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An Analysis of Mutagens in the Contents of the Biliary Tract in Pancreaticobiliary Maljunction

MASARU MIZUNO, TETSUO KATO, and KENJI KOYAMA

First Department of Surgery, Akita University School of Medicine, 1-1-1 Hondo, Akita 010, Japan

Abstract: A reflux of pancreatic juice into the biliary tract due to pancreaticobiliary maljunction has been considered to be an important factor in the development of biliary tract carcinogenesis. It is known that the contents of the biliary tract contain not only activated pancreatic enzymes but also certain mutagens. The purpose of this study was thus to isolate and identify such mutagenic substances. A 1:1 mixture of the control bile and pancreatic juice was mixed with bovine enterokinase (10 mg/ml), and the mixture was incubated at 37°C for 12 h. The mixture was demonstrated to be reproducibly mutagenic by the Ames test. The mutagenic substances in these mixtures, which were separated using organic analysis, were found to be included in the water-soluble fraction and to contain amino acids. Mutagenic substances are thought to have a molecular weight of 1500–3500 and to be a complex of low-molecular-weight, stable, mutagenic substances including amino acids and peptides.

Key Words: mutagenicity, biliary tract cancer, pancreaticobiliary maljunction

Introduction

Pancreaticobiliary maljunction, which is defined as a union of the pancreatic and biliary ducts located outside of the duodenal wall, has traditionally been regarded as a congenital anomaly accompanied by a dilatation of the common bile duct. However, maljunction without dilatation of the biliary tract has also recently been reported.[1–3] The causal relationship between the dilatation and the maljunction has not yet been clarified. The most critical pathological status in maljunction is the reflux of the pancreatic juice into the biliary tract. Various injurious substances are activated or produced by the mixture of pancreatic juice and bile, leading to secondary disorders such as cholangitis and pancreatitis. Furthermore, carcinoma of the biliary tract also frequently develops when such a maljunction has been present for a long time.[4–8] The mechanism of carinogenesis of the biliary tract in the presence of maljunction is still not clear. The purpose of this study was to isolate and identify any mutagenic substances in the biliary tract contents of patients with pancreaticobiliary maljunction.

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Materials and Methods

The following nine specimen types were analyzed:

1. The contents of the biliary tract collected during laparotomy in 9 patients with pancreaticobiliary maljunction
2. The bile of patients without maljunction collected via a bile drainage tube or during laparotomy in 10 patients
3. The pancreatic juice collected via a pancreatic duct drainage tube in 5 patients
4. A 1:1 mixture of the bile of patients without maljunction and pancreatic juice, incubated at 37°C for 12 h
5. A mixture of the bile (1 ml) of patients without maljunction and bovine enterokinase (10 mg; Sigma Chemical, St. Louis, MO, USA), incubated at 37°C for 12 h
6. A mixture of pancreatic juice (1 ml) and enterokinase (10 mg), incubated under the same conditions
7. A mixture of bile (1 ml), pancreatic juice (1 ml), and enterokinase (10 mg), incubated under the same conditions
8. A solution of 10 mg enterokinase in 1 ml of saline, incubated under the same conditions

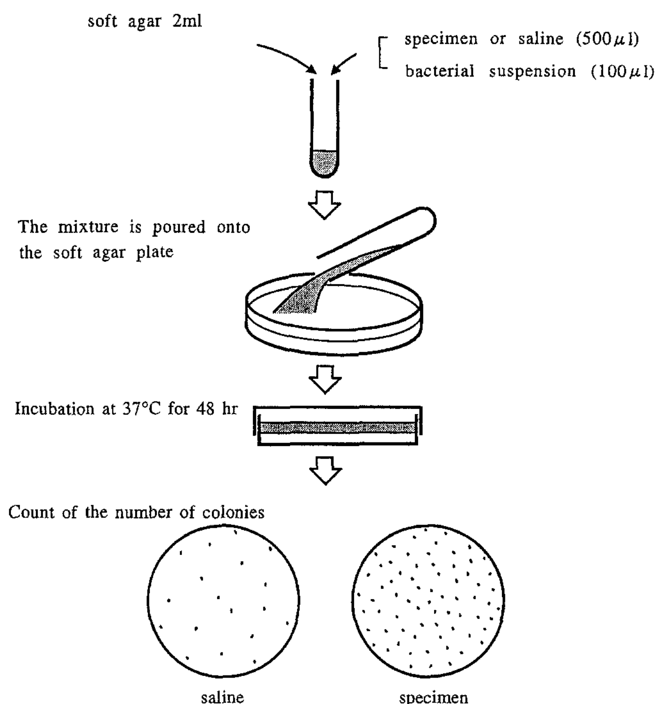

Fig. 1. Ames test for mutagenicity. Ames ratio-number of colonies in a specimen/that in saline. A ratio of over 2.0 is regarded as positive for mutagenicity

