ORIGINAL ARTICLE

Determination of a Safe Vascular Clamping Method for Liver Surgery

Evaluation by Measuring Activation of Calpain μ

Meng Wang, MD; Masato Sakon, MD; Koji Umeshita, MD; Hideyuki Miyoshi, MD; Kazunori Taniguchi, MD; Shin-ichi Kishimoto, MD; Shinobu Imajoh-Ohmi, PhD; Morito Monden, MD

Objective: To determine the safest method of hepatic vascular clamping associated with the least ischemia-reperfusion injury of the liver during liver surgery.

Setting: University laboratories.

Subjects: Sixty-five adult male Wistar rats.

Methods: The hilar area of the left lateral and median lobes of rat liver was clamped for 10 minutes (group 1), 15 minutes (group 2), or 20 minutes (group 3) followed by 5 minutes of reperfusion. The procedure was repeated for a total period of ischemia of 60 minutes in each group. Control rats underwent laparotomy without vascular clamping. In addition to histological examination, we determined calpain μ activity, a marker of liver injury, by Western blotting using specific antibodies against the intermediate (activated) and proactivated forms of calpain μ. Measurements were performed at the end of ischemia and after 2 hours of reperfusion. We also determined the degradation of talin, an intracellular substrate of calpain μ, by Western blotting.

Results: The level of adenosine triphosphate and energy charge at 2 hours after reperfusion did not change after ischemia-reperfusion irrespective of the duration of ischemic cycle. After 60 minutes of intermittent ischemia followed by 2 hours of reperfusion, cell membrane bleb formation, calpain μ activation, and talin degradation were detected in groups 2 and 3 but not in group 1.

Conclusion: The safest method of hepatic vascular clamping that produces a minimum or no ischemia-reperfusion injury is 60 minutes of 6 cycles of 10-minute vascular clamping interrupted by 5 minutes of reperfusion.

Arch Surg. 1998;133:983-987

From the Department of Surgery II, Osaka University Medical School, Osaka (Drs Wang, Sakon, Umeshita, Miyoshi, Taniguchi, Kishimoto, and Monden), and Institute of Medical Science, University of Tokyo, Tokyo (Dr Imajoh-Ohmi), Japan.

BLOOD LOSS is a major problem in liver surgery, contributing to postoperative mortality and morbidity.[1-3] Pringle[4] was first to describe total occlusion of the portal hilum during hepatic surgery to arrest hepatic hemorrhage. Complete or partial vascular clamping at the hepatic hilum has been a standard procedure for reducing blood loss during hepatic resection. However, hepatic inflow occlusion may induce ischemia-reperfusion injury of the liver. Recently, several investigators reported that intermittent vascular clamping for short periods is better than continuous clamping,[5-7] based on the presumption that a brief reperfusion after a short period of ischemia causes less severe liver damage.[8] Several studies have determined the safe time limits of hepatic vascular occlusion by evaluating transaminase levels or the clinical outcome.[9] However, the critical period of vascular clamping remains to be elucidated because the status of liver function of patients examined in these studies (eg, extent of hepatectomy, presence of liver cirrhosis or hepatitis) varied from one study to another. To our knowledge, there are no studies that have examined the molecular mechanisms of hepatic ischemia-reperfusion injury. Thus, the best method of hepatic vascular occlusion to obviate liver injury is still unknown at present.

Recent studies from our laboratory and those from other investigators have demonstrated that calpain μ, a Ca^{2+}-activated neutral protease (EC 3, 4, 22, 17), is mainly involved in ischemia-reperfusion injury of the liver.[10-12] We have recently reported that calpain μ, a highly Ca^{2+}-sensitive form of calpain, is activated during hepatocyte injury and that it degrades various cytoskeletal proteins as evident histopathologically by cell mem-

