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Induction of DNA Replication and Cell Growth in Rat Liver by Obstructive Jaundice

Masaki Terasaki,[1] Hiroshi Kuriki,[1] Yuji Nimura,[1] Shigehiko Shionoya,[1] Kiyohide Kojima[2] and Shonen Yoshida[2,3]

[1]*The First Department of Surgery and* [2]*Laboratory of Cancer Cell Biology, Institute for Disease Mechanism and Control, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466*

Obstructive jaundice, produced by ligating the common bile duct, induced a transient DNA replication followed by cell proliferation in rat liver. At 48 h after the operation, DNA polymerase α activity started to increase and reached its maximum level (more than twice the control) at day 4. At day 7, the enzyme level had decreased to the control level. Pulse-labeling experiment using radioactive thymidine showed that the rate of DNA synthesis increased approximately 2.5-fold in the same pattern as that of DNA polymerase α. The mitotic index in hepatocytes also increased 10-fold at day 4 and then decreased. The proliferation of liver cells induced by obstructive jaundice mimics the regeneration of partially hepatectomized liver, although the response was slightly delayed and the proliferation was transient.

Key words: Obstructive jaundice — Liver cell growth — DNA polymerase α — DNA replication

Recently, technical progress in hepatic surgery has enabled us to treat hepatic malignancy with obstructive jaundice, e.g., carcinoma of the hepatic hilus or the gallbladder. Several groups have reported that the existence of preoperative obstructive jaundice tended to cause postoperative complications[1-4] and recommended the use of percutaneous transhepatic biliary drainage (PTBD) to reduce the serum bilirubin level to a normal level before hepatic resection.[5] However, hepatectomy has occasionally been carried out without preoperative biliary drainage by other groups.[6,7] In connection with this, it was also reported that PTBD did not reduce the operative risk in hepatectomy.[8,9] On the other hand, many studies have been performed using experimental animals to evaluate the effect of obstructive jaundice on liver regeneration.[10-15] However, it is still a matter of debate whether or not obstructive jaundice itself inhibits liver cell proliferation. To solve this problem, we have started a systemic study to evaluate the regenerating capacity of rat liver with obstructive jaundice. The liver regeneration induced by partial hepatectomy is associated with the induction of a number of enzymes involved in DNA replication. Since DNA polymerase α plays a central role in eukaryotic DNA replication, the activity of this enzyme can be used as a marker for proliferation capacity. First, we measured the DNA polymerase α activity in rat liver with obstructive jaundice induced by ligation of the common bile duct. Contrary to our expectation, DNA polymerase α activity significantly increased after ligation of the common bile duct and the elevated level was maintained for several days. Here, our study was focused on this unusual phenomenon, and we found that the obstruction of the bile duct induced DNA replication and mitosis of hepatocytes without partial hepatectomy, in agreement with previous reports by Ferguson *et al.*[16] and Andrus *et al.*[17] The significance of this phenomenon in surgical treatment of the liver is discussed.

MATERIALS AND METHODS

Animals Adult male rats (200–250 g) of the Donryu strain were purchased from Chubu Shiryo Co. (Nagoya). Food was withheld 12 h before the operation. The rats were divided into two groups. The laparotomy was performed through a midline incision under ether anesthesia. In the operation group, the common bile ducts were ligated and divided to prevent recanalization. Sham operation was carried out with the other group, i.e., laparotomy and manipulation of the liver and the common bile duct except ligation. The day of the operation was defined as day 0. In both groups, three or five rats were killed between 10 and 12 a.m. at days 1, 2, 3, 4, 5 and 7 after the operation, respectively, and the livers were removed, immediately weighed and stored at −80°C.

Enzyme extraction One g of the liver was homogenized with a Dounce homogenizer and sonicated for 30 s in 9 volumes of 50 m*M* Tris-HCl (pH 7.5) containing 10% glycerol, 1 m*M* dithiothreitol (DTT) (Nakarai Chemical Co., Kyoto), 1 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO), leupeptin (1 μg/ml, Peptide Institute Inc., Osaka), 0.5% Triton

[3] To whom correspondence should be addressed.