9. A solution of 50 mg of each of the following nine bile acids in 1 ml of saline: cholic, lithocholic (Wako Pure Chemical Industries, Osaka, Japan), chenodeoxycholic, ursodeoxycholic, deoxycholic, taurocholic, taurochenodeoxycholic, taurodeoxycholic, and taurolithocholic (Sigma chemical) acids

Trypsin Activity

The specimens were examined for trypsin activity by the radio-immunoassay double antibody technique before freezing.

Ames Test (Fig. 1)

This mutagenicity test determines the mutation rate of *Salmonella typhimurium* TA100 (supplied by the Iwate Municipal Institute of Public Health), a bacterium requiring histidine, to a non-histidine-requiring bacterium when exposed to mutagenic substances.

The specimen (0.5 ml) and 0.1 ml of the bacterial suspension (1–2 $\times$ 10^9 *S. typhimurium* TA100/ml) were transferred to a test tube, and 2 ml of melted soft agar containing 0.05 mM of L-histidine (Sigma Chemical) was added to the tube. After being mixed, the contents of the tube were poured onto an agar plate containing no histidine. After incubation at 37°C for 48 h, the colonies were counted. The ratio of the number of colonies in the specimen to that in the saline solution (Ames ratio) was determined; a specimen showing a ratio of 2.0 or more was regarded as positive. The material used as a positive control was a food additive, AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)acryl-amid] (Otsuka Assay Laboratories, Tokushima, Japan).[9–11]

Isolation of Mutagenic Substances

The starting material was a mixture of bile, pancreatic juice, and enterokinase, which had previously been confirmed to have strong mutagenicity. The specimens were subjected to separation by biochemical analysis using a chloroform and methanol technique, gel chromatography, amino acid analysis, and gas chromatography/mass spectrometry.

Biochemical Analysis

The specimen (1 ml) was mixed with 3.75 ml of a mixture of chloroform (99.8%, Wako Pure Chemical Industries) and methanol (99.8%, Wako) (1:2). After the mixture was stirred and kept at room temperature for 1 h, it was centrifuged at 3000 rpm for 15 min. The supernatant was removed, and 4.75 ml of a mixture of chloroform, methanol, and water (1:2:0.8) was added to the pellet. The contents were stirred and centrifuged, and the supernatant was mixed with the supernatant from the previous centrifugation. The resultant supernatant was mixed with 2.5 ml of chloroform and 2.5 ml of water, and centrifuged. After the two fractions were condensed with a nitrogen air stream, the aqueous fraction (upper layer) was used to prepare an aqueous solution, and the chloroform fraction (lower layer) was used to prepare a dimethyl sulfoxide solution (Wako).[12] Each solution was examined for mutagenicity.

Gel Chromatography

The mutagen-positive specimens, i.e., the aqueous fraction obtained as described in the preceding paragraph, were subjected to gel chromatography with the Sephadex G-25 (Pharmacia, Uppsala, Sweden) to estimate the molecular weights of mutagen-positive fractions. The gel was dissolved with deionized water, degassed, and then transferred to a column 30 mm in diameter and 450 mm in length. The solvent used was deionized water, and the flow rate was 1 ml/min. During the determination of absorbance [optical density (OD): 280 nm] in the samples, each fraction was collected with a fraction collector. The markers used for molecular weight were vitamin B_{12} (molecular weight ca. 1500: Banyu Pharmaceutical, Tokyo, Japan) and glucagon (molecular weight ca. 3500: Kodama, Tokyo, Japan). The fractions isolated were examined for mutagenicity

Table 1. Trypsin activity

Specimens (n)	Activity (ng/ml)
Contents of the biliary tract in patients with maljunction (5)	17833 ± 7758
Bile of patients without maljunction (10)	562 ± 170
Pancreatic juice (5)	5750 ± 1769
Mixture of bile and pancreatic juice (5)	2120 ± 1141
Mixture of pancreatic juice and enterokinase (5)	>31500
Mixture of bile, pancreatic juice, and enterokinase (5)	17820 ± 1256

