

Bile-induced DNA strand breaks and biochemical analysis of bile acids in an experimental model of anomalous arrangement of the pancreaticobiliary ducts

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Abstract : A canine experimental model for the anomalous arrangement of the pancreaticobiliary ducts (APBD) was made to investigate the effects of bile acids on carcinogenesis. Seven adult mongrel dogs underwent dorsal pancreatico-cholecystostomy to serve as a functional model for APBD, and six dogs underwent the same procedure with the pancreatic duct ligated as a control group. Bile from the gallbladder was taken 14 months after surgery for bile acid analysis by HPLC. DNA strand breaks in HeLa cells induced by the bile were also investigated *in situ* by nick translation method. As a result, the fraction of cholic acid tended to be lower, and that of deoxycholic acid slightly higher in APBD-dogs (N.S.). The ursodeoxycholic acid percentage in APBD-dogs significantly decreased compared with that in the control and normal dogs ($p < 0.05$). Extremely high frequency of DNA strand breaks was shown in only two out of seven APBD-dogs. In those two dogs, the cholic acid percentage decreased and that of deoxycholic acid increased extremely. These findings suggest that the alteration of the bile composition in APBD caused frequent DNA strand breaks and repair which might lead to gene mutation and biliary tract carcinoma. *J. Med. Invest.* 44 : 47-51, 1997

Key Words : anomalous arrangement of the pancreaticobiliary ducts, bile acid fractions, DNA strand breaks, *in situ* nick translation

INTRODUCTION

Anomalous arrangement of the pancreaticobiliary ducts (APBD) is recognized as a condition commonly associated with congenital biliary dilatation (CBD, so-called choledochal cyst). The incidence of biliary tract carcinoma in patients with APBD is higher than in the general population, and onset of biliary carcinoma occurs at a younger age in patients with APBD than in those without APBD(1-3). Histopathological examination of excised choledochal cysts has frequently demonstrated the presence of metaplasia of the epithelium, and the prevalence of such metaplasia increases with age(4). However, despite these observations, there is no direct evidence linking metaplasia with the development of biliary carcinoma(4,5).

Our previous studies on experimental APBD in dogs suggested that pancreatic juice enzymes and bacteria

infecting the bile deconjugated detoxified mutagens in the bile and induced bile mutagenicity(6), and also demonstrated the presence of atypical biliary tract epithelium and DNA ploidy abnormalities(7). The present study was undertaken to investigate the etiological factors of biliary carcinoma by determining the frequency of DNA strand breaks in HeLa cells induced by bile and the biochemical analysis of bile acids in a canine model for APBD.

The nick translation method has become popular in radioisotope-labeling of DNA probes(8). The principle of this technique is based on the activity of E.coli DNA polymerase I to bind to a nick containing 3'-OH terminus and substitute the nucleotides one by one with externally added radioactive nucleotides in the 5'to 3'direction. The amount of this reaction is dependent on the number of nicks and hence on the action of DNase I which is usually added to the reaction mixture. This technique has also been used to detect DNA strand breaks in carcinogen-treated cells(9) and bleomycinexposed HeLa cells(10). Anai and Maehara, *et al.* (11,12) applied this technique to detect DNA strand scission in HeLa cells following heat

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treatment and induced radiation. In the present study, we used this method for *in situ* detection of the single DNA strand scission in HeLa cells induced by bile.

MATERIALS AND METHODS

Canine model and samples

Eight male and five female adult mongrel dogs weighing between 9.3-15.6 kg were used. The animals were divided into two groups. Group A consisted of seven dogs which were prepared as the APBD-model. After they were anesthetized by intravenous injection of 25 mg/kg body weight of sodium pentobarbital, the duodenum was incised to make a full thickness flap of the wall (8-10 mm in diameter), including the papilla of the dorsal pancreatic duct. The flap was then anastomosed with the gallbladder with interrupted sutures to obtain a functional model for APBD (pancreatico-cholecystostomy, Figure 1). Group B consisted of six dogs as the control group. They were also subjected to the pancreatico-cholecystostomy as described above, however the dorsal pancreatic duct was doubly ligated proximal to the anastomosis to cut off the flow of pancreatic juice into the gallbladder. The dogs were fed a regular pellet diet (Marubeni Animal Food Co., Ltd., Tokyo, Japan) and sacrificed 14 months after surgery. Samples of normal gallbladder bile were obtained from an additional seven normal dogs with a 21 gauge needle immediately after laparotomy. All bile samples were passed through a Millipore filter (pore size 0.45 μm) to remove bacteria and stored at -80°C until use.