X-100, 0.1 m*M* EDTA, and 0.5 *M* KCl. A half volume of each sample was used for measuring DNA content and protein concentration. The remainder of each sample was clarified by centrifugation at 18,000*g* for 30 min at 4°C. The supernatant was carefully collected and used for the assay of DNA polymerase activity. In measuring DNA polymerase α activity, the supernatant was diluted 5-fold with a solution containing 1 mg/ml bovine serum albumin (BSA), 25 m*M* Tris-HCl (pH 7.5), 1 m*M* DTT, and 10% glycerol. In determining DNA polymerase β activity, the supernatant was diluted 100-fold with the same solution used for DNA polymerase α.

Assays of DNA polymerases The reaction mixture for DNA polymerase α (50 μl) contained 80 m*M* potassium phosphate (pH 7.2), 8 m*M* 2-mercaptoethanol (2-ME), 200 μg/ml activated calf thymus DNA, 80 μM each of dATP, dGTP, and dCTP, 40 μM [^{3}H]dTTP (1,000 cpm/pmol), 8 m*M* $MgCl_2$, 200 μg/ml BSA and enzyme. The reaction mixture for DNA polymerase β (50 μl) contained 0.1 *M* Tris-HCl (pH 8.8), 5 μg of $(dA)_n/(dT)_{12\text{-}18}$ (base ratio of A/T=5), 80 μM dTTP, 40 μM [^{3}H]dTTP (1,000 cpm/pmol), 0.1 *M* NaCl, 4 m*M* MalNet, 0.5 m*M* $MnCl_2$ and 0.2 mg/ml BSA. Incubation was carried out at 37°C for 30 min. The reaction was terminated by the addition of 0.5 ml of cold 10% trichloroacetic acid (TCA) containing 0.2% sodium pyrophosphate and acid-insoluble radioactivity was measured as described previously.[18]

Others Protein concentration was measured by the method of Lowry *et al.*[19] using BSA as a standard and DNA content was determined by fluorescence assay, using Hoechst 33258.[20] Total DNA or total protein per liver was calculated from the total wet weight of the isolated livers. Glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP) were measured as described.[21, 22]

Measurement of DNA synthesis *in vivo* Rats were operated or sham-operated as described above. In both groups, at days 2, 4 and 7 after operation, [^{3}H]thymidine (specific activity, 2 Ci/mmol) was given intraperitoneally at a dose of 0.4–0.6 μCi/g body weight. Animals were killed 1 h after injection. The liver was removed and the blood in the liver was washed out immediately with physiological saline through the hepatic veins. One g of the liver was homogenized with a Dounce homogenizer in 9 volumes of the same buffer used for enzyme extraction and then sonicated for about 30 s. Sodium dodecyl sulfate (1%) and proteinase K (mg/ml) were added to each sample and incubation was carried out at 37°C for 1 h. Acid-insoluble radioactivity was measured in the same way as described for DNA polymerase assay using 0.5 ml of the sample.

Mitotic index A part of the median lobe of each liver used for DNA polymerase assay was fixed in formalin and stained with hematoxylin-eosin. With each slide, 30–50 visual fields (5,000–10,000 hepatocytes) were examined under the microscope at 400× magnifications and the hepatocytes with mitotic figures were counted.

RESULTS

Bilirubin and enzymes in blood As shown in Fig. 1(A), the level of the serum total bilirubin began to increase at day 1 after surgery and attained the level of about 5 mg/dl at day 2. This high level continued until 7 days after surgery. On the other hand, the levels of GPT and ALP rapidly increased at 1 day after surgery and then gradually decreased (Fig. 1(B)).

