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A New Model for Pancreaticobiliary Maljunction without Bile Duct Dilatation: Demonstration of Cell Proliferation in the Gallbladder Epithelium

胆管拡張を伴わない膵胆管合流異常の新しいモデル：胆嚢上皮における細胞増殖の実証

Running Title: Pancreaticobiliary maljunction

Key Words: Choledochal cyst, Bile ducts, Pancreatic ducts, Gallbladder neoplasms, Cell cycle, PCNA

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Abstract

Patients with pancreaticobiliary maljunction without bile duct dilatation develop gallbladder carcinoma frequently. No models of pancreaticobiliary maljunction without bile duct dilatation exist, and no previous studies have clearly demonstrated changes in the gallbladder relating to carcinogenesis. We, therefore, examined the cell kinetics of the gallbladder epithelium in a new experimental model of pancreaticobiliary maljunction without bile duct dilatation. A cat model was produced by performing choledocho-pancreatic side-to-side ductal anastomosis in nine animals. Five cats, in which the choledocho-pancreatic ducts were exposed only, served as controls. After 6 months, the gallbladders of these cats were removed and stained with anti-proliferating cell nuclear antigen (PCNA) antibody (PC10, Dako). The labeling index (LI) was determined from the percentage of positive nuclei in three microscopic fields. The diameter of the common bile duct was not different between the models and the controls. In the models, the number of PCNA positive cells was significantly increased. The mean (± standard deviation) PCNA LI was 28.1 ± 12.2% in the models and 4.3 ± 1.6% in the controls ($p<0.01$). These studies clearly indicate that this model is suitable for studying pancreaticobiliary maljunction without bile duct dilatation and that pancreaticobiliary maljunction has a prominent proliferating effect on the gallbladder epithelium, which may play an important role in gallbladder carcinogenesis.

Introduction

Pancreaticobiliary maljunction, an anomalous arrangement of the pancreaticobiliary ductal system, is a congenital anomaly defined as the union of the pancreatic and biliary ducts, outside of the duodenal wall. Consequently, the sphincter of Oddi fails to prevent mutual reflux of bile and pancreatic juice into the pancreatic and bile ducts. Despite the knowledge that a majority of patients with choledochal cysts have pancreaticobiliary maljunction, some patients with pancreaticobiliary maljunction do not have bile duct dilatation [1-3]. These patients are known to have a higher risk of developing carcinoma of the gallbladder [2,4-6]. In spite of the possible close relation between progressive changes in the epithelium of the gallbladder and malignancy under the influence of pancreaticobiliary maljunction, only a few studies have attempted to clarify epithelial changes in the gallbladder [4,7], and no attempt has succeeded in making a model of pancreaticobiliary maljunction without bile duct dilatation. We have succeeded in developing an experimental model of pancreaticobiliary maljunction without bile duct dilatation. Using this model, we studied the effect of pancreaticobiliary maljunction without bile duct dilatation on cell kinetics of the gallbladder epithelium using an antibody to the proliferating cell nuclear antigen (PCNA). PCNA is a nuclear protein that is synthesized in the late G1 and S phase of the cell cycle and is an auxiliary protein of DNA polymerase-delta [8]. PCNA expression correlates with a cell's proliferative state [9].

Materials and Methods

1. Pancreaticobiliary maljunction cat model

Fifteen cats weighing 3.5-5.0 kg underwent side-to-side anastomosis between the common bile duct and the pancreatic duct near the duodenum. A 4 - 6 mm incision was made in the distal pancreatic duct and the distal common bile duct under general anesthesia. The incised edges were anastomosed with a continuous 8-0 nylon monofilament suture to make a wide and long communication between the pancreatic duct and the common bile duct, resembling the common channel of maljunction of the pancreatico-biliary tract in humans (Fig. 1). The procedure did not result in dilatation of the common bile duct.

Five cats, in which the common bile duct and the pancreatic duct were exposed only, were used as controls.

All experiments were performed in accordance with the Animal Experimental Guide - Nagoya University School of Medicine (Nagoya University, revised in 1989).

2. Methods

Cholangiography was performed in five experimental group cats and five control cats which had survived for 6 months, and in four experimental group cats which had survived for 12 months. A cannula was introduced into the gallbladder after bile had been obtained for determination of amylase. A high amylase concentration documented reflux of pancreatic juice into the biliary tract.

The liver, gallbladder, hepatoduodenal ligament, pancreas, and duodenum were removed en bloc in the nine experimental group cats and five controls after cholangiography. These organs were preserved in 10% buffered formaldehyde solution for 2 days, embedded in paraffin, and sliced 2 μm thick for histologic and PCNA histochemical studies.

Sections were dewaxed in xylene, rehydrated through alcohol, and then immersed in 3% hydrogen peroxide in methanol for 30 minutes to block endogenous

peroxidase activity. Sections were subsequently washed in phosphate buffered saline (PBS) three times. Bovine serum albumin was applied for 10 minutes to reduce non-specific antibody binding. The sections were incubated with anti-PCNA antibody (PC10, Dako) at a dilution of 1:100 at 4°C overnight. After further washing, sections were covered with biotinylated rabbit anti-mouse immunoglobulin (diluted 1:200, Dako) for 60 minutes, washed three times in PBS, and then covered with peroxidase-conjugated streptavidine (Dako). After washing three more times, peroxidase activity was developed by diaminobenzidine. The sections were counterstained with methylgreen.

The labeling index (LI) was defined as the percentage of epithelial cell nuclei that expressed PCNA per total epithelial cells. Three randomly selected microscopic fields containing more than 1000 epithelial cells were observed in each section without prior knowledge of whether the section was from the experimental or control group.

