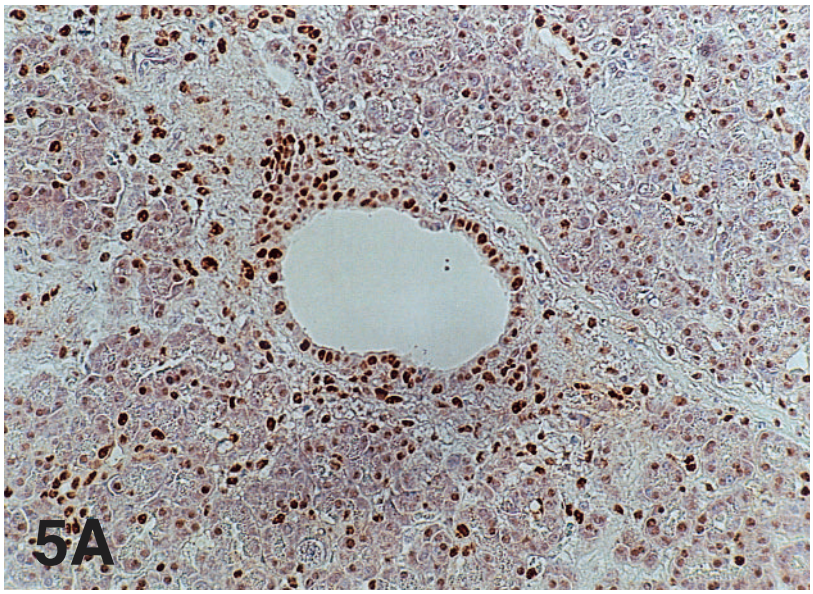


Fig. 4. Light microscopic view of pancreatic tissue stained using the TUNEL method. Two days after ligation, many TUNEL positive acinar nuclei are found. x 200



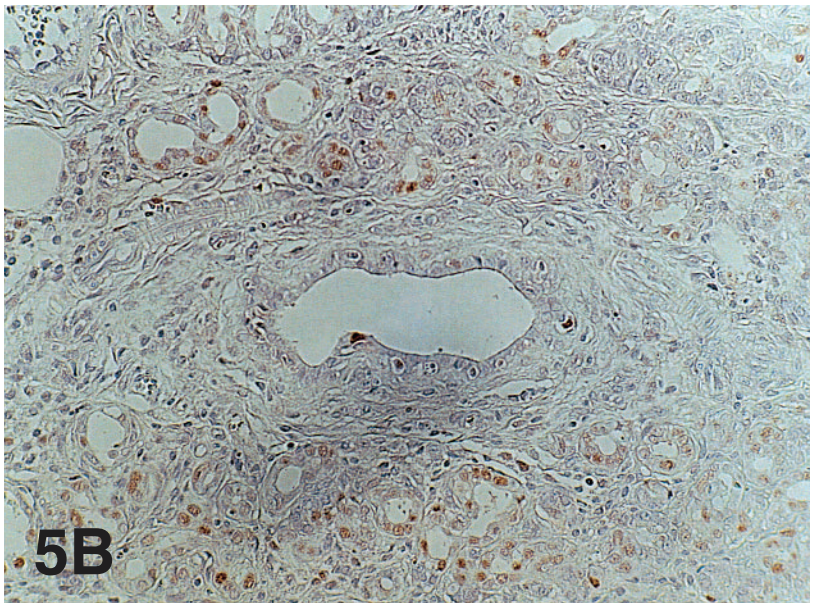
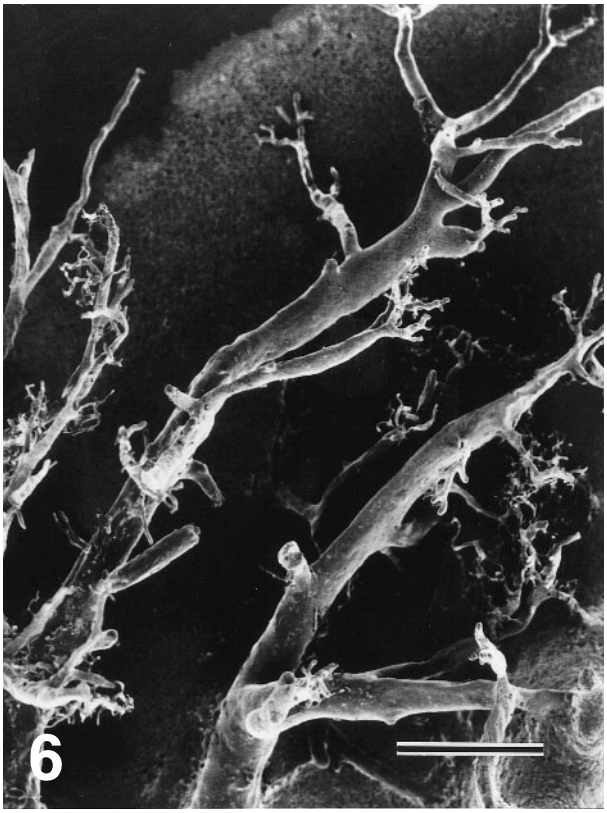