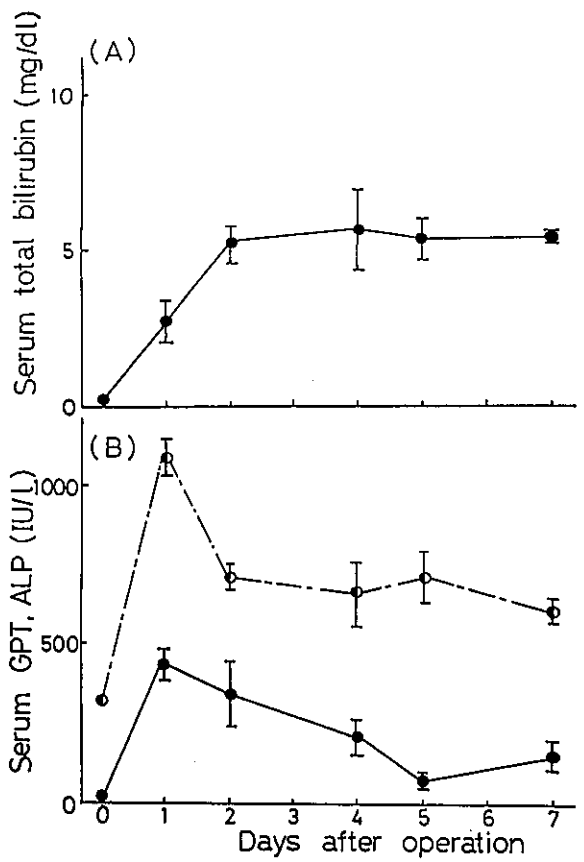

Fig. 1. Changes of bilirubin concentration and enzyme activities in rat serum after ligation and division of the common bile duct. Blood was withdrawn from the inferior vena cava under ether anesthesia and serum was obtained by centrifugation (3,000 rpm, 20 min). Total bilirubin concentration (A) and activities of GPT and ALP (B) were measured as described in "Materials and Methods" at the times indicated. ●, GPT; ◐, ALP.

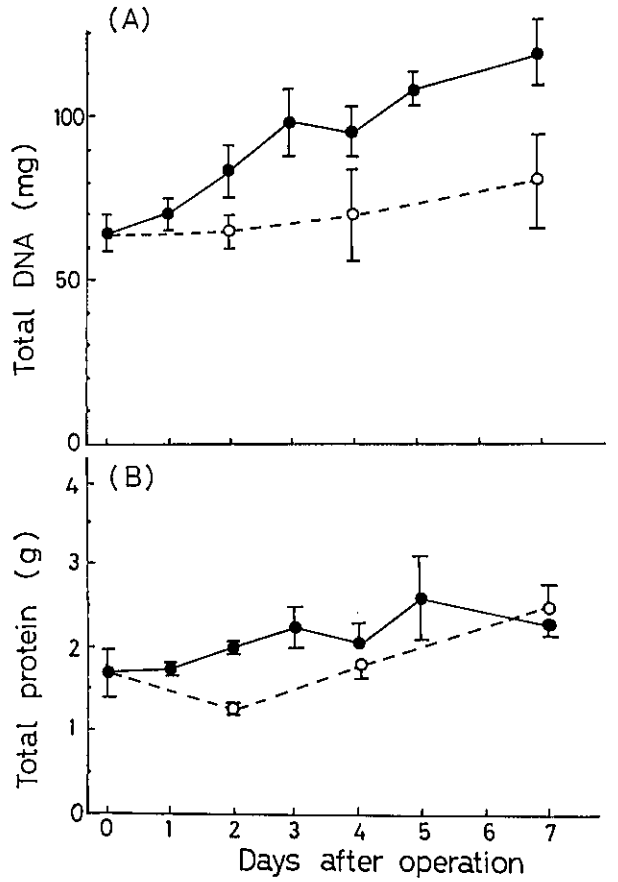

Fig. 2. DNA and protein contents. Contents of DNA (A) and protein (B) in the whole livers were measured with rats after biliary obstruction (●) and sham-operated rats (○), according to the methods described in "Materials and Methods."