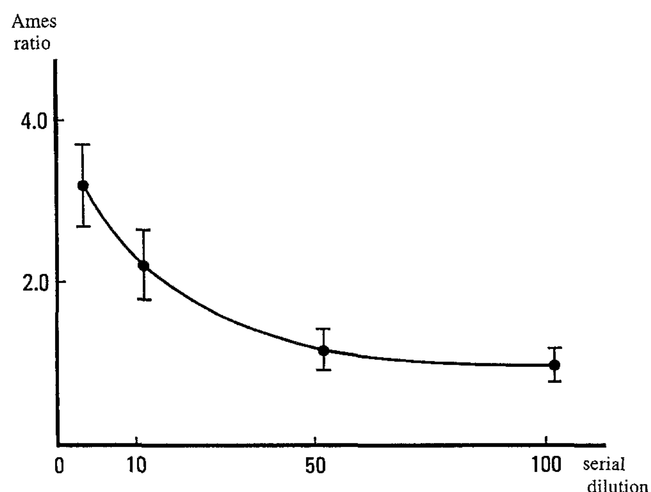

Fig. 2. Concentration-dependent dose-response curve with the Ames ratio of the mixture of bile, pancreatic juice, and enterokinase. (n = 5)

after condensation with a vacuum centrifugation condenser (CC-180: Tomy Seiko, Tokyo, Japan).

Amino Acid Analysis

The concentration of each mutagen-positive specimen prepared as described in the preceding paragraph was adjusted to 400μg/ml with a protein assay reagent (Pierce, Rockford, IL, USA), and 40 amino acid fractions were analyzed with an automatic amino acid analyzer (835: Hitachi, Hitachinaka, Japan).

Gas Chromatograaphy/Mass Spectrometry

The mutagen-positive specimens prepared as described under "Gel Chromatography" were centrifuged, condensed, dried, and dissolved in a small amount of methanol. The solution was subjected to gas chromatography (HP5971A: Hewlett-Packard, Palo Alto, CA, USA) at 60°–240°C and 10pSi² with a DB-1 column (15m × 0.25mm; J&W Scientific, Forsom, CA, USA).

Table 2. Mutagenicity test *1*

Specimens (n)	Ames ratio
Saline solution	1.00
AF-2	3.82 ± 0.30
Contents of the biliary tract in patients with maljunction (9)	2.47 ± 0.57*
Control bile of patients without maljunction (10)	1.07 ± 0.16*

AF-2, [2-(2-furyl)-3-(5-nitro-2-furyl)acryl-amid]
*$P < 0.01$

Table 3. Mutagenicity test *2*

Specimens (n)	Ames ratio
Control bile of patients without maljunction (10)	1.07 ± 0.16
Pancreatic juice (5)	1.01 ± 0.16
1:1 mixture of bile and pancreatic juice (10)	1.09 ± 0.17
Solution of 10mg enterokinase per ml of saline solution (5)	1.10 ± 0.12
Mixture of bile (1ml) and enterokinase (10mg) (5)	1.03 ± 0.05
Mixture of pancreatic juice (1ml) and enterokinase (10mg) (5)	1.16 ± 0.05
Mixture of bile (1ml), pancreatic juice (1ml), and enterokinase (10mg) (25)	2.21 ± 0.67*

*$P < 0.01$

Results

Trypsin Activity (Table 1)

Trypsin hyperactivity of more than 10000ng/ml was observed in all five specimens collected from the patients with maljunction. The activity was under 1000ng/ml in all 10 patients without maljunction. The activity level was 17820 ± 1256ng/ml in the mixture of bile, pancreatic juice, and enterokinase, and over 31500ng/ml in the mixture of pancreatic juice and enterokinase, thus indicating strong activation. The activity level of the mixture of bile, pancreatic juice, and enterokinase was very similar to that of the specimens collected from the patients with maljunction. On the other hand, it was 5750 ± 1769ng/ml in the pancreatic juice, and 2120 ± 1141ng/ml in the mixture of bile and pancreatic juice, thus indicating weak activation.

Mutagenicity Test

The Ames ratio for AF-2, the positive control, was 3.82 ± 0.3 per microgram. The ratio for the biliary contents of the patients with maljunction (2.47 ± 0.57) was significantly ($P < 0.01$) higher than that for the bile of patients without maljunction (1.07 ± 0.16) (Table 2).

