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ORIGINAL ARTICLE

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Evaluation of warm ischemia-reperfusion injury using heat shock protein in the rat liver

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Abstract We focused on heat shock protein 70 (HSP70) as a marker of viability in hepatic warm ischemia-reperfusion. Segmental hepatic warm ischemia was produced in rats for 15, 30, 60, 90, 120, or 180 min. Liver sections were evaluated at 30, 60, and 120 min of reperfusion. Expression of HSP70 and messenger RNA (mRNA), apoptosis, and apoptosis-associated genes such as Bcl-2 and Bax were studied. Expression of HSP70 and mRNA was augmented as warm ischemia was prolonged, but was markedly suppressed in livers with more than 120 min of ischemia. The highest accumulation of HSP70 was observed in the nucleus. In livers subjected to longer duration of warm ischemia, necrosis and apoptosis were evident and Bcl-2 mRNA expression and Bcl-2/Bax protein ratio were markedly diminished. Apoptosis may be related to the process of cellular injury induced by warm ischemia-reperfusion. Expression of HSP70 and the Bcl-2 family can be effective markers of viability in hepatic warm ischemia-reperfusion.

Keywords Heat shock protein · Viability · Warm ischemia-reperfusion · Necrosis · Apoptosis · Bcl-2

Introduction

Refinements in surgical techniques, organ preservation, patient management, and the understanding of immune responses have significantly expanded the application of liver transplantation to a greater range of patients in recent years. Despite widespread public information to encourage increased organ donation, successful clinical use of University of Wisconsin (UW) solution, and expansion of the donor pool with living-related, split, and reduced-size liver donors, many patients still die while waiting for a liver graft [31]. The current organ shortage has caused the transplant community to examine critically its donor criteria [5]. Organ shortage has also led some teams to use livers obtained from donors who have suffered irreversible cardiopulmonary arrest [6], also called non-heart-beating donors (NHBDs). The compromised viability of the graft due to warm ischemia-related cardiopulmonary arrest may lead to primary dysfunction or nonfunction [8]. Although successful kidney transplantations have been reported for organs procured from NHBDs, encouraging results have not yet been observed in liver transplantation [6, 10].

The use of several parameters such as hepatic enzymes, arterial ketone body ratio, hyaluronic acid in the reperfusion effluent, ATP level, and others, have been proposed to assess the condition of the liver after reperfusion [16, 22, 26, 27]. However, these parameters were not satisfactory in evaluating or predicting the viability of liver grafts obtained from NHBDs.

Recently, it has become clear that all cells share a common molecular response to noxious stimuli, such as ischemia, hypoxia, increased temperatures, infections, and toxins, whereby they increase the synthesis of a family of proteins known as stress proteins or heat shock proteins (HSPs) [23]. Among these, the HSP70 family is

one of the most fundamental. Some studies have demonstrated their importance, not only under noxious conditions but also in normal cellular processes such as protein folding, transport, assembly, and degeneration [36]. Furthermore, it has been shown that the production of HSPs could protect the organism against second exposure to lethal hyperthermia, which has been described as the thermotolerance phenomenon [18]. Heat shock preconditioning reduces ischemia-reperfusion injury in normal and steatotic livers [20, 38]. Ischemic preconditioning also attenuates liver damage in subsequent ischemia-reperfusion injury, improving the restoration of hepatic function during reperfusion and resulting in higher post-ischemic survival [20].

In addition, a recent report suggested that HSP70 was involved in the prevention of apoptosis [21]. It has become clear that HSP70 is a general anti-apoptotic protein that protects cells from cytotoxicity induced by tumor necrosis factors, monocytes, oxidative stress, chemotherapeutic agents, ceramide, and radiation [13, 14, 15, 17, 24, 30]. However, the mechanism of action through which HSP70 exerts its anti-apoptotic effects is still obscure.

After brief brain ischemia, a striking induction of HSP70 immunoreactivity was demonstrated in cell populations that were destined to survive transient ischemia insult [33]. Prolonged expression of HSP70 messenger RNA (mRNA) provides a useful marker for vulnerable neuron populations that may be useful in evaluating the progression of ischemia injury [25]. Accumulation of HSP70 mRNA reflects the severity of ischemia-reperfusion injury in the liver and may reflect the shortage in the HSP70 pool for adaptation against injurious conditions at real time rather than the amount of HSP70. Synthesis of HSP70 is regulated both transcriptionally and post-transcriptionally through induction of mRNA synthesis and degeneration of the transcripts, and protein accumulation represents the consequences during a relatively long period [1].

We focused on HSP70 as a marker of viability of the liver subjected to warm ischemia-reperfusion injury. We studied expression of HSP70 and mRNA after warm ischemia-reperfusion. In addition, we investigated hepatocellular injury including apoptosis, expression of apoptosis-associated genes, and their relation to the expression of HSP70 in the liver subjected to warm ischemia-reperfusion.

Materials and methods

Treatment of animals

A model of segmental (70%) hepatic warm ischemia was used in all experiments. Male Wistar rats weighing 200–300 g were obtained from JCL (Miyagi, Japan) and were used for all experiments. All animal experiments in this study followed the Guidelines for Animal Experimentation, Hirosaki University. Rats were housed in a climatized environment with a 12-h dark–light cycle and had free access to water and standard rat chow (MF Oriental Yeast, Tokyo, Japan) prior to experiments. Rats were anesthetized through inhalation of diethyl ether (Wako, Osaka, Japan) and intraperitoneal pentobarbital sodium (Abbott, Chicago, Ill.) at a dose of 50 mg/kg. After a midline laparotomy, all structures in the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes were occluded with a microvascular clamp for 15, 30, 60, 90, 120, or 180 min, and 70% of the liver was subjected to warm ischemia. This method of segmental hepatic ischemia prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. Reperfusion was initiated by removal of the clamp. Liver sections were taken at 30, 60, and 120 min after reperfusion. The sample of post-ischemic lobe was freeze-clamped and stored in liquid nitrogen. Another part of the sample was fixed in 10% phosphate-buffered formalin. As a control group, rats were treated in the same way as the experimental animals, except that the portal vein and hepatic artery were exposed but not clamped, and liver extirpation was performed immediately after treatment.