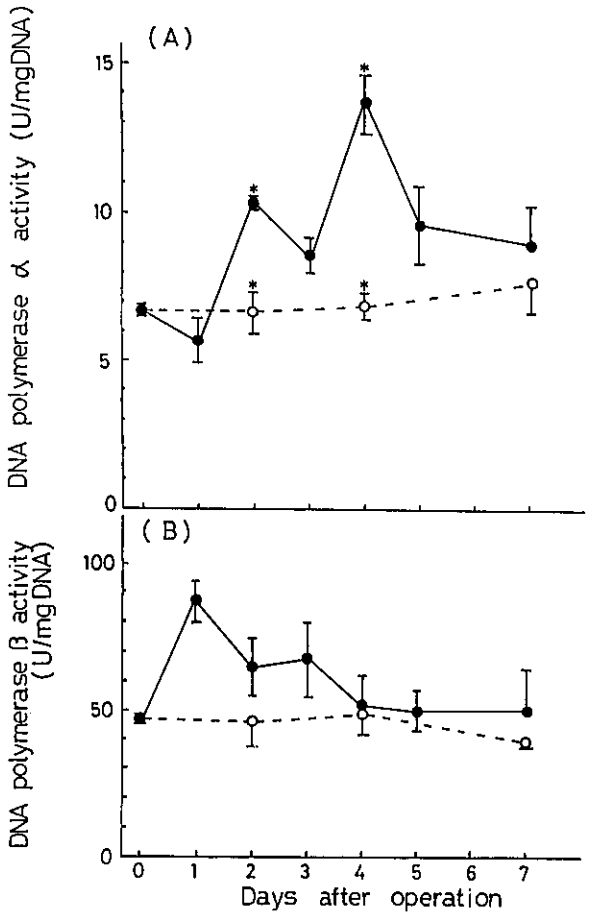

Fig. 3. Changes in the activities of DNA polymerases after obstruction of the common bile duct. DNA polymerase α (A) and β (B) activities were measured by the methods described in "Materials and Methods." Each point represents the mean ± SD for 3 or 5 animals. * $P < 0.01$, Student's t test. ○, sham-operated liver; ●, obstructed liver.

DNA and protein amounts Total DNA and protein in whole livers increased gradually with growth. A significant increase was observed in the DNA content in obstructed livers compared with those in the sham-operated livers (Fig. 2(A)), amounting to about 1.5-fold at day 7.

Activity of DNA polymerase α The level of DNA polymerase α activity was measured as a marker for cell proliferation. As shown in Fig. 3(A), DNA polymerase α activity in livers started to increase at day 2 after the operation and attained its maximal level at day 4, decreasing thereafter. At day 4 after the operation, the activity of DNA polymerase α was increased approximately 2-fold compared with that in the sham-operated group. However, at day 7 after surgery, no significant difference was observed compared with sham-operated livers (Fig. 3(A)).

Activity of DNA polymerase β DNA polymerase β activity increased rapidly at 1 day after operation and then decreased. This change was similar to those of the serum GPT and ALP (Fig. 3(B)).

Incorporation of [^{3}H]thymidine into DNA In order to know whether DNA replication is really enhanced by biliary obstruction or not, a pulse-labeling experiment was carried out. As shown in Fig. 4, the rate of incorporation of [^{3}H]thymidine into cellular DNA in the obstructed liver increased significantly compared with that in sham-operated liver, at days 2 and 4 after surgery. However, at day 7 after operation, there was no significant difference in [^{3}H]thymidine incorporation between the two groups (Fig. 4). At day 4 after surgery, the rate of DNA synthesis was 2.5 times higher in the obstructed liver than that in the control group. This large

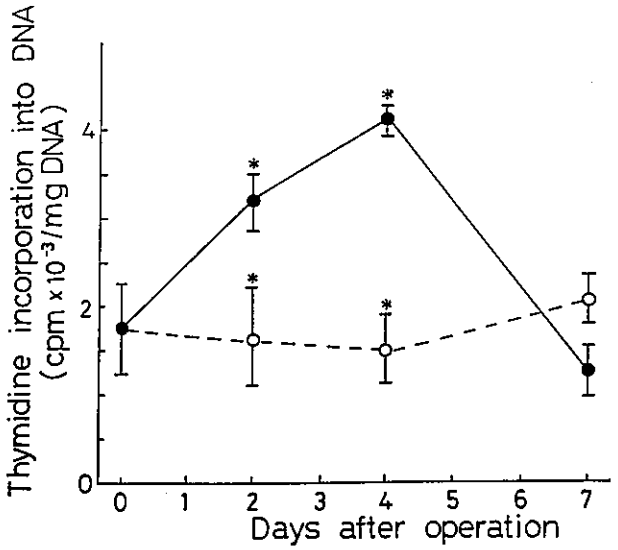

Fig. 4. Increase in incorporation of [^{3}H]thymidine into rat liver DNA after obstruction of the common bile duct. [^{3}H]-Thymidine was given intraperitoneally at a dose of 0.4–0.6 μCi/g body weight at the times indicated and labeled for 1 h. DNA synthesis was measured as described in "Materials and Methods." Each point indicates the mean±SD for 3 or 5 animals. $*P<0.01$, Student's t test. ○, sham-operated liver; ●, obstructed liver.

