Demonstration of Deoxyribonuclease I in Bile Juice by a Non-Radioactive Zymographical Method

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ABSTRACT. Deoxyribonuclease I (DNase I) is a digestive enzyme which distributes in digestive fluids such as pancreatic juice and saliva. The distribution of DNase I in bile juice is still unknown.

This is the first report to prove the existence of DNase I and its origin in bile juice, using human and rat bile juice obtained during decompression and ligation of the common bile duct (CBD).

DNase I was detected by a non-radioactive zymographical method modified activity blotting method with renaturation and blotting buffer systems. The identification as human and rat DNase I was based on their characteristics.

We showed that human and rat bile juice DNase I were specific enzymes hydrolyzing double-stranded DNA down to 5'phospho-oligonucleotides at each optimal condition, and that G-actin completely inhibited enzyme activities. These characteristics were compatible with the DNase I previously reported in humans and rats. In addition, the human bile juice DNase I increased following the recovery of biliary stasis by percutaneous transhepatic cholangio drainage (PTCD).

These results prove that DNase I exists in bile juice with obstructive jaundice, and suggest the possibility that it originates from liver tissue.

Key words: deoxyribonuclease I — obstructive jaundice — bile juice — apoptosis

Deoxyribonuclease I (DNase I) is an endonuclease that hydrolyzes double-stranded DNA down to 5'phospho-(tri-and/or tetra-) oligonucleotides.[1-3] Human and rat DNase I activities are demonstrated in some organs and body fluids by several zymographical methods such as the singleradical enzyme diffusion method,[4] overlay gel method[5] and activity gel method.[6] DNase I activities are found in digestive organs such as the pancreas, small intestine, and salivary gland, and body fluids; i.e., the pancreatic juice and saliva, and are also found in non-digestive organs such as the liver, kidney, spleen, placenta, heart, brain, lung, and thymus, and blood cells (leukocytes, erythrocytes) and body fluids; i.e., semen, breast milk, serum and urine.[4-6] However, it is still unclear whether DNase I exists in bile juice.

In vivo, DNase I has been regarded as a digestive enzyme released into the alimentary tract from the pancreas and salivary gland for degradation of alimentary DNA.[7,8]

Recently, it has been thought that DNase I is related to apoptosis as a regulatory event in every organ.[9-11] Biochemically, apoptosis is characterized

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by the internucleosomal DNA cleavage in which DNase I activity increases in apoptotic cells.[12-14] Thus, intracellular DNase I activity is a useful indicator of apoptosis.[15] Furthermore, DNase I in urine also appears as the result of apoptosis in distal tubular and collecting duct cells.[16-18] DNase I is thought to transfer into the nucleus from cytoplasm in its original cell by apoptosis-inducing factors, moreover it is also released into the extracellular fluid.[19] Therefore, the elevation of extracellular DNase I is also thought to be associated with apoptosis in its original cells.

Bhathal *et al*[20] showed that hyperplastic liver cells during CBD obstruction were deleted by apoptosis after CBD decompression.

Therefore, DNase I in bile juice may be associated not only with the degradation of alimentary DNA, but also with apoptosis in liver tissue under pathogenesis-like obstructive jaundice.

In this study, we demonstrated the existence of DNase I in bile juice and also investigated its origin.

MATERIALS AND METHODS

Chemicals

A Klenow fragment (Klenow polymerase) of E. coli DNA polymerase I was purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Highly polymerized calf thymus DNA and deoxynucleotide triphosphates (dNTPs) were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Nylon membrane(Hybond-N) was purchased from Amersham International Plc (Buckinghamshire, England). Digoxygenin(DIG)-labeled deoxyuridine triphosphate (dUTP) and the DIG Luminescent Detection Kit for nucleic acid were obtained from Boehringer Mannheim (Mannheim, Germany).

Preparation of bovine pancreatic DNase I

Purified bovine pancreatic DNase I was obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan). The sample was eluted in 100 mM sodium acetate, pH 5.0, 5 mM $MgSO_4$ and 50% glycerol, and was stored at -20°C until use.