Amylase levels in the gallbladder bile were measured by enzymatic assay to confirm the patency of the pancreatico-cholecystostomy. The bile acid fractions were obtained by an immobilized enzyme column method, and measured by high

performance liquid chromatography (HPLC) (13). Statistical analysis was performed using Student's or Welch's *t*-test.

Cell culture and treatment

HeLa cells were maintained as monolayers on plastic dishes in Eagle's minimum essential medium (MEM) containing L-glutamine (292 mg/l) and 10% fetal calf serum supplemented with streptomycin, penicillin and amphotericin B. For the nick translation assay (11,12) 1×10^5 cells were plated in two chambered Lab-Tek tissue culture chamber/slide (Nunc, Inc., Naperville, IL, USA) and cultured for 48 hours in an atmosphere containing 5% CO_2 and 95% air at 37°C . The cells were then exposed to bile samples diluted 10^4 times with MEM for three hours at 37°C . The plastic chambers were removed from the slides after the exposure, and the cells were rinsed with phosphate-buffered saline and fixed with ethanol/acetic acid (3 : 1, v/v) for 10 minutes at room temperature.

In situ nick translation

After fixation, the slides were overlaid with $15 \mu\text{l}$ of a nick translation mixture and incubated for 10 minutes at room temperature. The nick translation mixture contained 50 mM Tris-HCl pH 7.5, 5 mM MgCl_2 , 10 mM 2-mercaptoethanol, 200 U/ml of *E. coli* DNA polymerase I (endonuclease-free; Boehringer Mannheim GmbH Biochemica, Mannheim, Germany), 30 μM each of dATP, dGTP, dCTP and dTTP (Sigma Chemical Co., St. Louis, MO, USA) and 0.1 μM ^3H -labeled dTTP (104 Ci/mmol; Amersham Japan, Tokyo, Japan). The reaction for nick translation was terminated by washing the slides with 50 mM Tris-HCl pH 7.5. The slides were then dehydrated in ethanol and allowed to dry. The dried slides were coated with a nuclear emulsion (Sakura NR-M 2, Konica Co., Tokyo, Japan) and stored in a dark box at 4°C for 3 days. The autoradiographs were developed and the nuclei counter stain with hematoxylin was added. The grain density of 100 nuclei selected at random was measured microscopically using an image processing and analyzing system (Software: NIH Image; online-software, hardware: Macintosh IIfx; Apple Computer Japan, Inc., Tokyo, Japan). Grain density was expressed as a ratio compared with the density of 100 untreated cells (grain-density ratio).

RESULTS

Amylase level in gallbladder bile

The level of amylase in the gallbladder bile ranged from 41800 to 176500 (mean \pm S.D. :