MATERIALS AND METHODS

RAT MODEL OF HEPATIC ISCHEMIA-REPERFUSION INJURY

Sixty-five male Wistar rats, each weighing 220 to 260 g, were used in the present study. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our institutions. Each animal was anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg of body weight) followed by laparotomy and intravenous injection of 50 units of heparin sodium to prevent blood coagulation. A small atraumatic vascular clamp was applied at the hilar area of the left lateral and median lobes for 60 minutes. The animals were divided into the following 3 groups based on the duration of ischemic cycle: 6 cycles of 10-minute ischemia (group 1, n = 18), 4 cycles of 15-minute ischemia (group 2, n = 18), or 3 cycles of 20-minute ischemia (group 3, n = 18). Ischemia was interrupted by 5 minutes of declamping (reperfusion) between each period. Control rats underwent laparotomy but not vascular clamping (n = 11) (**Figure 1**). All procedures were performed in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.

ADENOSINE TRIPHOSPHATE LEVELS AND ENERGY CHARGE

Liver tissue was harvested at the end of ischemia and after 2 hours of reperfusion. The concentration of adenine nucleotides was measured as described previously.[13,14] Briefly, the tissue specimen was immersed immediately in liquid nitrogen and lyophilized overnight. Freeze-dried specimens were stored at –80°C for 7 days. To determine the level of adenine nucleotides, the specimen was thawed and then homogenized with 6 mL of 5% perchloric acid. After centrifugation, the supernatant was neutralized with 2.5 N potassium hydroxide, and the concentration of adenine nucleotides, including adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), was assayed by high-pressure liquid chromatography using an ion-exchange column (DEAE-2SW, Tosoh Corp, Tokyo, Japan). Energy charge was calculated according to the Atkinson equation: (ATP + ½ ADP)/(ATP + ADP + AMP).[15]

HISTOLOGICAL EXAMINATION

In a separate set of experiments similar to those described above, tissue samples were obtained for histological examination after in situ perfusion, fixation with 0.1% glutaraldehyde, and infusion of 4% paraformaldehyde via the portal vein. Biopsy specimens were processed for routine histopathologic examination using a light microscope.

ANTIBODIES AGAINST INTERMEDIATE (ACTIVATED) AND PROACTIVATED FORMS OF CALPAIN μ

Antibodies against peptides NH_2-AQVQKQC-COOH and NH_2-SEEIITPVYCTGVSAQVQKQRARELG-COOH, which correspond to the amino-terminal regions of the intermediate (activated) (78 kd) and proactivated (80 kd) forms of calpain μ, were prepared as described previously.[11,16,17]

WESTERN BLOT ANALYSIS

Liver tissues were immediately frozen by liquid nitrogen and stored at –80°C for 3 days. For Western blot analysis, samples were homogenized in an ice-water bath using a radioimmune protein assay buffer (1.0% Nonidet P-40 [Iwai Kagaku Co Ltd, Tokyo, Japan]; 0.1% deoxycholic acid; 150-mmol/L sodium chloride; 1.0-mmol/L phenylmethylsulfonyl fluoride; 50-mmol/L Tris hydrochloride, pH 7.5) containing 5-mmol/L ethylene glycol-bis (β-aminoethyl ether)-N,N,N′, N′-tetraacetic acid (EGTA), and 5-μmol/L leupeptin. After centrifugation at 3000 rpm for 20 minutes at 4°C, the supernatant (25 μg of protein) was subjected to Western blot analysis using specific antibodies against the intermediate and proactivated forms of calpain μ or an anti-talin antibody (Sigma Chemical Co, St Louis, Mo). This method allowed the detection of active calpain μ induced by autodigestion.

STATISTICAL ANALYSIS

Data were expressed as mean ± SD. Differences in ATP concentration and energy charge were tested for statistical significance using the Student *t* test and Dunnett multiple comparison test. A *P* value of less than .05 denoted the presence of a statistically significant difference.

brane bleb formation, an early step in cellular ischemia-reperfusion injury.[11] By analyzing the activation of calpain μ in liver tissue, we investigated the safest method of hepatic vascular clamping in an experimental model using rat liver.