Preparation of crude samples from human bile juice

Three patients with obstructive jaundice (one pancreatic cancer patient and two CBD cancer patients) were included in this study after their informed consents were obtained for the use of bile juice samples for the detection of DNase I. Each bile juice sample was collected from the PTCD tube. These samples were obtained immediately after PTCD and during the first, second and third weeks after PTCD. They were centrifuged at 3,000 x g for 10 min, and then each supernatant was frozen, dried and eluted in 100 mM sodium acetate, pH 5.0 containing 0.3 mM phenylmethylsulfonylfluoride (PMSF) of a twofold volume to weight-gram. Thereafter, they were stored at -80°C until use.

Preparation of crude samples from rat bile juice

The protocol for this experiment was reviewed and approved by the Ethical Committee for Animal Studies at Kawasaki Medical School (No. 96-129). Ten Wistar male rats (200 g) were operated under ether anesthesia. The CBD in each rat was ligated by placing three ties (3-0 surgical silk) between

its confluence with the most caudal hepatic duct and the pancreas. Bile juice samples were obtained on the seventh day after CBD ligation, and were centrifuged at 3,000 x g for 10 min. Each supernatant was frozen, dried and eluted in 100 mM sodium acetate, pH 5.0, containing 0.3 mM PMSF of a twofold volume to dried weight-gram. The samples were stored at -80°C until use.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was essentially carried out using the method reported by Laemmli[21] with minor modifications. The electrode buffer contained 0.025 M Tris-base, 0.192 M glycine and 0.1% SDS, pH 8.3. The stacking gel contained 3% acrylamide, 0.08% N,N′-methylene-bisacrylamide, 0.125 M TrisHCl, pH 6.8, 0.1% SDS, 0.1% N,N,N′,N′-tetramethylethylenediamide(TEMED) and 0.03% ammonium persulfate. The separation gel contained 12% acrylamide, 0.33% N,N′-methylene-bisacrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% TEMED and 0.05% ammonium persulfate. The samples in the gel loading buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 2.5% β-mercaptoethanol (β-ME), 0.25 M sucrose and 0.01% bromphenol blue) were incubated at 100°C for 2 min., and loaded onto the gel. Electrophoresis was carried out at room temperature at a constant current of 20 mA. An LMW Electrophoresis Calibration Kit obtained from Pharmacia Biotech (Uppsala, Sweden) was used for the molecular mass marker for SDS gel electrophoresis.

Non-radioactive Zymographical Method

DNase I was essentially assayed using the activity blotting method reported by Seki *et al*,[22,23] with some modifications. The zymographical method consisted of the following six steps ; 1) preparation of crude samples and purified samples ; 2) SDS-(denatured)-PAGE for protein separation ; 3) renaturation of the proteins separated on SDS-PAGE ; 4) preparation of native DNA fixed membranes ; 5) protein blotting onto the native DNA-fixed membrane at 30°C, for 48 hr., a process during which incision is introduced to the native DNA by DNase I ; 6) detection of the activity-blotted sites to localize the DNase I .

The incision provides priming sites for DNA synthesis. The blotted membrane is incubated with DNA polymerase in the presence of non-radioactively (digoxygenin-) labeled substrates. The site of substrate incorporation on the membrane that reflects the molecular weight of the enzyme is finally visualized on an X-ray film by fluorescence of digoxygenin.

Among these steps, we changed the renaturation and blotting buffer systems. For the detection of DNase I in human bile juice samples, the renaturation buffer system was changed to 100 mM sodium cacodylate, pH 6.5 containing 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.01% NaN_3 and 0.01% TritonX-100, and the blotting buffer system was changed to 100 mM sodium cacodylate, pH 6.5 containing 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.01% NaN_3 and 7 mM β-ME. For the detection of DNase I in rat bile juice samples, the renaturation buffer system was changed to 40 mM Tris-HCl, pH 7.6 containing 10 mM $MgCl_2$, 1mM $CaCl_2$, 0.01% NaN_3 and 0.01% Triton-X100, and the blotting buffer system was changed to 40 mM Tris-HCl, pH 7.6 containing 10 mM $MgCl_2$,

1mM $CaCl_2$, 0.01% NaN_3 and 7 mM β-ME.