Fig. 5. Light microscopic view of pancreatic tissue stained with PCNA antibody. **A.** Three days after ligation, most interlobular duct cells have PCNA-positive nuclei, and there are some PCNA-positive nuclei in the intralobular regions. **B.** Five days after ligation, there are few PCNA-positive nuclei in the interlobular duct cells, and many PCNA-positive nuclei are seen in the intralobular regions containing ductular proliferation. x 200



shallow depressions from which long cilia originated were observed on the inner surface one day after ligation. In small ducts with a diameter of less than 30 μm, crater-like deep depressions were found on the 2nd day (Fig. 9). On the other hand, large interlobular ducts over 80 μm in diameter showed different time course changes. On the 2nd and 3rd day after ligation, the apical surfaces of their epithelial cells showed a bulge with a long cilium (Fig. 10). Five days after ligation, the bulges disappeared and crater-like deep depressions with long cilia were observed (Fig. 11).

Discussion

In the present study, proportional increases in the diameter of common bile-pancreatic ducts following ligation were seen as a result of increased hydrostatic pressure, which clarified the dramatic morphological

Fig. 6. SEM view of a pancreatic duct corrosion cast two days after sham operation. The pancreatic duct system has a tree-like shape and every pancreatic ductal lumen is nearly cylindric. The corrosion cast of small pancreatic duct demonstrate smooth inner surface. x 50, bar: 300 μm.