RESULTS

LEVELS OF ATP AND ENERGY CHARGE

In all groups, ATP levels significantly decreased after ischemia-reperfusion ($P<.05$), but returned almost to baseline after 2 hours of reperfusion. There was no significant difference in ATP levels between the 3 groups (**Figure 2**). Similar changes were observed in energy charge, and no differences were present between the groups after reperfusion (**Figure 3**).

HISTOLOGICAL FINDINGS

Histological examination showed the formation of numerous cell membrane blebs and microparticles after cyclic ischemia in group 3 (**Figure 4**). These changes were more pronounced 2 hours after reperfusion in groups 2 and 3 than in group 1 (**Figure 5**). Furthermore, following cyclic ischemia-reperfusion, the cells became smaller, and the space between cells increased in groups 2 and 3. However, no significant changes in cell size or shape were observed in group 1.

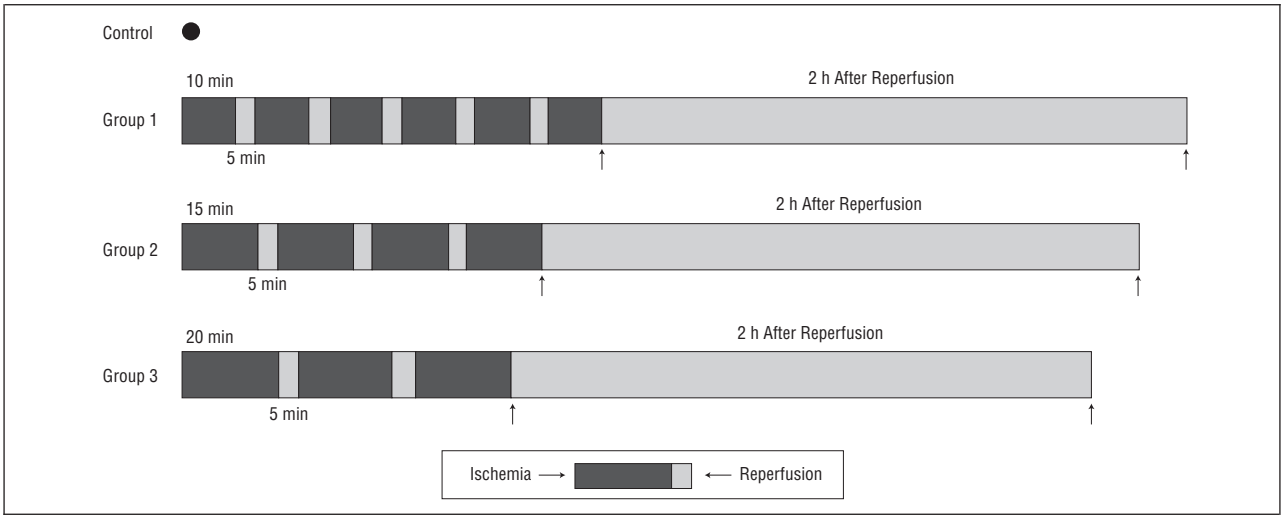

Figure 1. *Experimental model. Explanation of groups is as follows: control, without ischemia; group 1, six 10-minute cycles of ischemia; group 2, four 15-minute cycles of ischemia; and group 3, three 20-minute cycles of ischemia. Arrows indicate time at which liver tissue was harvested. Samples were immersed immediately in liquid nitrogen.*