Statistical analysis was performed using Student's t-test for the degree of bile duct dilatation, the MannWhitney test for amylase values, and the Welch test for the PCNA LI, with significance levels of $p<0.05$. Numbers are expressed as the mean ± standard deviation.

Results

1. Pancreaticobiliary maljunction cat model

Nine out of 15 cats survived surgery and were alive for more than 6 months. Three cats died of pancreatitis within the first postoperative week. The causes of death in the remainder were unknown. There was no evidence of pancreatitis in the surviving cats at autopsy.

2. Amylase in bile

The Amylase concentration in the gallbladder bile of the experimental group cats was significantly higher than that of the control cats ($p<0.01$) (Fig. 2).

3. Cholangiography

The pancreatic duct was visualized in all the experimental group cats, but was not visualized in the control cats. The mean diameter of the common bile duct was 2.72 $\pm$ 0.60 mm in the experimental group cats and 2.33 $\pm$ 0.76 mm in the controls. This difference was not significant (Fig. 3).

4. Histologic findings of the gallbladder

The gallbladder epithelium became villous or spongioid in appearance in the experimental group cats, although the epithelium of control cats was flat with few folds (Fig. 4). This hyperplastic change was very prominent in seven of the nine experimental group cats, but was rather mild in two experimental group cats. Inflammatory changes, such as infiltration by lymphocytes, were found in four experimental group cats, and were not found in five experimental group cats or all the control cats. Inflammation was considered chronic in three experimental group cats, and chronic and acute in one experimental group cat. Metaplastic changes such as pseudo-pyloric glands and goblet cells were not found in either the experimental group cats or the controls.

5. PCNA immunohistochemical staining

PCNA positive cells were increased significantly in the gallbladder epithelium

of the experimental group cats, compared with a few scattered positive cells in the controls (Fig. 4). In four out of the nine experimental group cats, the population of PCNA positive cells increased at the base of the epithelial folds, suggestive of the "proliferation compartment" [10] that is observed in the intestinal epithelium. The increase in PCNA positive cells was observed in the experimental group cats independent of any inflammatory changes in the gallbladder.

6. PCNA labeling index

The mean PCNA LI of the experimental group cats was 28.1 ± 12.2% at the fundus, 25.1 ± 9.6% at the body, and 21.6 ± 11.3% at the neck. The mean LI of control cats was 4.3 ± 1.6% at the fundus, 4.9 ± 3.3% at the body, 3.6 ± 2.9% at the neck. The mean LI was 22.5 ± 4.5% in the experimental group cats with inflammation, and 32.5 ± 15.0% in the experimental group cats without inflammation. The LI of the experimental group cats was significantly larger in all three portions of the gallbladder than that of control cats (Fig. 5). However, the LI was not significantly different between 6-month experimental group cats and 12-month experimental group cats, or between experimental group cats with inflammation and experimental group cats without inflammation.

Discussion

There is very close relation between pancreaticobiliary maljunction and biliary carcinoma: bile duct carcinoma develops in patients with choledochal cysts which are almost uniformly associated with pancreaticobiliary maljunction[2,11,12]. Recently, it has been reported that patients with pancreaticobiliary maljunction without bile duct dilatation frequently develop gallbladder carcinoma, and patients with gallbladder carcinoma frequently have pancreaticobiliary maljunction [2,4-6]. In short, pancreaticobiliary maljunction without bile duct dilatation is now widely accepted as a risk factor for gallbladder carcinoma.

Although dogs are popular in studies of pancreaticobiliary maljunction [2,13,14], the model has some anatomical and thus technical disadvantages: dogs have their major drainage of pancreatic juice via the accessory pancreatic duct, which enters the duodenum separately without joining the common bile duct. The smaller pancreatic duct joins the common bile duct [15]. There is a long distance between the bile and pancreatic duct, and the anastomosis of these ducts has to be made in an end-to-side fashion [13,14], which tends to develop anastomotic stenosis resulting in biliary dilatation and subsequent cholangitis. On the contrary, in cats, a long and wide side-to-side anastomosis can be fashioned with little possibility of anastomotic stricture, since the main pancreatic duct is the main drainage of pancreatic juice, and the main pancreatic duct and the bile duct, positioned in parallel, enter into the major papilla as they do in humans [16]. Since there was no difference in the diameter of the bile duct between the models and the controls, our model is superior to the dog model for studying pancreaticobiliary maljunction without bile duct dilatation.

The epithelium of the gallbladder in patients with pancreaticobiliary maljunction is characterized histologically by hyperplasia and/or metaplasia. Metaplasia was emphasized in the respect of a precancerous condition [4]. Yamamoto has reported that the gallbladder epithelium displayed metaplastic changes in 26 out of 39

pancreaticobiliary maljunction patients, and hyperplasia in 15. The metaplastic changes were focal, in contrast to the hyperplastic changes which were diffuse. Thus, the initial effect of reflux of pancreatic juice may be epithelial proliferation resulting in later epithelial hyperplasia [7].

Our study demonstrated that in normal gallbladder epithelium, only a few PCNA positive cells were identified, and their location was random. In contrast, abundant PCNA positive cells were detected in all our pancreaticobiliary maljunction models (Figs. 4, 5). We experimentally confirmed for the first time that reflux of pancreatic juice produces a hyper-kinetic state in the gallbladder epithelium, although this relation has been suggested by the clinical data of others [7,17-19].