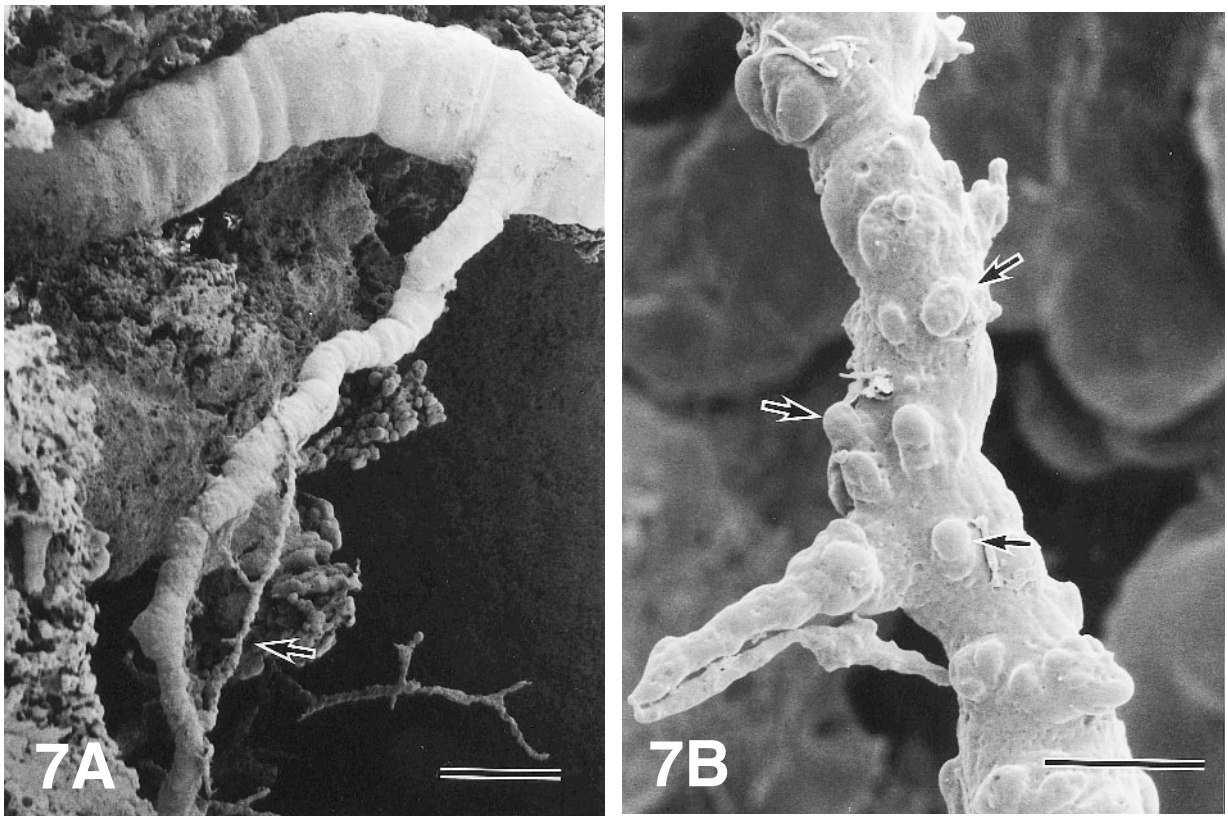


Fig. 7. SEM views of a pancreatic duct corrosion cast two days after ligation. **A.** Interlobular ducts showing tortuous ductal channels or helical deformity. x 100, bar: 150 μm. **B.** Higher magnification of the arrow in A. Small pancreatic duct (approximately 10 μm in diameter) has many luminal blebs (arrows). x 2,000, bar: 10 μm.

changes of pancreatic tissue that occur after increased pancreatic duct pressure. These changes can be divided into those in the pancreatic acini and those in the ducts. In the acini, many acinar cells become apoptotic with a resulting decrease in acinar cells. Interlobular or intralobular ductules, on the other hand, had many proliferating cells detected by PCNA immunostaining, though PCNA might be involved in DNA repair synthesis in some duct cells (Celis et al., 1987; Zuber et al., 1989; Nichols and Sancar, 1992; Shivji et al., 1992; Wada et al., 1997). Since we found a proliferation of intralobular ductules along with apoptotic changes of acinar cells, the number of ductular cells in the lobules increased in comparison with that of acinar cells after ligation of the pancreatic ducts.

Our results were almost the same as in previous reports for the immunohistochemical detection of apoptotic cells and proliferating cells (Kaiser et al., 1995; Doi et al., 1997; Wada et al., 1997). Since proteolytic and lipolytic enzymes are abundantly present in acinar cells and pancreatic juice, leakage of pancreatic juice out of the ductal system damages pancreatic acinar cells and vascular systems. Injured necrotic acinar cells may further damage surrounding tissue, because of the leakage of enzymes and subsequent activation. The spaces between the intra-acinar secretory canaliculi and the basement membrane are especially vulnerable to leakage of pancreatic juice when intraductal pressure is elevated. The decreased production of pancreatic juice and decreased vulnerable portion caused by apoptosis in acinar cells may be a pancreatic tissue self-defense system. Stimulated proliferation of duct cells may also be a defense against high pancreatic duct pressure.