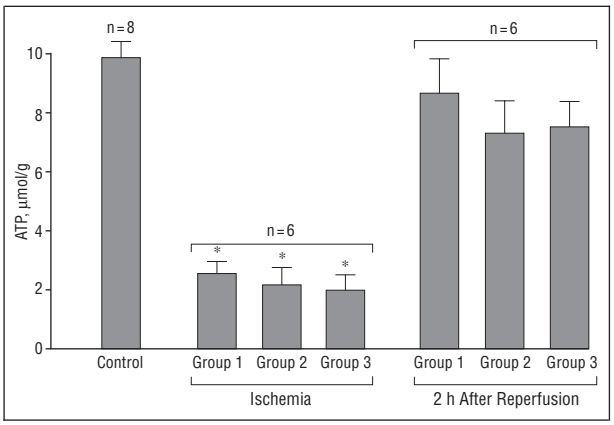

Figure 2. *Adenosine triphosphate (ATP) levels after 60 minutes of intermittent ischemia followed by 2 hours of reperfusion. The ATP levels markedly decreased after 60 minutes of intermittent ischemia, but almost returned to baseline 2 hours after reperfusion in all groups. There was no significant difference in ATP levels among these 3 groups. Data are mean ± SD (n = 6). Asterisk indicates significantly different from the control value (P<.05). See legend to Figure 1 for an explanation of the groups.*

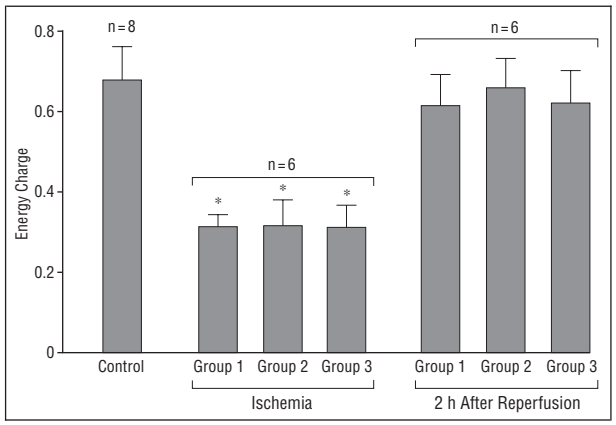

Figure 3. *Energy charge examined after 60 minutes of intermittent ischemia followed by 2 hours of reperfusion. Energy charge after reperfusion did not differ from the control value in all groups. Data are mean ± SD (n = 6). Asterisk indicates significantly different from the control value (P<.05). See legend to Figure 1 for an explanation of the groups.*

ACTIVATION OF CALPAIN µ AND DEGRADATION OF TALIN

Using specific antibodies to the intermediate (activated) (78 kd) and proactivated (80 kd) forms of calpain µ, Western blotting was performed to examine whether activation of calpain µ was different in the 3 groups. As shown in **Figure 6**, the intermediate form of calpain µ appeared 2 hours after reperfusion in groups 2 and 3, but not in group 1. However, the amount of procalpain µ (80 kd) did not change in any of these groups. To further confirm the activation of calpain µ, we investigated the proteolysis of talin, an intracellular substrate of calpain µ, by Western blotting using an anti-talin antibody. As shown in **Figure 7**, talin was significantly degraded at 2 hours after cyclic ischemia-reperfusion in groups 2 and 3, although no significant changes were observed in group 1.

COMMENT

Along with recent advances in surgical techniques, the introduction of total or hemihepatic vascular occlusion has reduced blood loss during liver resection. However, the best method for hepatic vascular occlusion remains controversial. The critical duration of occlusion of hepatic blood flow differs widely from one study to another, ranging from 15 minutes to more than 60 minutes.[18-22] These different conclusions may result, at least in part, from differences in experimental or clinical settings, such as the location and technique of vascular occlusion, extent of hepatectomy, and the presence of liver cirrhosis or hepatitis. Furthermore, the effect of ischemia-reperfusion on hepatocyte cell function has not been previously examined, particularly intracellular molecular changes. Ischemia-reperfusion injury of the liver is a complex process that involves several intracellular and extracellular events.[10] Various factors such as reactive oxygen species,[23-25] platelet-activating factor,[26,27] thromboxane