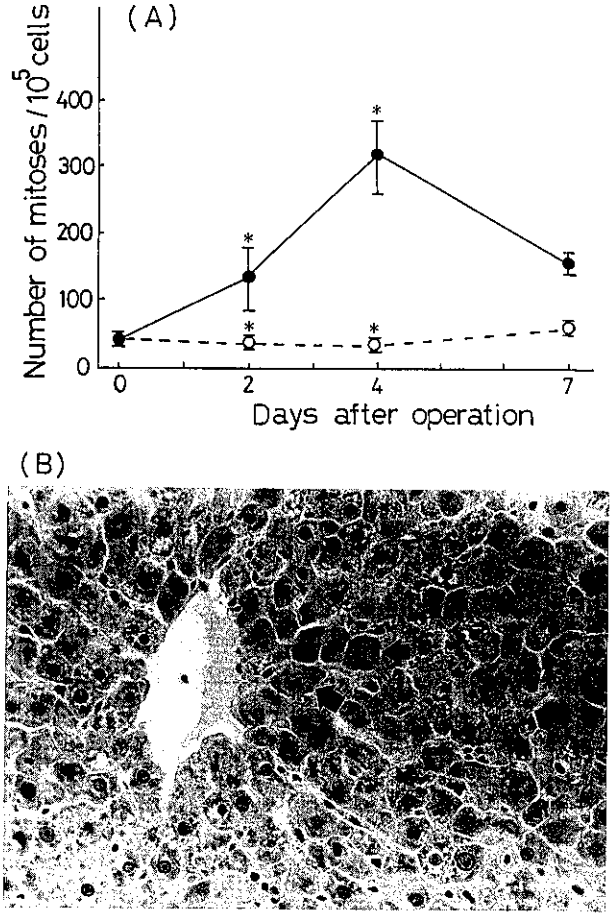

Fig. 5. Increase in the mitotic activity of hepatocytes after obstruction of the common bile duct. Removed livers were fixed and stained as described in "Materials and Methods." (A) Numbers of mitoses were expressed per 10^5 cells. $*P<0.01$, Student's t test. ○, sham-operated liver; ●, obstructed liver. (B) Photomicrograph of the lobules at day 4 after ligation and division of the common bile duct. Arrows indicate hepatocytes in the mitotic phase. 400× magnification.

amount of incorporation may be due to S phase cells rather than repair DNA synthesis. This was confirmed by the following experiment.

Mitotic activity of hepatocytes A part of each liver was fixed and stained to observe mitosis of hepatocytes. As shown in Fig. 5(A), the mitotic index (MI) of liver specimens from obstructed liver increased remarkably after surgery; it was 10 times higher than that of the sham-operated control at day 4 after surgery. As shown in Fig. 5(B), hepatocytic mitosis was clearly found in each lobule examined.

DISCUSSION

In the present study, we showed that experimental biliary obstruction of rat liver induced transient proliferation of hepatic cells, which was detected as increased DNA polymerase α activity, as well as an increase in the rate of incorporation of radioactive thymidine into DNA, an increase in total DNA per organ and finally, an increase in mitotic index. Since the cells in the mitotic phase were largely hepatic parenchymal cells rather than interstitial cells, the induction of cell growth might mimic the liver regeneration induced by partial hepatectomy. We have observed that DNA polymerase α, after two-thirds partial hepatectomy of normal liver, showed about 7-fold increase compared with the control level at 24 h after operation (data not shown). At the same time, it was observed that DNA polymerase β activity was also increased 2- to 2.5-fold at 5 h after operation and the high level was maintained for at least 24 h (data not shown). Thus, the pattern of induction of DNA polymerase α after partial hepatectomy slightly differed from that after biliary obstruction in both the time of response and the extent of induction. The induction of mitosis started at day 2 and reached maximum at day 4 after biliary obstruction. At these time points, hepatic cells of operated rats did not show