Interlobular ducts showed three kinds of morphological changes after ligation. The most prominent change was a helical deformity of the pancreatic ducts that was accompanied by bulges in the apical epithelial surfaces of the large interlobular ducts. Since these two morphological changes are associated with the remarkable increase of proliferating cells in ductal epithelium, they may be necessary to accommodate the increased number of epithelial cells on the ductal surface (Garrett et al., 1973). The third morphological change after pancreatic duct ligation was observed on the inner surfaces of the interlobular ducts. On the apical surface of the epithelium, many crater-like depressions and long cilia had developed, though the causative mechanism is unclear. These three morphological changes, helical deformity of the ducts, bulges on the apical surfaces of the epithelial cells in

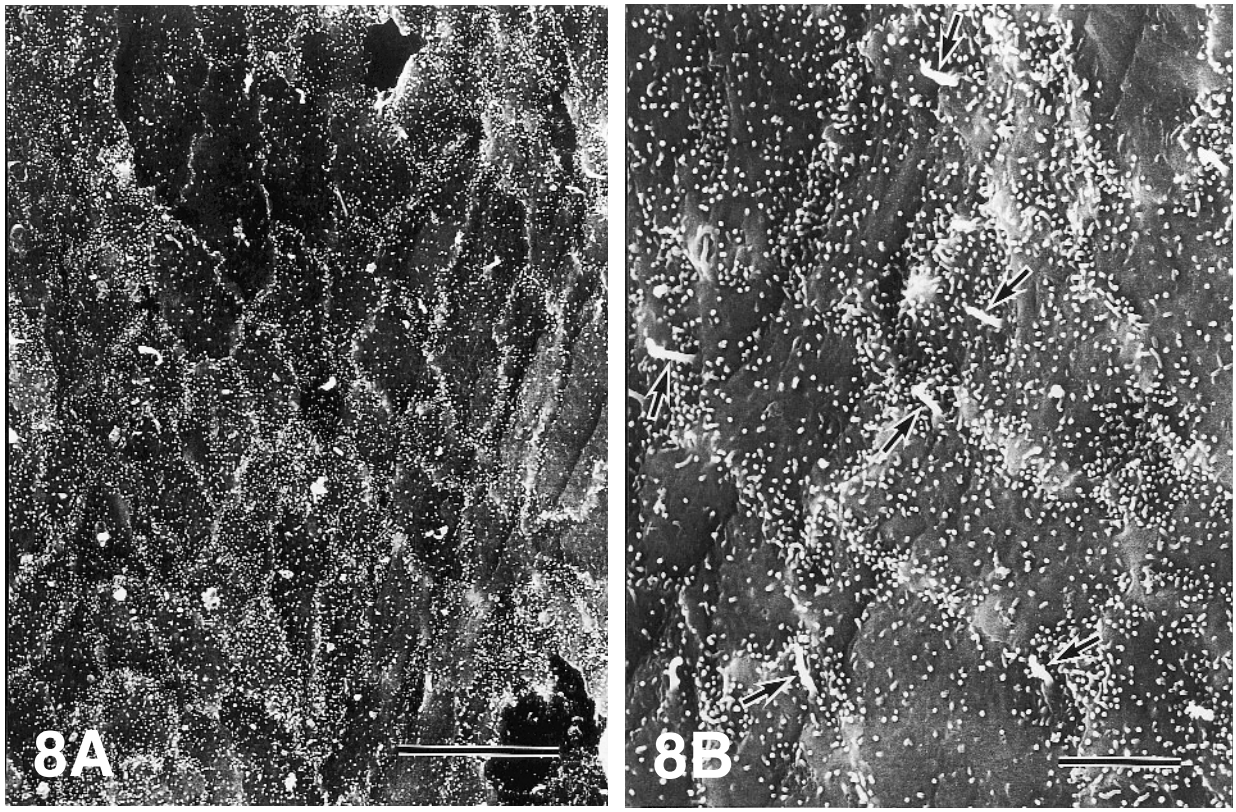


Fig. 8. SEM view of the inner surface of a large interlobular duct (approximately 100 mm in diameter) in conventionally-fixed pancreatic tissue at two days after sham operation. **A.** Smooth inner surface showing microvilli, short cilia, and a polygonal pattern, which is seen along the epithelial cell boundaries. Original magnification: x 2,000, bar: 10 μm. **B.** Higher magnification of A. Each epithelial cell has a single cilium (arrow). x 5,000, bar: 3 μm.