The proliferative action of the regurgitated pancreatic juice induces the morphologically hyperplastic changes in the epithelium observed in pancreaticobiliary maljunction patients. The proliferative effect may be important in relation to carcinogenesis, since an abnormal cell proliferation is known to be an early biological alteration in the carcinogenic process. Increased cell turnover rate may either lead to an increased number of cells susceptible to the action of carcinogens, or may amplify cell defects induced by carcinogens [20].

The PCNA positive cells was scattered throughout the normal epithelium, however, in four experimental group cats, PCNA positive cells were found at the base of the folds, representing organization of the proliferation compartment. It is likely that new cells derived from the proliferation compartment migrate toward the top of the fold. Cell proliferation kinetics in the gallbladder are similar to those of the intestinal epithelium [10,20]. Therefore, this change in distribution of the proliferative cells can be considered as a kind of metaplasia.

Chronic inflammation may cause increased cell proliferation. However, our five experimental group cats without inflammation had high PCNA labeling index values as did the four with inflammation. This suggests that inflammation may have

little impact on cell proliferation. Bile in the condition of pancreaticobiliary maljunction is reported to contain activated pancreatic enzymes such as trypsin and phospholipase A [13,14], increased secondary bile acids, increased unconjugated bile acids [13,21], and mutagens [22]. Secondary bile acids may cause increased proliferation as they possess co-mutagenic activity [21], but we detected no such increase in secondary bile acids in the bile within the gallbladder of models (unpublished data). Further investigation is needed to determine which substance causes the proliferative effect.

In conclusion, a new model of pancreaticobiliary maljunction without bile duct dilatation was produced using cats. Increased PCNA positive cells were demonstrated in the gallbladder epithelium immunohistochemically. Our study revealed that pancreaticobiliary maljunction without bile duct dilatation has a proliferative effect on the gallbladder epithelium, which may be related to carcinogenesis.

Acknowledgment

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Figure Legends

Fig. 1. Schema of a pancreaticobiliary maljunction cat model produced by side-to-side anastomosis of the common bile duct (BD) and the main pancreatic duct (PD). In this model the common bile duct proximal to an anastomosis does not dilate postoperatively.

Fig. 2. Amylase concentrations in gallbladder bile: The amylase concentration was significantly higher in the models than in the controls ($p<0.01$).

Fig. 3. Cholangiography showing the pancreatic duct (PD) in an experimental group cat (A) but not in a control cat (B). The common bile duct (BD) was not significantly increased in diameter between the models (A: arrowheads) and the controls (B: arrowheads).

Fig. 4. PCNA immunostaining of gallbladders: The gallbladder epithelium in the models (A, B) was villous in form compared to the flat epithelium of the controls (C). PCNA positive cells were significantly increased in the models (A, B). The population of positive cells was found mainly at the base of the folds in four experimental group cats (B) and diffusely in the other five experimental group cats (A). In contrast, positive cells were scattered and few in the controls (C). (Original magnification, A: X33, B: X33, C: X50.)

Fig. 5. Labeling Index: The PCNA LI of the experimental group cats was significantly larger at all three portions of the gallbladder than that of controls.