any morphological change. At present, the mechanism of induction of cell growth by the ligation of the common bile duct is unknown. However, it is tempting to speculate that, although there is no obvious morphological change, the hepatic cells could be damaged biochemically and physiologically by stagnated bile[23] and the damaged cells could generate a growth signal[24] to the intact cells. Alternatively, bilirubin or bile acids themselves might act as growth stimuli for hepatic cells. We are now testing the latter possibility by introducing bilirubin or bile acids into normal liver via the portal vein. Since the discovery of liver regeneration in partially hepatectomized rats by Higgins and Anderson in 1931,[10] many studies have been performed concerning cell growth, cell cycle and DNA replication using this system.[11] Several investigators examined the effects of obstructive jaundice on the regeneration capacity of partially hepatectomized liver.[12-14, 16, 17] Their conclusions were apparently contradictory. Mann and co-workers[12] found that obstructive jaundice inhibited liver regeneration and this conclusion was supported by Higgins and Anderson[13] and Cameron.[15] On the contrary, Ferguson *et al.*[16] reported that liver regeneration was better in bile duct-ligated rats than that of normal liver, and this was supported by Weinbren.[14] In 1961, Andrus *et al.*[17] found that obstructive jaundice itself induced liver cells to proliferate at days 1 and 2 after biliary obstruction. Since than, this interesting finding has not been studied further. The present data clearly indicate that biliary obstruction in rats induced proliferation of liver cells, but at day 7, the rate of proliferation decreased. It is conceivable that, in the course of prolonged obstructive jaundice for more than 7 days, liver cells become refractory to proliferation stimuli, due to biochemical and physical disturbances caused by stagnated bile. It may be important to know whether or not liver cells lose their regeneration capacity after prolonged obstructive jaundice and, if so, whether they can recover their capacity to regenerate after biliary drainage. Here we used DNA polymerase α activity as a marker for cell proliferation. It was shown in this study that the induction pattern of DNA polymerase α corresponded well with DNA synthesis measured in terms of the incorporation of [^{3}H]thymidine (Fig. 4), in agreement with our previous results with cultured human fibroblasts.[25] The assay of DNA polymerase α may be a simple and convenient way to assess cell growth in liver regeneration. It should be noted, however, that the assay conditions for DNA polymerase α employed here might also detect DNA polymerase δ,[26] which shows characteristics similar to those of the α-enzyme but is a distinct enzyme involved in chromosomal DNA replication as well. At present it is not certain how much of the total incorporation is due to DNA polymerase δ. Therefore we tentatively designated the incorporation as DNA polymerase α activity. DNA polymerase β activity rapidly increased at day 1 and then gradually decreased. This change was similar to those of serum GPT and ALP, indicators for submicroscopic damage of liver cells and bile duct cells. The elevation of DNA polymerase β might reflect the early repair processes induced by cell damage caused by ligation of the common bile duct, although the physiological role of DNA polymerase β has not been fully established yet.

In conclusion, the liver cells respond well to obstructive jaundice as a proliferation stimulus and proliferate transiently. This process may modulate the regeneration of liver after partial hepatectomy under pathological conditions causing obstructive jaundice. It is suggested that the duration of jaundice may be critical.

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REFERENCES

1) Nakayama, T., Ikeda, A. and Okuda, K. Percutaneous transhepatic drainage of the biliary tract. *Gastroenterology*, **74**, 554–559 (1978).
2) Pollock, T. W., Ring, E. R., Oleaga, J. A., Freiman, D. B., Mullen, J. L. and Rosato, E. F. Percutaneous decompression of benign and malignant obstruction. *Arch. Surg.*, **114**, 148–151 (1979).
3) Hansson, J. A., Hoevels, J., Simert, G., Tylén, U. and Vang, J. Clinical aspects of nonsurgical percutaneous transhepatic bile drainage in obstructive lesions of the extrahepatic bile ducts. *Ann. Surg.*, **189**, 58–61 (1979).
4) Denning, D. A., Ellison, E. C. and Carey, L. C. Preoperative percutaneous transhepatic biliary decompression lowers operative morbidity in patients with obstructive jaundice. *Am. J. Surg.*, **141**, 61–65 (1981).
5) Nimura, Y., Hayakawa, N., Kamiya, J., Kondo, S. and Shionoya, S. Hepatic segmentectomy with caudate lobe resection for bile duct carcinoma of the hepatic hilus. *World J. Surg.*, **14**, 535–544 (1990).
6) McPherson, G. A. D., Benjamin, I. S., Hodgson, H. J. F.,