The DNase I in each sample was detected with bovine pancreatic DNase I as the control. To determine the DNase I activity in each sample, each band detected on an X-ray film was measured as a spot density by a spectrophotometer. These were expressed as relative scores for Kunitz units of bovine pancreatic DNase I.[24]

Protein determination

Proteins were determined by the Bio-Rad protein assay (Hercules, CA) using bovine serum albumin as a standard.

RESULTS

Detection of DNase I in human bile juice

Figure 1 shows the bands detected in each human bile juice sample obtained from three obstructive jaundice patients with a bovine pancreatic DNase I sample as the control (Fig 1). A band in lane 1 (purified bovine pancreatic DNase I) indicated a molecular weight of 31 KDa, and also each band in lanes 2, 3 and 4 (lane 2; a bile juice sample from a pancreatic cancer patient, lanes 3 and 4; bile juice samples from two CBD cancer patients) indicated a molecular weight of 34 KDa (Fig 1).

Figure 2A shows the optimal pH for the band of 34 KDa detected in human bile juice by changing each renaturation and blotting buffer system. Lanes 1, 2 and 3 were detected in each renaturation and blotting buffer system made at pH 6.0, 6.5 and 7.5. A stronger band was observed at pH 6.5 than at pH 6.0 or 7.5 (Fig 2A). Figure 2B shows the ion requirement for the band of 34 KDa detected in human bile juice by changing divalent cations in each renaturation and blotting buffer system. Each band was detected in each renaturation and blotting buffer system including 20 mM $MgCl_2$ alone in lane 1, 10 mM $MgCl_2$ and 1mM $CaCl_2$ in lane 2, and 20 mM $CaCl_2$ alone in lane

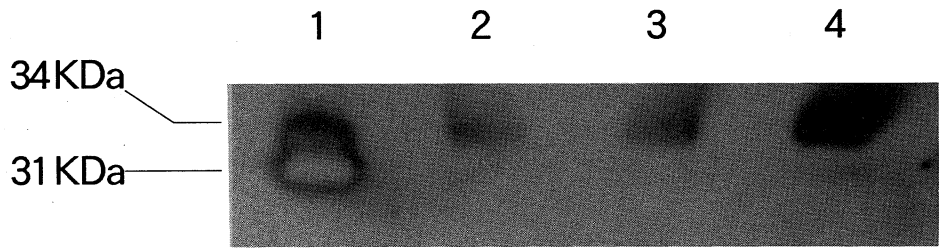

Fig 1. Detection of DNase I in human bile juice
Purified bovine pancreatic DNase I and crude human bile juice samples were electrophoresed on an SDS-polyacrylamide gel. Then, the separated proteins in the gel were renatured, and blotted onto a native DNA fixed membrane in the buffer systems as described in MATERIALS AND METHODS. The membrane was incubated for DNA synthesis and the sites of substrate incorporation (primed sites) on the membrane were visualized by digoxygenin luminescent detection. Proteins were applied as follows; lane 1, purified bovine pancreatic DNase I, 12 μg; lane 2, a crude human bile juice sample from a pancreatic cancer patient, 120 μg; lanes 3 and 4, crude human bile juice samples from CBD cancer patients, 120 μg.

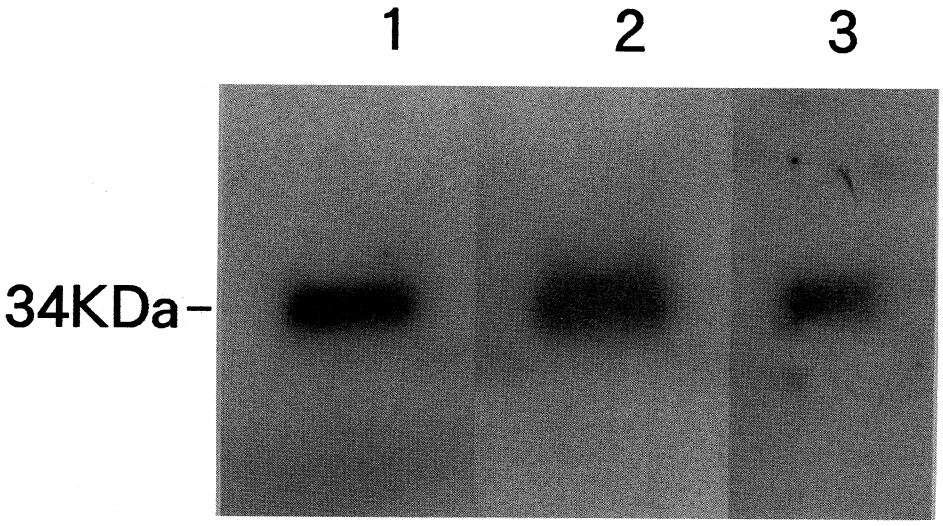

Fig 2A.