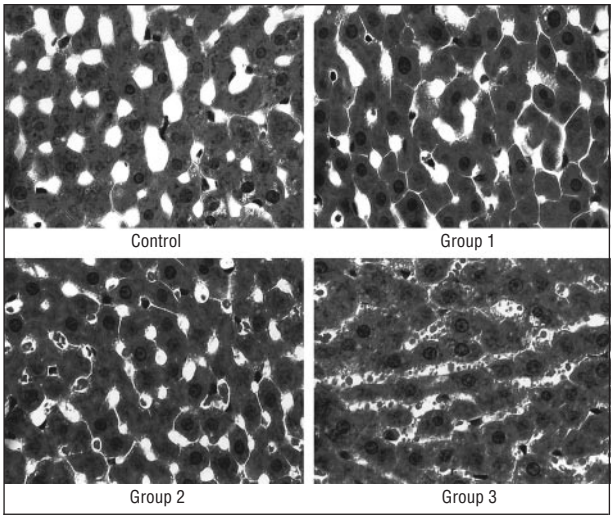

Figure 4. *Photomicrograph of the histological changes after 60 minutes of intermittent ischemia. Note the presence of numerous cell membrane blebs and microparticles at the end of ischemia in group 3 (hematoxylin-eosin, original magnification ×400). See legend to Figure 1 for an explanation of the groups.*

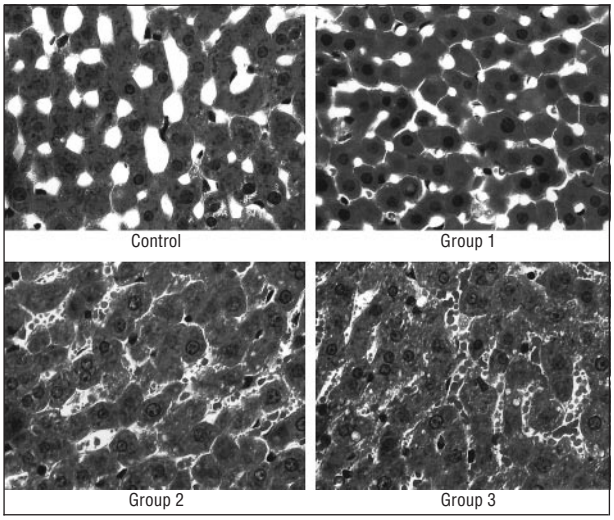

Figure 5. *Photomicrograph of the histological changes in the liver after 60 minutes of intermittent ischemia followed by 2 hours of reperfusion. Cell membrane bleb and microparticle formation was prominent in groups 2 and 3 but was only negligible in group 1. Note also that after cyclic ischemia-reperfusion, hepatocytes are smaller and the gaps between cells increased in groups 2 and 3 (hematoxylin-eosin, original magnification ×400). See legend to Figure 1 for an explanation of the groups.*

A_2,[28] leukotriene B_4,[29] and endothelin-1[30] are thought to be involved in ischemia-reperfusion cell injury. These extracellular mediators stimulate hepatocytes directly or indirectly and increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), the initial step in this process, followed by a variety of intracellular events such as mitochondrial dysfunction. These changes suggest that the pathologic insult of ischemia-reperfusion can be evaluated by measuring $[Ca^{2+}]_i$. However, accurate determination of $[Ca^{2+}]_i$ in hepatocytes in vivo or ex vivo is still difficult. To resolve this problem, we examined activation of calpain μ during intermittent ischemia. Calpain μ, a Ca^{2+}-sensitive form of calpain, is activated in hepatocytes following Ca^{2+} influx induced by various stimuli and is considered to play a prominent role in cell injury due to the associated degradation of cytoskeletal proteins such as talin and α-actinin.[11,31-33] Thus, analysis of calpain μ activation in liver tissue is a good marker for the evaluation of the pathologic insult of ischemia-reperfusion on hepatocytes.