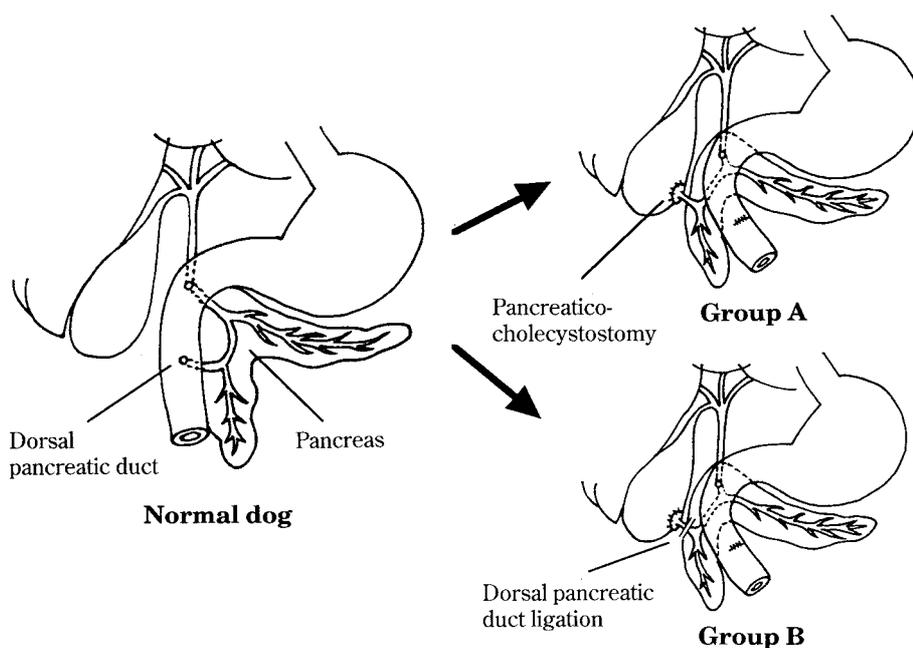


Fig.1. Preparation of experimental models in dogs: group A was prepared for APBD by pancreatico-cholecystostomy. Group B was also subjected to pancreatico-cholecystostomy, however the dorsal pancreatic duct was doubly ligated proximal to the anastomosis to cut off the flow of pancreatic juice into the gallbladder.

84600±45373) IU/L in group A (n=7), from 12 to 583(79 ±212)IU/L in group B (n=6) and from 28 to 1760 (104±634) IU/L in the normal bile samples (n=7). Amylase level was significantly higher in group A than in the other groups (p<0.01).

In situ nick translation

DNA strand breaks in bile-treated cells were visualized as tiny silver grains as shown in Figure 2. Grain-density ratios of normal dogs, groups A, and B are shown in Figure 3. Group B showed no difference in the ratio to normal dogs. However, the ratio was dispersed and two samples showed an extremely high ratio in group A.

Bile acid fractions in gallbladder bile

As shown in Figure 4, the level of cholic acid fraction (CA) tended to be lower, and that of deoxycholic acid (DCA) slightly higher in group A than in normal dogs and group B (not significant). The level of ursodeoxycholic acid (UDCA) was significantly decreased in group A compared with that in normal dogs (p<0.05), whereas the level of chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) levels did not change significantly.

Relationship between the grain-density ratio and CA or DCA

There was a significant negative correlation between the level of CA and the grain-density ratio (r=0.596, p<0.01) (Figure 5 a). In contrast, there was a significant positive correlation between the level of DCA, and the grain-density ratio showed (r=0.567, p<0.01) (Figure 5 b). In the two samples showing high grain-density ratios, the cholic acid percentage decreased, and that of deoxycholic acid increased extremely. No significant correlation found between the grain-density ratio and the level of CDCA, UDCA or LCA.

Histopathological findings

The results of the histopathological examination of gallbladder specimens in group A and group B were as follows: hyperplasia was found in 7/7 (100%) of group A and in 3/6 (50%) of group B; mucosal desquamation was found in 2/7 (29%) of group A; lymphoid cell infiltration was found in 7/7 (100%) of group A and in 6/6 (100%) of group B; lymphatic follicle was found in 5/7 (71%) of group A and

in 5/6 (84%) of group B; intramural glandular structure was found in 4/7 (57%) of group A and in 1/6 (17%) of group B; goblet cell appearance was found in 2/7 (29%) of group A.

DISCUSSION

Since Cook *et al.* (14) pointed out the chemical similarity between bile acids and methylcholanthrene, a well-known carcinogenic agent, many investigators have reported that bile acids, especially secondary bile acids, have tumor