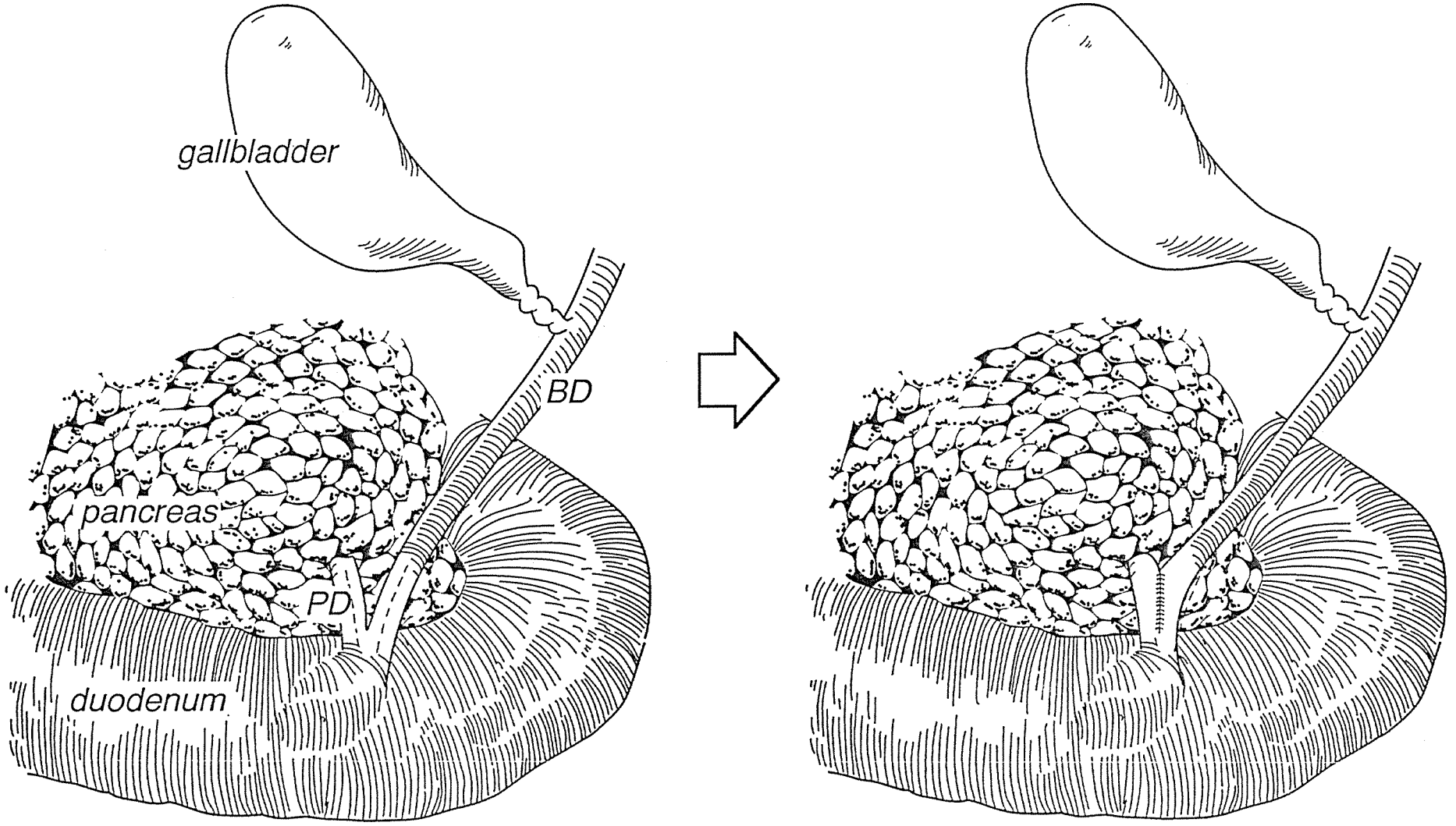

Fig. 1.

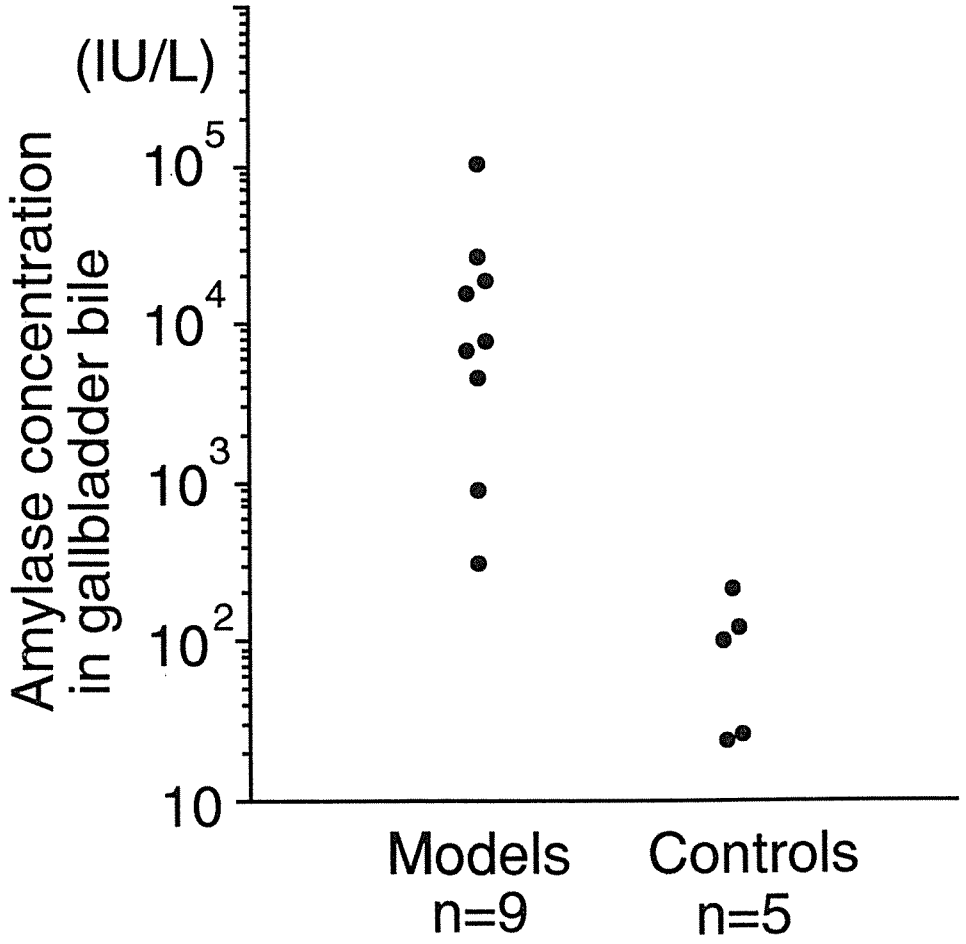
Amylase concentration in gallbladder bile
(IU/L)
10^5
10^4
10^3
10^2
10
Models
n=9
Controls
n=5

Fig. 2.