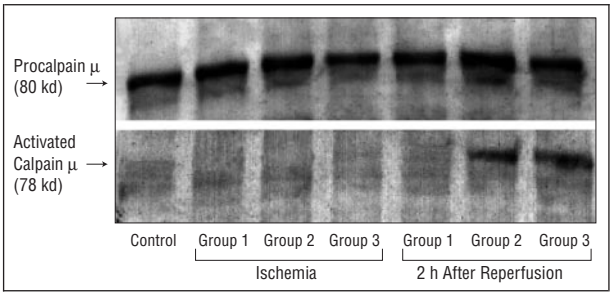

Figure 6. *Western blot analysis of activation of calpain μ in ischemia-reperfusion injury of the rat liver. The intermediate (activated) form of calpain μ appeared at 2 hours after reperfusion in groups 2 and 3 but not in group 1. However, the amount of procalpain μ (80 kd) was not markedly decreased in any group. See legend to Figure 1 for an explanation of the groups.*

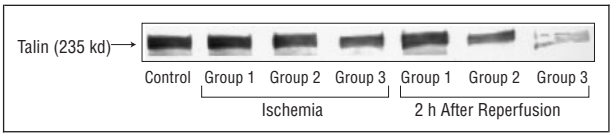

Figure 7. *Western blot analysis of talin in ischemia-reperfusion injury of the rat liver. Talin was significantly degraded 2 hours after cyclic ischemia-reperfusion in group 2 and even more degraded in group 3. However, no significant change was observed in group 1. See legend to Figure 1 for an explanation of the groups.*

In the present study, activation of calpain μ was evaluated by Western blotting using specific antibodies against the intermediate (activated) form of calpain μ and its endogenous substrate. As shown in Figure 6, activation of calpain μ occurred in rats subjected to 4 cycles of 15-minute intermittent ischemia (group 2) or 3 cycles of 20-minute intermittent ischemia (group 3), but not in those subjected to 6 cycles of 10-minute intermittent ischemia (group 1) or in the control group (without ischemia). These results were consistent with those of talin degradation (Figure 7). In addition, activation of calpain μ and degradation of talin was also marked following 60 minutes of continuous ischemia.[34] Thus, the safest method for a 60-minute duration of ischemia was six 10-minute cycles. The results of calpain μ activation were also confirmed by those of histopathologic examination. In these studies, cell membrane bleb formation was prominent at the end of the ischemia cycles in group 3 (Figure 4) and after reperfusion in groups 2 and 3 but was only negligible in group 1 (Figure 5), suggesting that $[Ca^{2+}]_i$ is not significantly elevated in hepatocytes. Since elevation of $[Ca^{2+}]_i$ is an early intracellular step in the process that leads to ischemia-reperfusion injury, these results confirm that six 10-minute cycles of intermittent ischemia do not induce pathologic changes in hepatocytes.

Our results also showed a lack of significant differences in ATP levels and energy charge between the experimental groups (Figures 2 and 3). Both measures returned to the preischemia levels at 2 hours after vascular

declamping, even in groups 2 and 3. This finding suggests that activation of calpain μ occurs even when mitochondrial function does not deteriorate.[10] Thus, activation of calpain μ may be a more sensitive measure of ischemia-reperfusion injury of the liver.

Our experiments were performed in healthy rats and, therefore, may be clinically irrelevant as liver surgery is usually performed in humans with a variety of liver diseases. Nevertheless, our studies formed the basis for our understanding of the cellular changes induced by various forms of intermittent ischemia. Future studies using animal models with different liver diseases should be performed to examine the impact of similar cycles of ischemia on hepatocellular injury. On the other hand, preliminary studies in our laboratory in patients with hepatic dysfunction (ie, obstructive jaundice, liver cirrhosis, or chronic active hepatitis) or in those undergoing extended hepatectomy, indicate that analysis of activation of calpain μ may be particularly useful for determination of the effect of vascular clamping time on surgical outcome and liver function. Thus, such evaluation is important since the functional reserve of the liver is generally limited and postoperative liver failure is still a frequent complication in these patients.[35,36]

In conclusion, by measuring activation of calpain μ, representing the initial step in cell membrane disruption due to ischemia-reperfusion, we determined that vascular clamping in rat liver for 60 minutes in the form of 6 cycles of 10-minute ischemia interrupted by 5 minutes of reperfusion is a safe method to prevent arresting hepatic hemorrhage.