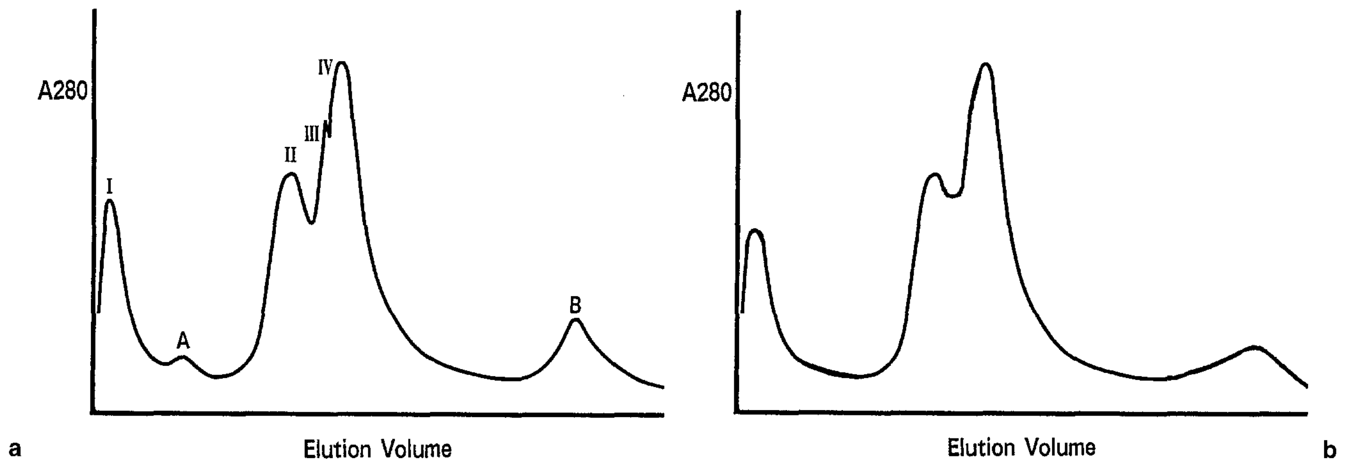

Fig. 3. a Chromatogram of the aqueous fraction. Four fractions (*I–IV*) were obtained. *A*, glucagon (molecular weight about 3500); *B*, vitamin B_{12} (M.W. about 1500). **b** Aqueous fraction of the contents of the biliary tract in a patient with pancreaticobiliary maljunction demonstrating a similar chromatogram

On the other hand, the ratio for the mixture of bile, pancreatic juice, and enterokinase was 2.21 ± 0.67, which was significantly ($P < 0.01$) higher than that of any otehr specimen (Table 3). A concentration-dependent dose-response curve for the mixture of bile, pancreatic juice, and enterokinase was obtained (Fig. 2).

All of the commercially available bile acid solutions were mutagen-negative (Table 4).

Isolation and Identification of Mutagenic Substances

The Ames ratio for the aqueous layer of the mixture of bile, pancreatic juice, and enterokinase was significantly ($P < 0.01$) higher than that for the chloroform layer (Table 5).

Table 4. Mutagenicity test *3*

Specimens	Ames ratio
Cholic acid	1.05 ± 0.15
Chenodeoxycholic acid	1.05 ± 0.13
Deoxycholic acid	1.07 ± 0.08
Lithocholic acid	0.99 ± 0.09
Ursodeoxycholic acid	1.06 ± 0.08
Taurocholic acid	1.07 ± 0.12
Taurochenodeoxycholic acid	1.06 ± 0.09
Taurodeoxycholic acid	1.04 ± 0.11
Taurolithocholic acid	1.09 ± 0.07

Table 5. Mutagenicity of chloroform and methanol fractions

Fractions (*n*)	Ames ratio
Chloroform (10)	1.29 ± 0.28*
Aqueous (10)	2.16 ± 0.53*

$*P < 0.01$

Gel chromatography of the aqueous fraction with the Sephadex G-25 provided the chromatogram shown in Fig. 3a; four fractions were obtained. The Ames ratio for fraction II was 2.19 ± 0.59, which was significantly higher than that for the other fractions ($P < 0.01$; Table 6). The molecular weight of fraction II was estimated to range from 1500 to 3500. The chromatography findings using the contents of the biliary tract of patients with

Table 6. Mutagenicity of fractions separated by gel chromatography

Fractions (n = 10)	Ames ratio
I	1.04 ± 0.06
II	2.19 ± 0.52*
III	1.20 ± 0.15
IV	1.21 ± 0.18

$*P < 0.01$

Table 7. Amino acid analysis of fraction II

Amino acid	%
Glu	3.03 ± 2.15
Ala	3.20 ± 2.27
Val	0.87 ± 0.62
Gly	12.70 ± 7.41
Lys	3.83 ± 2.71
Thr	1.97 ± 1.46
Leu	3.00 ± 2.12
Ser	5.33 ± 5.80
His	4.20 ± 0.57
Arg	7.23 ± 2.58
Ile	1.73 ± 1.47
Tyr	9.23 ± 0.82
Phe	8.43 ± 1.17
Orn	1.10 ± 0.86
Asp	5.10 ± 5.10
$GluNH_2$	1.46 ± 2.07
Met	1.40 ± 1.07

pancreaticobiliary maljunction showed a similar pattern. Fraction II was considered to contain amino acids because it showed high absorbance at OD 280nm.