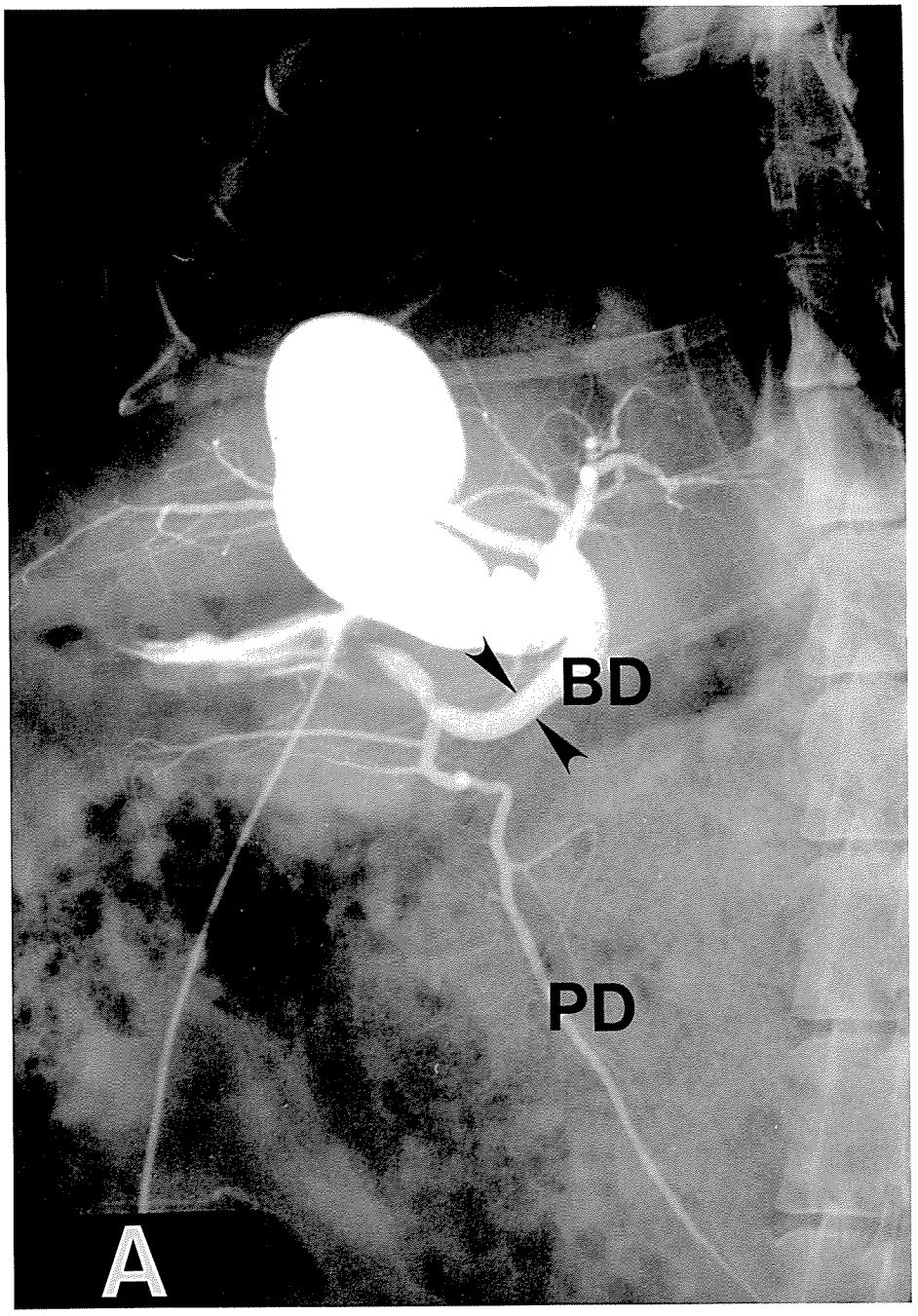

Fig. 3. (A)