Reprints: Masato Sakon, MD, Department of Surgery II, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan (e-mail: msakon@surg2.med.osaka-u.ac.jp).

REFERENCES

1. Nagao T, Inoue S, Goto S, et al. Hepatic resection for hepatocellular carcinoma: clinical features and long-term prognosis. *Ann Surg.* 1987;205:33-40.
2. Pachter HL, Spencer FC, Hofstetter SR, Coppa GF. Experience with the finger fracture technique to achieve intra-hepatic haemostasis in 75 patients with severe injuries of the liver. *Ann Surg.* 1983;197:771-778.
3. Delva E, Barberousse J, Nordlinger B, et al. Hemodynamic and biochemical monitoring during major liver resection with use of hepatic vascular exclusion. *Surgery.* 1984;95:309-318.
4. Pringle JH. Notes on the arrest of hepatic hemorrhage due to trauma. *Ann Surg.* 1908;48:541-549.
5. Isozaki H, Adam R, Gigou M, Szekly M, Shen M, Bismuth H. Experimental study of the protective effect of intermittent hepatic pedicle clamping in the rat. *Br J Surg.* 1992;79:310-313.
6. Okuyama K. Experimental and clinical studies on hepatic resection by intermittent interception of hepatic hilus under normothermic state. *Jpn J Gastroenterol Surg.* 1987;20:1883-1891.
7. Fuse A. Experimental studies on influences of intermittent hemihepatic vascular occlusion. *J Jpn Surg Soc.* 1989;90:863-873.
8. Horiuchi T, Muraoka R, Tabo T, Uchinami M, Kimura N, Tanigawa N. Optimal cycles of hepatic ischemia and reperfusion for intermittent pedicle clamping during liver surgery. *Arch Surg.* 1995;130:754-758.
9. Wu CC, Hwang CR, Liu TJ, P'eng FK. Effects and limitations of prolonged intermittent ischemia for hepatic resection of the cirrhotic liver. *Br J Surg.* 1996;83:121-124.
10. Rosser BG, Gores GJ. Liver cell necrosis: cellular mechanisms and clinical implications. *Gastroenterology.* 1995;108:252-275.
11. Miyoshi H, Umeshita K, Sakon M, et al. Calpain activation in plasma membrane bleb formation during tert-butyl hydroperoxide-induced rat hepatocyte injury. *Gastroenterology.* 1996;110:1897-1904.
12. Arora AS, Groen PC, Croall DE, Emori Y, Gores GJ. Hepatocellular carcinoma cell resist necrosis during anoxia by preventing phospholipase-mediated calpain activation. *J Cell Physiol.* 1996;167:434-442.
13. Hashimoto T, Negawa Y, Tagawa K. Properties of binding sites for adenine nucleotides on ATPase from yeast mitochondria. *J Biochem.* 1981;90:1141-1150.
14. Ukei T, Monden M, Umeshita K, et al. Electron spin resonance signals of nonheme iron as an indicator of ischemia-reperfusion injury of the liver. *Transplantation.* 1994;58:287-292.
15. Atkinson DE. The energy charge of the adenylate pool as a regulatory parameter: interaction with feedback modifiers. *Biochemistry.* 1968;7:4030-4034.
16. Kikuchi H, Imajoh-Ohmi S. Activation and possible involvement of calpain, a calcium-activated cysteine protease, in down-regulation of apoptosis of human monoblast U937 cells. *Cell Death Differ.* 1995;2:195-199.
17. Kikuchi H, Imajoh-Ohmi S, Kanegasaki S. Novel antibodies specific for proteolysed forms of protein kinase C: production of antipeptide antibodies available for in situ analysis of intracellular limited proteolysis. *Biochim Biophys Acta.* 1993;1162:171-176.