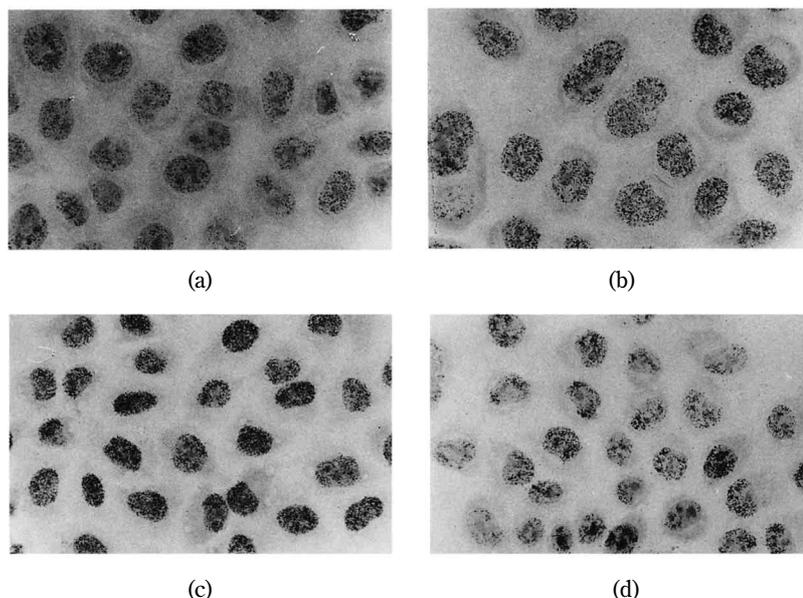


Fig. 2. Visualization of *in situ* nick translation in HeLa cells. After being nicktranslated *in situ* with ³H-labeled dTTP, the cells were autoradiographed and counter stained with hematoxylin. (a) Untreated cells; (b) Cells exposed to gallbladder bile from a normal dog; (c) Cells exposed to gallbladder bile from a dog in group A; (d) Cells exposed to gallbladder bile from a dog in group B. (original magnification, ×400)

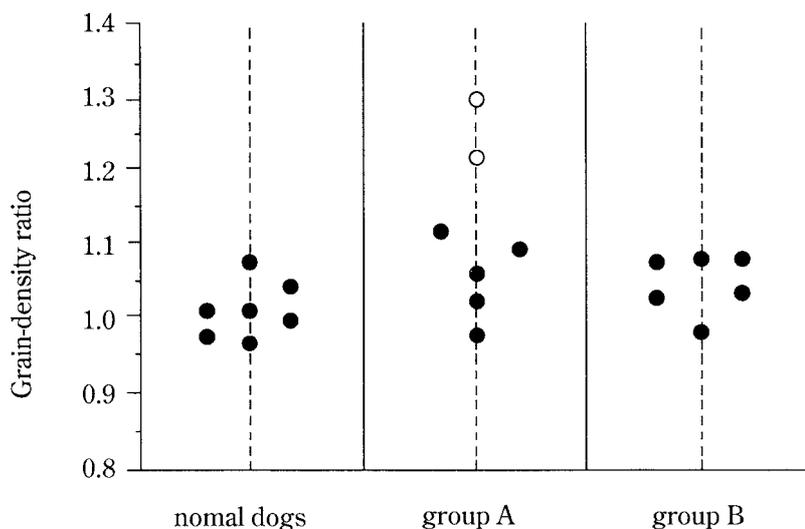


Fig. 3. DNA strand breaks in HeLa cells treated with gallbladder bile from the normal dogs, group A, and group B. Grain density was determined by light microscope observation using an image processing and analyzing system. The relative ratio between grain density of bile-treated cells and that of untreated cells was calculated as a grain-density ratio (treated cells/untreated cells). One hundred cells selected randomly from each sample were used to count grain-density. Two samples (○) showed extremely higher grain density ratios than the others (●).

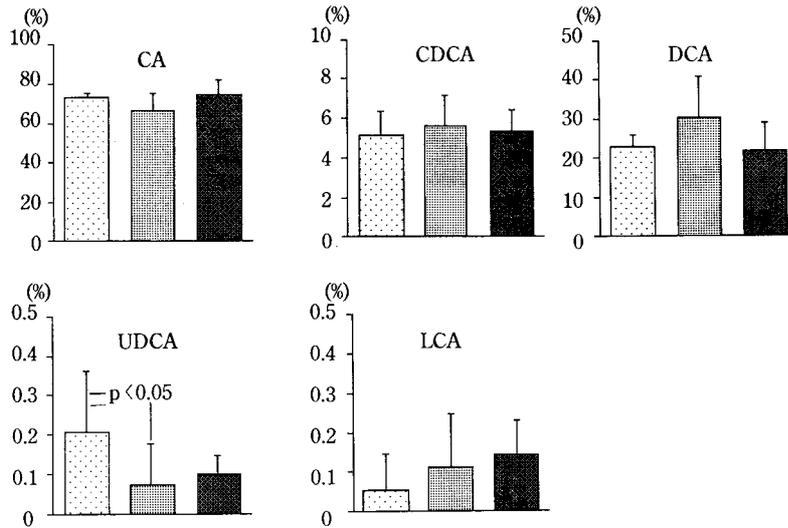


Fig. 4. Bile acid fractions in gallbladder bile of the normal dogs, group A, and group B. □, normal dogs (n=7); ▨, group A (n=7); ▩, group B (n=6).