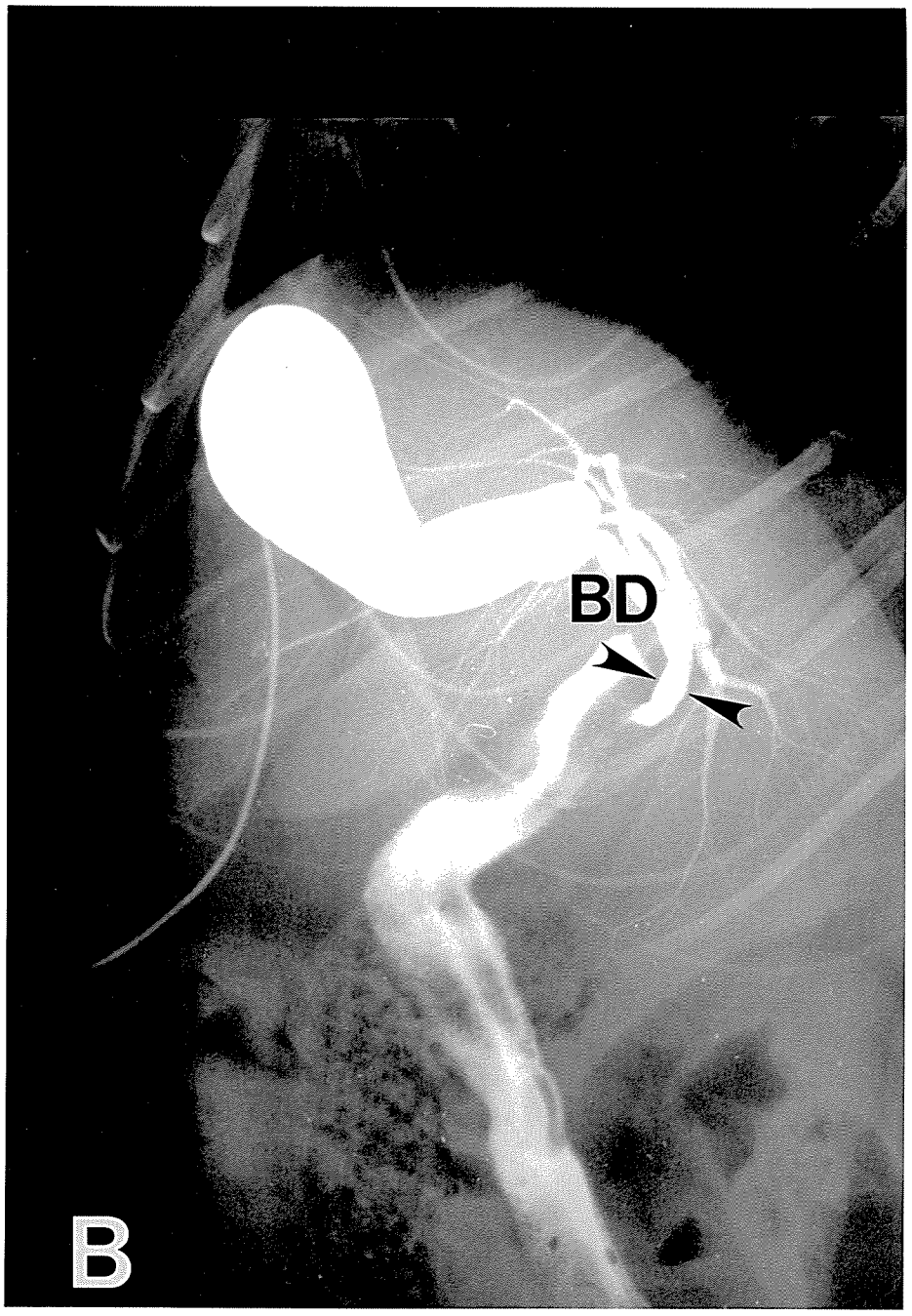

Fig. 3. (B)

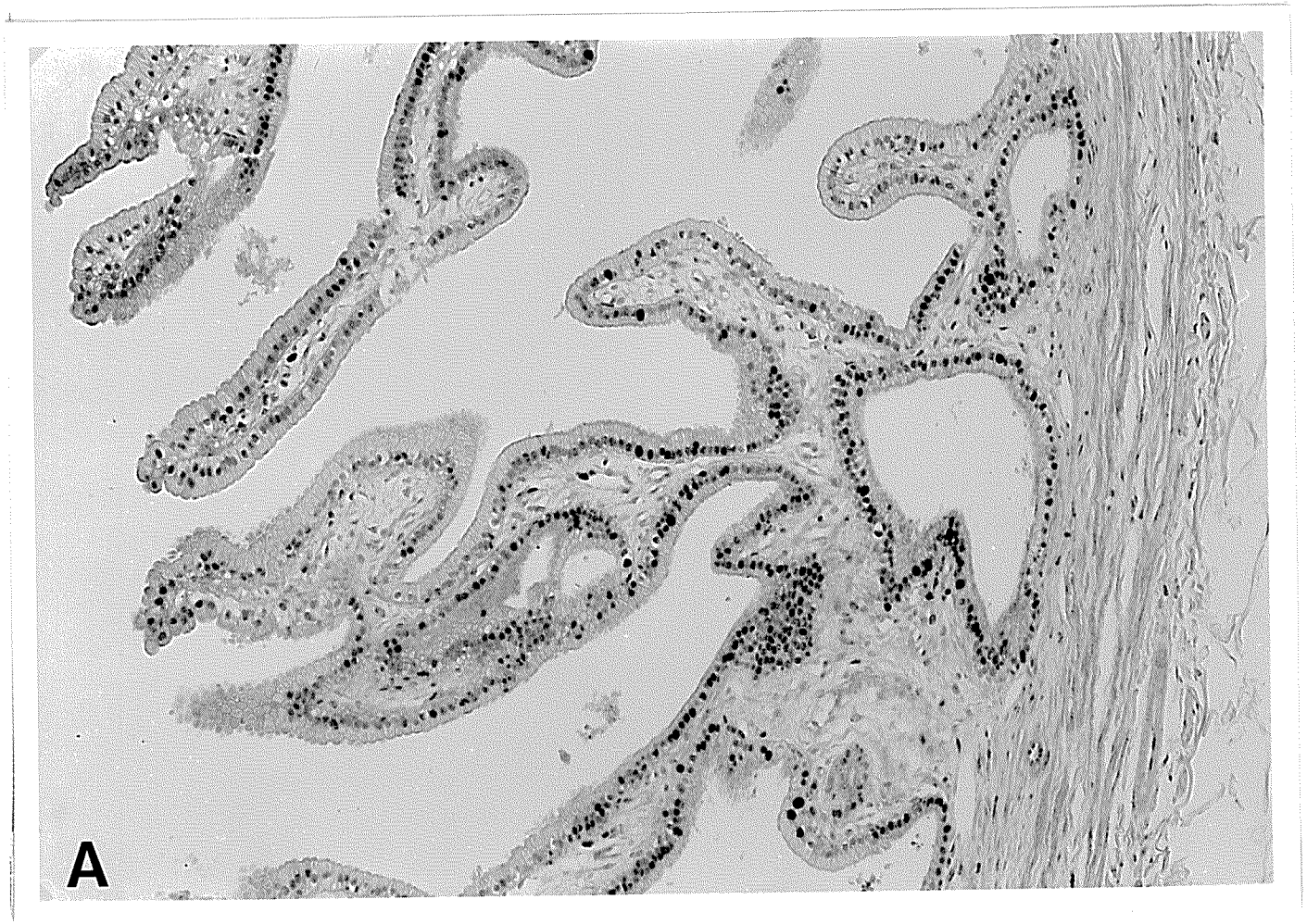

Fig. 4. (A)