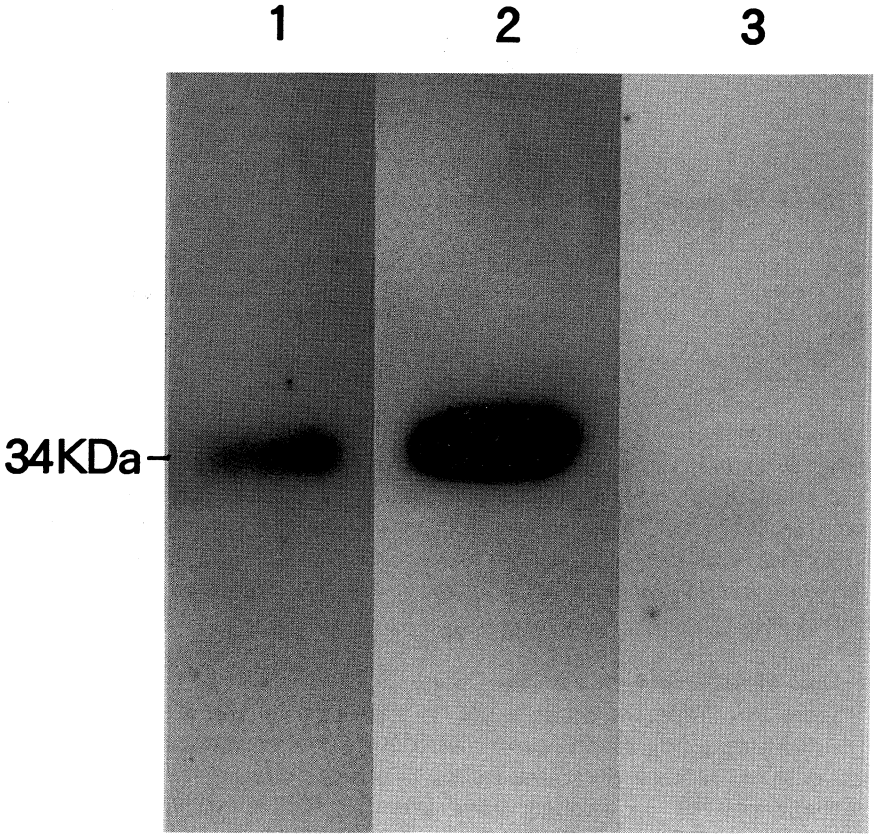

Fig 2B.