The amino acid analysis of fraction II revealed relatively large quantities of glycine, tyrosine, and phenylalanine (Table 7), and this fraction was not denatured by phenol.

Gas chromatography of fraction II showed so many peaks that we could not make any conclusive remarks based on these findings. However, it is interesting to note that the results partially matched the findings of known mutagens.

Discussion

Under the conditions associated with pancreaticobiliary maljunction, carcinogenesis of the biliary tract occurs at a high incidence. Reflux of the activated pancreatic juice into the biliary tract is considered to be involved in carcinogenesis of the biliary tract.[13,14] Pancreatic enzymes other than amylase and lipase are essentially excreted as nonactive zymogen, and are first activated with enterokinase in the duodenum. During maljunction, however, trypsin is already activated in the biliary tract in most cases, as demonstrated in the present study. The mechanism of activation has not yet been clarified.[14–17] Under this condition, injurious pancreatic enzymes — specifically, phospholipase A_2 and elastase I — are activated. Activated phospholipase A_2 hydrolyzes lecithin, a major component of lipids in the bile, and then produces lysolecithin, which has a more injurious effect on the cell membrane. Deconjugated bile acids and β-glucuronidase may also develop into injurious substances in the presence of a biliary tract infection. Retention of these injurious substances in the dilated bile duct and gallbladder may induce chronic inflammation of the biliary tract, and may cause metaplasia or atypia of the biliary epithelial cells, thus resulting in cancer. Since many carcinogens are mutagenic substances that cause injury to DNA, this mutagenicity test is considered to be useful for screening carcinogens.

Kato et al. investigated the mutagnicity of the biliary contents using a sport-rec assay,[4] and reported a high rate of mutagenicity. In the present study, we used the Ames test,[18] which has a high sensitivity and permits the detection of both base repair substitution and frameshift types of mutation. The biliary contents of patients with maljunction showed a high frequency of mutagenicity, which is consistent with previous reports.[4,8,14] To further identify mutagenic substances, we reproduced the biliary contents in maljunction by mixing bile, pancreatic juice, and enterokinase, and used this mixture as a starting material for the isolation of mutgenic substances. It is likely that such bile acids as lithocholic acid, which have been shown to promote carcinogenesis of the colon, are included as mutagenic substances in the starting material.[19–21] However, no significant difference in the total bile acids was observed between the patients with maljunction and those with a normal junction. Furthermore, free cholic acid and taurine and glycine conjugate were negative for mutagenicity.[22] These results suggest that mutagenic substances other than bile acids are present in the biliary contents of patients with maljunction.

The mixture of bile, pancreatic juice, and enterokinase that was used as the starting material was subjected to biochemical analysis using both the chloroform and methanol technique, and mutagenic substances were found to be contained in the water-soluble fraction.[23] Fraction II of the water-soluble fraction obtained with the Sephadex G-25 showed the greatest mutagenicity, and its molecular weight was estimated to be within the range of 1500–3500. According to previous reports, most mutagenic substances have a molecular weight of less than 1300, but some (gonadotropin and versatint blue) have a molecular weight of 2500 or more. An amino acid analysis of fraction II also revealed large quantities of aromatic amino acids, such as tyrosine and phenylalanine, which contain a benzene ring.

Gas chromatography/mass spectrometry of fraction II was unable to identify the mutagenic substances. Since typical mutagenic substances usually contain a benzene ring, a cyclohexene ring, a nitro radical, or an *N*-nitroso radical, these substances are considered to derive from bile acids (having a cyclohexene ring) or aromatic amino acids (having a benzene ring).[24,25] Mutagenic amino acids include Trp-P-1 (a tryptophan heating product), Glu-P-1 (a glutamine heating product), and MeIQ (2-amino-3,4-dimethylimidazo-[4,5-f]quinoline), which derive from heated animal proteins. However, the existence of substances that contain mutagens in the form of peptides or proteins has not yet been confirmed. Although the usual peptides and proteins are denatured by phenol, in our study, fraction II was found to be stable in phenol.

These findings suggests that the mutagenic substances in fraction II with a molecular weight of 1500–3500 are a complex of low-molecular-weight, stable mutagenic substances and amino acids or peptides.

This study clarified the existence of mutagens in the contents of the biliary tract of patients with pancreaticobiliary maljunction and also described the character of these mutagens. Mutagenicity, however, does not always mean carcinogenesis. Therefore, further examinations are called for to elucidate more conclusively the carcinogenesis of human bile duct cancer.

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