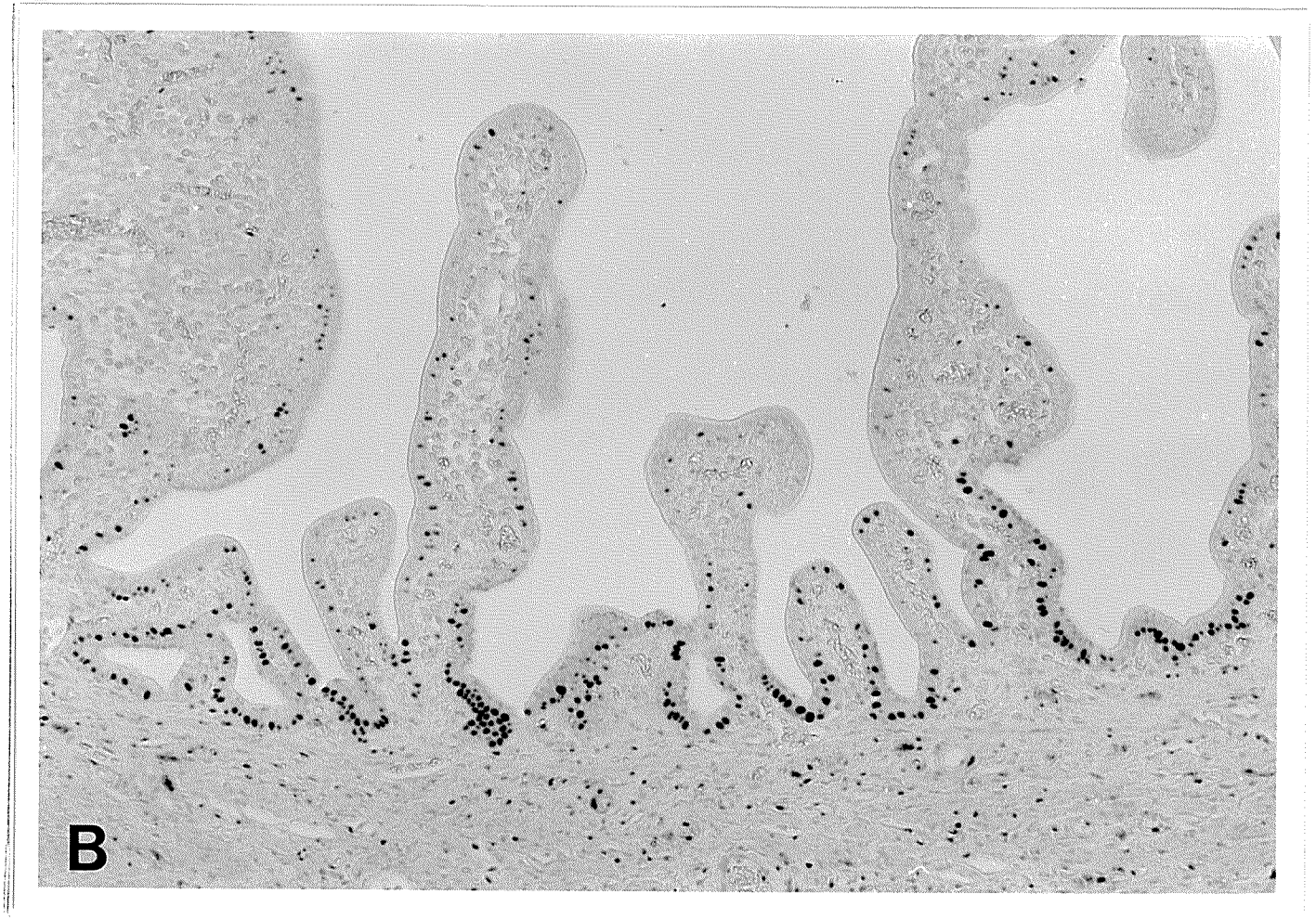

Fig. 4. (B)