18. Huguet C, Nordlinger B, Bloch P, Conard J. Tolerance of the human liver to prolonged normothermic ischemia. *Arch Surg.* 1978;113:1448-1451.
19. Huguet C, Nordlinger B, Galopin JJ, Bloch P, Gallot D. Normothermic hepatic vascular exclusion for extensive hepatectomy. *Surg Gynecol Obstet.* 1978;147:689-693.
20. Hannoun L, Borie D, Delva E, et al. Liver resection with normothermic ischemia exceeding 1 h. *Br J Surg.* 1993;80:1161-1165.
21. Delva E, Camus Y, Nordlinger B, et al. Vascular occlusions for liver resections. *Ann Surg.* 1989;209:211-228.
22. Huguet C, Gavelli A, Chieco PA, et al. Liver ischemia for hepatic resection: where is the limit? *Surgery.* 1992;111:251-259.
23. Weinbroum A, Nielsen VG, Tan S, et al. Liver ischemia-reperfusion increases pulmonary permeability in rat: role of circulating xanthine oxidase. *Am J Physiol.* 1995;268:G988-G996.
24. Jaeschke H. Reactive oxygen and ischemia/reperfusion injury of the liver. *Chem Biol Interact.* 1991;79:115-136.
25. Jaeschke H, Smith CV, Mitchell JR. Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver. *J Clin Invest.* 1988;81:1240-1246.
26. Wang KS, Monden M, Kanai T, et al. Protective effect of platelet-activating factor antagonist on ischemia-induced liver injury in rats. *Surgery.* 1993;113:76-83.
27. Serizawa A, Nakamura S, Suzuki S, Baba S, Nakano M. Involvement of platelet-activating factor in cytokine production and neutrophil activation after hepatic ischemia-reperfusion. *Hepatology.* 1996;23:1656-1663.
28. Hanazaki K, Kuroda T, Haba Y, Shiohara E, Kajikawa S, Iida F. Protective effect of the thromboxane A_2 receptor antagonist ONO 3708 on ischemia-reperfusion injury in the dog liver. *Surgery.* 1994;116:896-903.
29. Hughes H, Farhood A, Jaeschke H. Role of leukotriene B_4 in the pathogenesis of hepatic ischemia-reperfusion injury in the rat. *Prostaglandins Leukotriene Essent Fatty Acids.* 1992;45:113-119.
30. Kawamura E, Yamanaka N, Okamoto E, Tomoda F, Furukawa K. Response of plasma and tissue endothelin-1 to liver ischemia and its implication in ischemia-reperfusion injury. *Hepatology.* 1995;21:1138-1143.
31. Murata M, Monden M, Umeshita K, et al. Role of intracellular calcium in superoxide-induced hepatocyte injury. *Hepatology.* 1994;19:1223-1228.
32. Beckerle MC, O'Halloran T, Burridge K. Demonstration of a relationship between talin and P235, a major substrate of the calcium-dependent protease in platelets. *J Cell Biochem.* 1986;30:259-270.
33. Fox JEB, Goll DE, Reynolds CC, Phillips DR. Identification of two proteins (actin-binding protein and P235) that are hydrolyzed by endogenous Ca^{2+}-dependent protease during platelet aggregation. *J Biol Chem.* 1985;260:1060-1066.
34. Wang M, Sakon M, Miyoshi H, et al. Prostacyclin analogue suppressed ischemia-reperfusion injury of the rat liver: evaluation by calpain μ activation. *J Surg Res.* 1997;73:101-106.
35. Tanabe G, Sakamoto M, Akazawa K, et al. Intraoperative risk factors associated with hepatic resection. *Br J Surg.* 1995;82:1262-1265.
36. Bozzetti F, Gennari L, Regalia E, et al. Morbidity and mortality after surgical resection of liver tumors: analysis of 229 cases. *Hepatol Gastroenterol.* 1992;39:237-241.