large interlobular ducts, and formation of crater-like depressions and long cilia, have also been observed in the pancreatic interlobular ducts of WBN/Kob rats and SHRSP (Ashizawa et al., 1995, 1997, 1999; Hidaka et al., 1995). These two strains are reported to develop pancreatic fibrosis and are used as models of chronic pancreatitis. We previously found these morphological changes in the pancreatic interlobular ducts of these model rats and reported them as a characteristic change of chronic pancreatitis. In the present experiment, these three morphological changes could be observed as early as several days after pancreatic duct ligation. They may be adaptive changes by the pancreatic tissue to a pathological increase in intra-pancreatic duct pressure.

In summary, we clarified that ligation of the pancreatic duct caused proliferation of intralobular

Fig. 9. SEM view of the inner surface of a small interlobular duct (approximately 17 μm in diameter) in conventionally-fixed pancreatic tissue at two days after ligation. Note the deep crater-like depressions (arrows) and long cilium in the inner surface with helical deformity. These crater-like depressions correspond to the luminal blebs in Fig. 7B. x 2,000, bar: 10 μm.

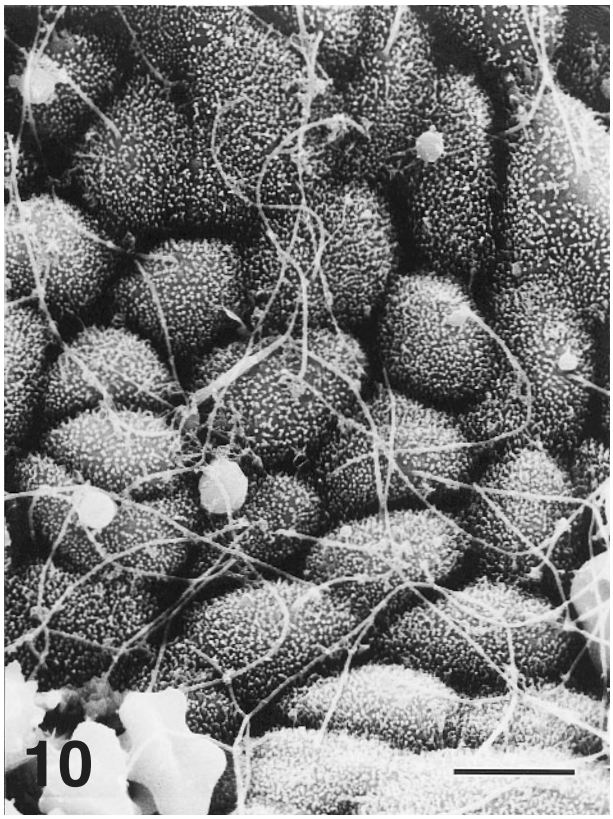


Fig. 10. SEM view of the inner surface of a large interlobular duct (approximately 85 μm in diameter) in conventionally-fixed pancreatic tissue two days after ligation. Each epithelial cell has a bulging apical surface with a long cilium. x 3,000, bar=5 μm.

Fig. 11. SEM view of the inner surface of a large interlobular duct (approximately 80 μm in diameter) in conventionally-fixed pancreatic tissue five days after ligation. There is no epithelial cell with a bulging apical surface seen. Note the deep crater-like depressions (large arrows) and long cilia (small arrows). x 3,000, bar: 5 μm.

ductules, helical deformity of the interlobular ducts, bulging of the apical epithelial surfaces of the large interlobular ducts, and crater-like depressions and long cilia on the apical epithelial surfaces of the pancreatic ducts. These morphological changes are also found in chronic pancreatitis model rats and increased intra-ductal pressure may be a causative factor.

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