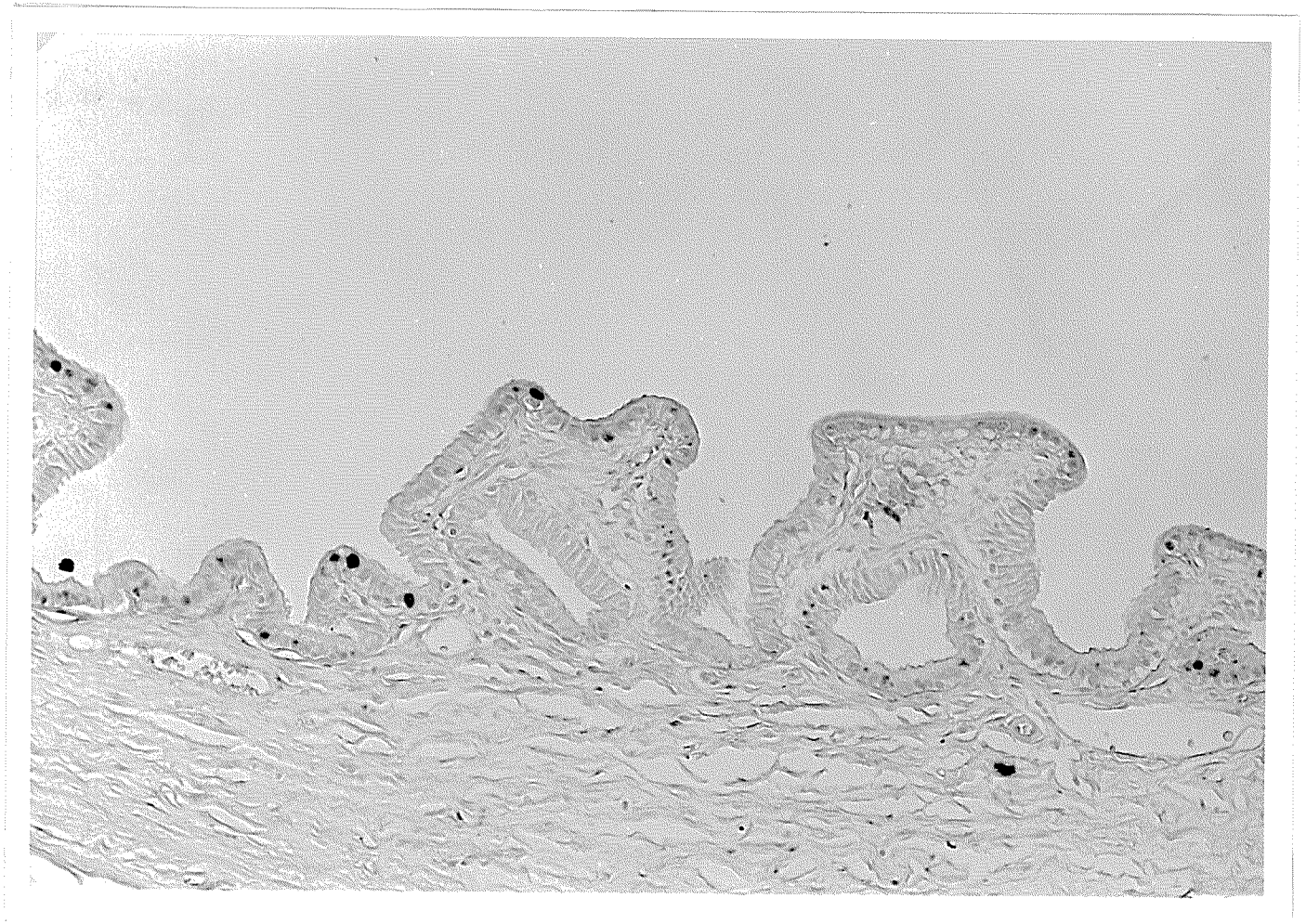

Fig. 4. (C)

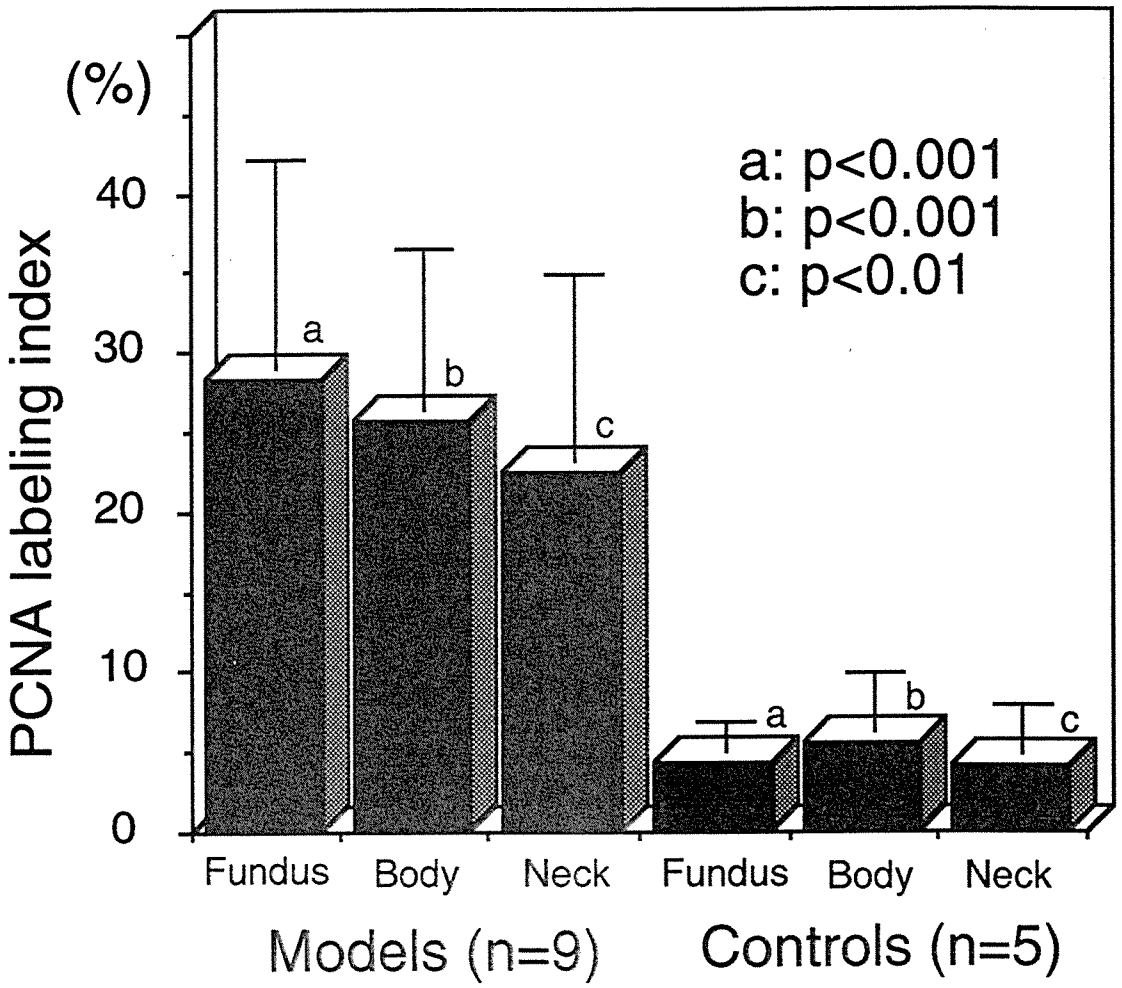
(%)
PCNA labeling index
40
30
20
10
0
a: p<0.001
b: p<0.001
c: p<0.01
a
b
c
a
b
c
Fundus
Body
Neck
Fundus
Body
Neck
Models (n=9)
Controls (n=5)

Fig. 5.