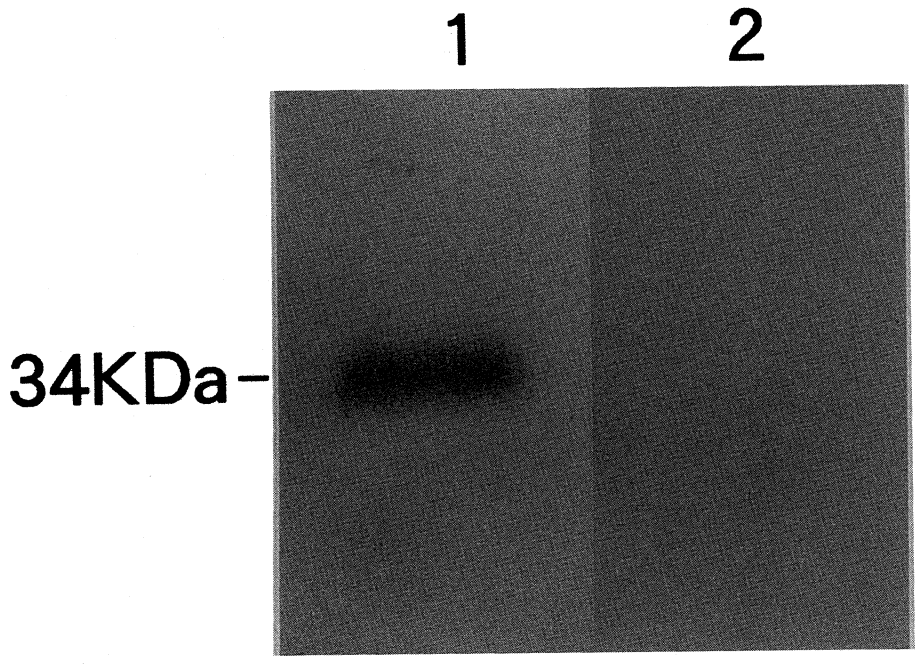

Fig 2C.

Fig 2. Human bile juice DNase I dependence on pH and divalent cations
Figure 2A shows the optimal pH for human bile juice DNase I activity. Lanes 1, 2 and 3 were detected in each renaturation and blotting buffer system of pH 6.0, 6.5 and 7.5. Figure 2B shows the ion dependency for human bile juice DNase I activity. All bands were detected in each renaturation and blotting buffer system including 20 mM $MgCl_2$ in lane 1, 10 mM $MgCl_2$ and 1mM $CaCl_2$ in lane 2, and 20 mM $CaCl_2$ in lane 3. Figure 2C shows the effect of G-actin for human bile juice DNase I activity. The band indicated a molecular weight of 34 KDa was detected in each renaturation and blotting buffer system added 10 μg/ml G-actin in lane 1 and 20 μg/ml G-actin in lane 2. In all lanes, 120 μg of protein was loaded into each lane.

3. A weaker band was observed with magnesium alone and no-band was observed with calcium alone (Fig 2B). Figure 2C shows the effect of G-actin for the band of 34 KDa detected in human bile juice. The band of 34 KDa detected in human bile juice was detected in each renaturation and blotting buffer system added 10 μg/ml G-actin in lane 1 and 20 μg/ml G-actin in lane 2. A weak band was observed in lane 1, and no-band was observed in lane 2 (Fig 2C).

Detection of DNase I in rat bile juice

Figure 3A shows the optimal pH for the band of 31.6 KDa detected in rat bile juice by changing renaturation and blotting buffer systems. Lanes 1, 2 and 3 were detected in each renaturation and blotting buffer system made at pH 7.0, pH 7.6 and pH 8.0. A stronger band was observed at pH 7.6 than at pH 7.0 or 8.0 (Fig 3A). Figure 3B shows the ion requirement for the band of 31.6 KDa in rat bile juice by changing divalent cations in each renaturation and

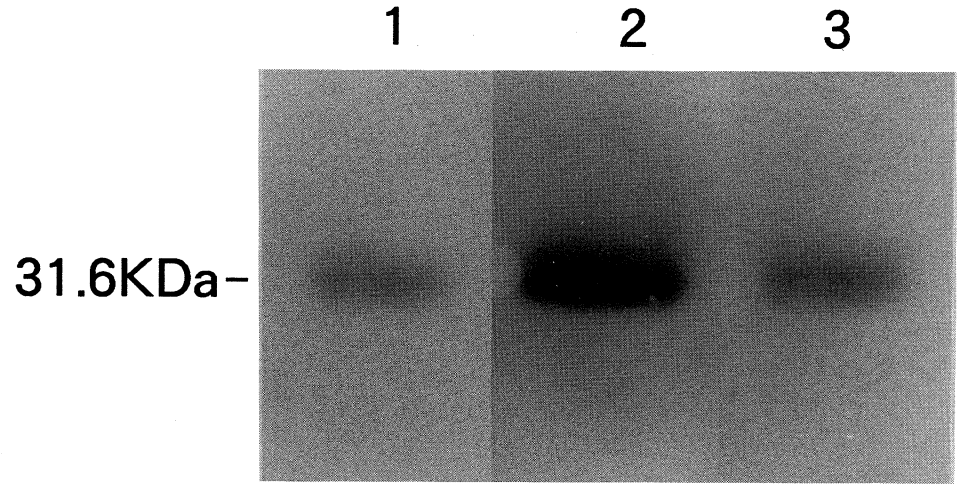

Fig 3A.