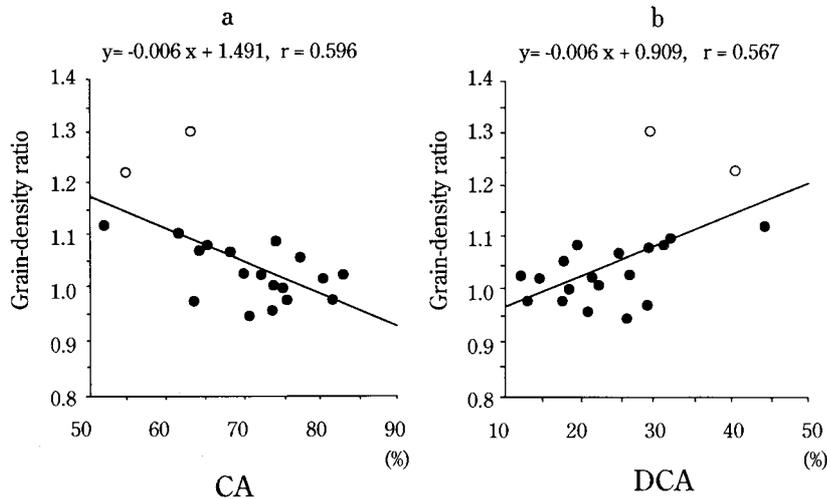


Fig. 5. Correlation between the grain-density ratio and CA (a) or DCA (b). ○, two samples showed higher grain density ratios; ●, the other samples.

enhancing effects (15-19). Revelle *et al.* (20) and Funabiki *et al.* (21) recently reported increased levels of secondary bile acids, DCA and LCA, in the bile from cystic dilated bile duct with APBD regardless of the presence or absence of malignancy. Kato *et al.* (22) also demonstrated that the whole bile duct contents from six out of 12 patients with APBD showed positive mutagenic activity by the *Bacillus subtilis* rec assay. These findings suggested that increased level of secondary bile acids in the bile was one of the etiological factors of biliary carcinoma in patients with APBD. APBD is a condition in which the common duct is abnormally long and the junction of the common bile duct and the pancreatic duct is located outside the duodenal wall. The primary physiological abnormalities in APBD are the reflux of pancreatic juice into the biliary tract and stagnation of the bile and pancreatic juice mixture. Our experimental model does not exactly duplicate the anatomical abnormality found in human APBD but it is similar from the pathophysiological point of view as shown by the significantly high level of

amylase in the bile from the experimental group. In the present study, the level of UDCA fraction was significantly decreased in the APBD-dogs. This phenomenon was interesting because the UDCA was reported to have a protecting effect against some mutagens in gastric mucosa (23) and liver cells (24-26). Only two dogs in the APBD-dogs showed a marked increase of DNA strand breaks. In addition, the level of CA decreased and that of DCA increased significantly in these two dogs. The grain-density ratio showed a negative correlation with the level of CA and a positive correlation with that of DCA in all dogs. Stasis of the bile may contribute to bacterial overgrowth and to the generation of unconjugated secondary bile acids. Furthermore, these bacteria and their products or unconjugated secondary bile acids or unidentified bile acid metabolites may participate in the development of biliary metaplasia and carcinoma in APBD (20). Histopathologically, hyperplasia was observed in all APBD-dogs and metaplasia in two, although there was no neoplastic growth. We cannot explain precisely the cause of alteration of bile acid composition, increase of DCA and decrease of CA and UDCA, in the present study. In only two dogs in group A, a markedly high grain-density ratio was shown by *in situ* nick translation. Bile acid composition in the two dogs was also altered significantly. DNA strand breaks do not cause carcinogenesis directly. However, fre-

quent DNA breaks, might be caused by the alteration of bile acid composition, and the resulting repairing action would increase the chance of gene mutation such as oncogene mutations which can lead to carcinogenesis in APBD.

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