Bowley, N. B., Allison, D. J. and Blumgart, L. H. Preoperative percutaneous transhepatic biliary drainage: the results of a controlled trial. *Br. J. Surg.*, **71**, 371–375 (1984).
7) Lai, E. C. S., Tompkins, R. K., Roslyn, J. J. and Mann, L. L. Proximal bile duct cancer. Quality of survival. *Ann. Surg.*, **205**, 111–118 (1987).
8) Blumgart, L. H., Hadjis, N. S., Benjamin, I. S. and Beazley, R. Surgical approaches to cholangiocarcinoma at confluence of hepatic ducts. *Lancet*, **i**, 66–69 (1984).
9) Bengmark, S., Ekberg, H., Evander, A., Klofver-Stahl, B. and Tranberg, K-G. Major liver resection for hilar cholangiocarcinoma. *Ann. Surg.*, **207**, 120–125 (1988).
10) Higgins, G. M. and Anderson, R. M. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch. Pathol.*, **12**, 186–202 (1931).
11) Bucher, N. L. and Swaffield, M. N. The rate of incorporation of labeled thymidine into the deoxyribonucleic acid of regenerating rat liver in relation to the amount of liver excised. *Cancer Res.*, **24**, 1611–1625 (1964).
12) Mann, F. C., Fishback, F. C., Gay, J. G. and Green, G. F. Experimental pathology of the liver. III, IV, V. *Arch. Pathol.*, **12**, 787–793 (1931).
13) Higgins, G. M. and Anderson, R. M. Experimental pathology of the liver. VII. Restoration of the liver after partial surgical removal and ligation of the bile duct in white rats. *Arch. Pathol.*, **14**, 42–49 (1932).
14) Weinbren, K. The effect of bile duct obstruction on regeneration of the rat's liver. *Br. J. Exp. Pathol.*, **34**, 280–289 (1953).
15) Cameron, G. R. Liver regeneration and biliary obstruction. *J. Pathol. Bacteriol.*, **41**, 283–287 (1935).
16) Ferguson, C. C., Rogers, C. S. and Vars, H. M. Liver regeneration in the presence of common bile duct obstruction. *Am. J. Physiol.*, **159**, 343–350 (1949).
17) Andrus, C. H., Pechet, G. and MacDonald, R. A. Bile duct obstruction and regeneration of the liver. *Proc. Soc. Exp. Biol. Med.*, **106**, 809–812 (1961).
18) Yoshida, S., Kondo, T. and Ando, T. Multiple molecular species of cytoplasmic DNA polymerase from calf thymus. *Biochem. Biophys. Acta*, **353**, 463–474 (1974).
19) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
20) Labarca, C. and Paigen, K. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.*, **102**, 344–352 (1980).
21) Bergmeyer, H. V., Kreutz, F. H., Pilz, W., Schmidt, F. W., Buttner, H., Lang, H., Rick, W., Stamm, D., Hillman, G., Laue, D., Schmidt, E. and Szasz, G. Recommendations of the German Society for Clinical Chemistry. *Z. Klin. Chem. Klin. Biochem.*, **10**, 281–291 (1972).
22) Bessy, O. A., Lowry, O. H. and Brock, M. J. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. Biol. Chem.*, **164**, 321–329 (1946).
23) Miyata, K. Delayed recovery of mitochondrial function in rat liver after releasing biliary obstruction. *Nagoya J. Med. Sci.*, **45**, 97–105 (1983).
24) Nakamura, T., Nawa, K. and Ichihara, A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.*, **122**, 1450–1459 (1984).
25) Nakamura, H., Morita, T., Masaki, S. and Yoshida, S. Intracellular localization and metabolism of DNA polymerase α in human cells visualized with monoclonal antibody. *Exp. Cell. Res.*, **151**, 123–133 (1984).
26) Byrnes, J. J., Downey, K. M., Black, V. L. and So, A. G. A new mammalian DNA polymerase with 3′ to 5′ exonuclease activity: DNA polymerase δ. *Biochemistry*, **15**, 2817–2823 (1976).