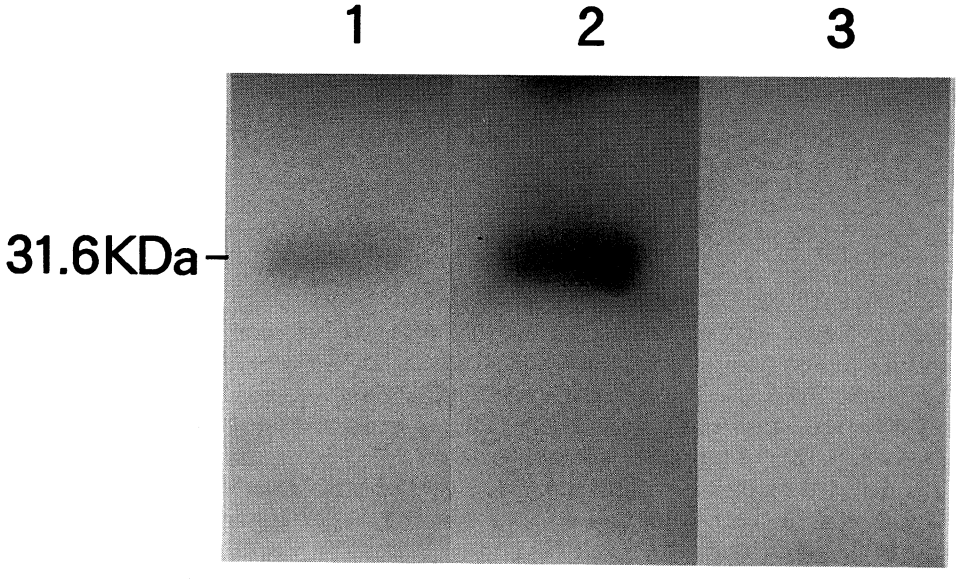

Fig 3B.

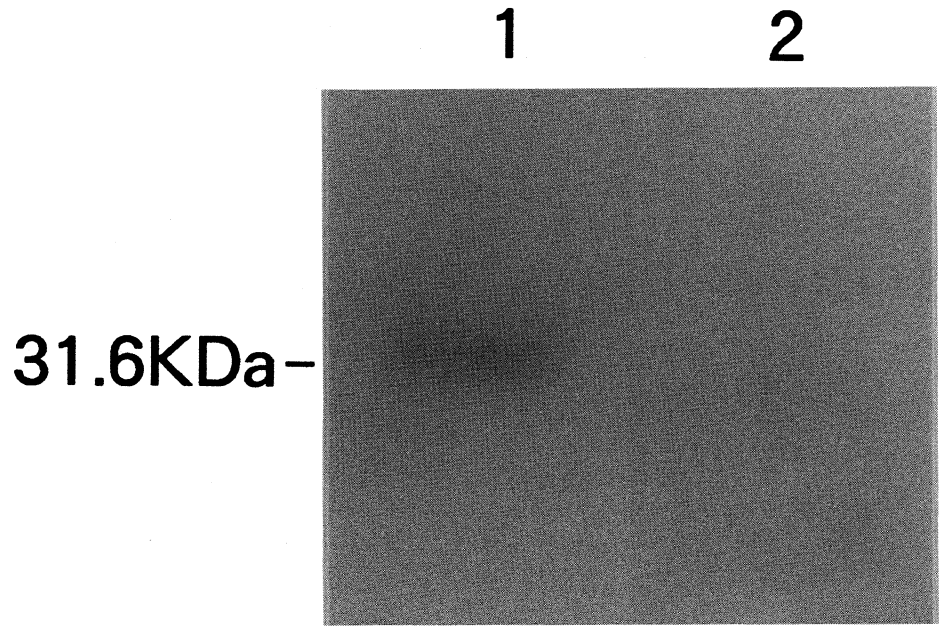

Fig 3C.

Fig 3. Rat bile juice DNase I dependence on pH and divalent cations
Figure 3A shows the optimal pH for rat bile juice DNase I activity. Lanes 1, 2 and 3 were detected in each renaturation and blotting buffer system of pH 7.0, 7.6 and 8.0. Figure 3B shows the ion dependency for rat bile juice DNase I activity. All bands were detected in each renaturation and blotting buffer systems including 20 mM $MgCl_2$ in lane 1, 10 mM $MgCl_2$ and 1 mM $CaCl_2$ in lane 2, and 20 mM $CaCl_2$ in lane 3. Figure 3C shows the effect of G-actin for rat bile juice DNase I activity. The band indicated a molecular weight of 31.6 KDa was detected in each renaturation and blotting buffer system added 5 μg/ml G-actin in lane 1 and 10 μg/ml G-actin in lane 2. In all lanes, 120 μg of protein was loaded into each lane.

blotting buffer system. Each band was detected in each renaturation and blotting buffer system including 20 mM $MgCl_2$ alone in lane 1, 10 mM $MgCl_2$ and 1 mM $CaCl_2$ in lane 2, and 20 mM $CaCl_2$ alone in lane 3. A weaker band was observed with magnesium alone and no band was observed with calcium alone (Fig 3B). Figure 3C shows the effect of G-actin for the band of 31.6 KDa detected in rat bile juice. The band of 31.6 KDa detected in rat bile juice was detected in each renaturation and blotting buffer system added 5 μg/ml G-actin in lane 1 and 10 μ g/ml G-actin in lane 2. A weak band was observed in lane 1, and no-band was observed in lane 2 (Fig 3C).

Serial changes of human bile juice DNase I after PTCD

Figure 4 shows the serial changes of DNase I in human bile juice and total bilirubin in serum after PTCD. The mean relative scores for Kunitz units of DNase I in three human bile juice samples were 1.32×10^{-4}, 1.36×10^{-4}, 2.42×10^{-4} and 3.45×10^{-4} ($/\mu$g) immediately after PTCD and at the first, second and third weeks after PTCD, respectively (Fig 4). The mean total bilirubin in the three human serum samples, on the contrary decreased from 10. 8 to 2.8 to 0.8 to 0.7 (mg/dl) at each week after PTCD (Fig 4).

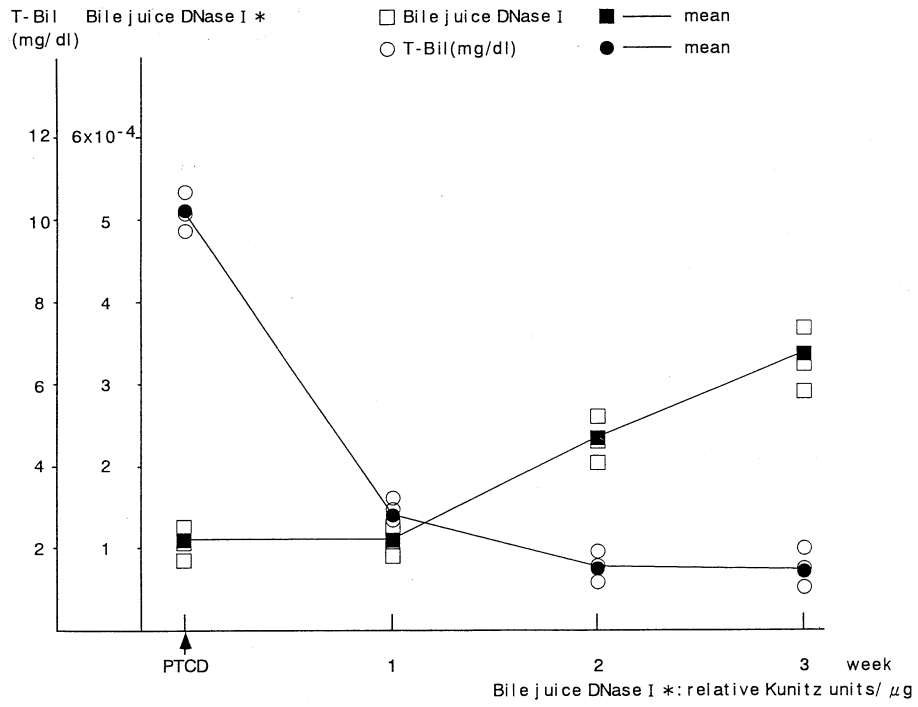

Fig 4. Serial changes in human bile juice DNase I and serum bilirubin after PTCD. Human bile juice DNase I increased in contrast to a decrease in serum bilirubin after PTCD.

DISCUSSION

This study demonstrated the existence of DNase I in human and rat bile juice by modifications of an activity blotting method.[22,23] Human bile juice samples were obtained from patients with pancreatic cancer or CBD cancer after PTCD. Rat bile juice samples were obtained by experimental CBD ligation. The identifications of DNase I were based on six criteria: substrate specificity, DNA nicking pattern, pH dependence, ion requirements, inhibition by actin and molecular weight.

The enzyme detected in human bile juice was a specific enzyme producing 3'-hydroxyl-termies as the priming site for DNA polymerase on native DNA. The enzyme activity was much stronger at pH 6.5 than at pH 6.0 or 7.5 with both magnesium and calcium ions. Furthermore, 20 μg/ml G-actin completely inhibited the enzyme activity. These characteristics were consistent with those of DNase I previously reported in human kidney and urine.[17,18] The enzyme detected in human bile juice indicated a molecular weight of 34 KDa on SDS-PAGE, whereas Yasuda *et al.* reported that their purified human urine and kidney DNase I indicated a molecular weight of 38 KDa on SDS-PAGE.[18] This discrepancy might be attributed to the difference in the preparations of each sample. Human urine DNase I under the crude condition has been shown to have a molecular weight of 34 KDa on SDS-PAGE.[18] Therefore, the enzyme detected in human bile juice is thought

to be compatible with the DNase I previously reported in human urine,[18] since human bile juice samples were prepared under the crude condition in this experiment. These results prove that DNase I exists in human bile juice under CBD obstruction.

To explore whether DNase I also exists in rat bile juice under CBD obstruction, we made a rat experimental model by CBD ligation. Rat bile juice was obtained on the seventh day after CBD ligation. Then, we tried to detect DNase I in rat bile juice by modifications of a non-radioactive activity blotting method.

The enzyme detected in rat bile juice was a specific enzyme for DNA as well as that human bile juice DNase I digested double-stranded DNA down to 3'-hydroxyl-termies. The enzyme activity was much stronger at pH 7.6 than at pH 7.0 or 8.0 with both magnesium and calcium ions. Furthermore, 10 μ g/ml G-actin completely inhibited the enzyme activity. The enzyme in rat bile juice indicated a molecular weight of 31.6 KDa. These characteristics differed from the characteristics of human bile juice DNase I. This discrepancy is thought to be due to the difference in species; i. e., humans and rats, since the characteristics of the enzyme detected in rat bile juice were compatible with those of DNase I previously reported in rat organs.[6] Therefore, the enzyme detected in rat bile juice is DNase I. This finding proves that DNase I also exists in rat bile juice under experimental CBD obstruction.

DNase I is generally regarded as an exocrine enzyme that is released into pancreatic juice from the pancreas and into saliva from the salivary gland.[25,26] Bile juice DNase I may be also released from liver tissue, since DNase I distributes in human and rat liver tissues.[6,27]

We also investigated the serial changes in human bile juice DNase I and serum bilirubin after PTCD. Human bile juice DNase I increased in contrast to a decrease in serum bilirubin after PTCD. These results indicate that changes in human bile juice DNase I depend on the degree of biliary stasis. This suggests the possibility that the levels of bile juice DNase I depend on the liver function following to the biliary stasis.

DNase I is also known to be an enzyme that produces internucleosomal DNA cleavage in apoptotic cells.[9-14] DNase I is not only transferred into the nucleus from cytoplasm in its original cells by apoptosis-inducing factors, but also into extracellular fluids.[19] Therefore, the level of extracellular DNase I is thought to be affected by apoptosis in DNase I's original cells.

Bhathal *et al*[20] have shown that many apoptotic cells appear in liver cells after CBD decompression in rat experimental models by CBD ligation. The elevation of human bile juice DNase I after PTCD was compatible with the phase of CBD decompression. This suggests the possibility that human bile juice DNase I increased after PTCD due to apoptosis in liver tissue induced by CBD decompression.

The future investigation of DNase I levels in liver tissue is important to clarify the significance of bile juice DNase I.

The above findings clarify that DNase I exists in bile juice and suggest the possibility that it originates from the liver tissue.

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