Quantification of HSP70 by enzyme-linked immunosorbent assay

The expression of HSP70 was analyzed by enzyme-linked immunosorbent assay (ELISA) at 30, 60, and 120 min after reperfusion following 15, 30, 60, 90, 120, or 180 min of warm ischemia, and likewise in the control group ($n = 6$ for each group). A sample of approximately 100 mg frozen liver tissue was homogenized in lysis buffer consisting of 50 mmol Tris-HCl (pH 7.4), 1 mmol EDTA, 1 mmol ethylene glycol-bis-(β-amino-ethylether)-tetra-acetic acid (EGTA), 50 mmol sodium fluoride, 1% Triton X-100, and 0.5% Tergitol NP-40 for extraction of protein. For protease inhibition, 1 mmol sodium orthovanadate, 1 mmol phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml pepstatin A were added to the lysis buffer. The lysate was clarified by centrifugation at 15,000 rpm for 30 min at 4 °C and the supernatant was used for analysis. The absolute amount of HSP70 in the samples was determined by bicinchonic acid protein reagent assay system (Pierce, Rockford, Ill.) with bovine serum albumin (BSA) as the standard (micrograms HSP70/milligram total protein). The diluents of the supernatant in carbonate buffer (50 mmol, pH 9.6) were used for HSP70 ELISA and protein assay.

Fifty microliters of samples and standards (H9776, Sigma, St. Louis, Mo.) were placed in wells of microtiter plates at 4 °C overnight, and the non-specific binding sites were saturated by PBS-1% BSA and the plate incubated at room temperature for 1 h. Fifty microliters of anti-HSP70 monoclonal antibody (BRM-22, Sigma), which is known to be cross-reactive with rat HSP70, diluted 1:2000 with PBS-BSA were added. After incubation for 1 h at room temperature, 50 μl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Lot 297, MBL, Nagoya, Japan) diluted 1:2000 with PBS-BSA was added. After 1 h of incubation at room temperature, TMB substrate (KPL, Guildford, UK) was added. After a few minutes, the reaction was terminated with 1 N sulfuric acid. The colored reaction product was quantified immediately with a microplate reader (Model 550, Bio-Rad, Hercules, Calif.) at dual wavelengths of 450/655 nm. HSP70 concentration was then determined by extrapolation from the standard curve.

RNA preparation and reverse transcription-polymerase chain reaction

The expression of HSP70, caspase-3, inhibitor of apoptosis protein-2 (IAP-2), Bcl-2, Bax, and p53 mRNAs was analyzed via the reverse transcription-polymerase chain reaction (RT-PCR) technique

at 30, 60, and 120 min after reperfusion following 15, 30, 60, 90, 120, or 180 min of warm ischemia, and likewise in the control group. Total RNA was prepared by use of the RNeasy kit (Qiagen, Hilden, Germany). Total RNA (1 μg) was reverse-transcribed by Omniscript reverse transcriptase (Qiagen). The resulting complementary DNA (cDNA) was used as a template for PCR. PCR was performed with the Taq PCR Master Mix Kit (Qiagen). Primer sequences were HSP70 up: 5′-GCAGACCGCAGCGACATGGC-3′; HSP70 down: 5′-GATGCTCTTGTTCAGGTCGC-3′ with 28 amplification cycles run; caspase-3 up: 5′-GGTATTGAGACA-GACAGTGG-3′; caspase-3 down: 5′-CATGGGATCTGTTTC-TTTGC-3′ with 30 amplification cycles run; IAP-2 up: 5′-GCTT-CTGTTGTGGCCTGATG-3′; IAP-2 down: 5′-CACCTTGGAAA-CCACTTGGC-3′ with 30 amplification cycles run; p53 up: 5′-GTGGCCTCTGTCATCTTCCG-3′; p53 down: 5′-CCGTCA CATCAGAGCAACG-3′ with 30 amplification cycles run; Bcl-2 up: 5′-CACCCCTGGCATCTTCTCCTT-3′; Bcl-2 down: 5′-AGC-GTCTTCAGAGACAGCCAG-3′ with 38 amplification cycles run; Bax up: 5′-CCACCAGCTCTGAACAGATCATGA-3′; Bax down: 5′-TCAGCCCATCTTCTTCCAGATGGT-3′ with 35 amplification cycles run. The cDNA for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as internal control with 22 amplification cycles run. Primers used to amplify G3PDH cDNA were 5′-ACCACAGTCCATGCCATCAC-3′ (sense primer) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense primer). Typical reaction conditions included denaturing for 1 min at 94 °C, annealing for 1 min at 55 °C, and primer extension for 1 min at 72 °C. The amplified products were resolved by gel electrophoresis on 2% agarose containing 0.05% ethidium bromide. The gel was visualized and photographed under ultraviolet transillumination.

Immunohistochemistry for HSP70

Immunohistochemistry for HSP70 was performed in the livers subjected to 60 or 180-min of warm ischemia followed by 120-min of reperfusion, as well as in the control group. Paraffin-embedded sections (4 μm) were mounted on amino-propyl-triethoxysilane-coated slides. The paraffin was removed and the sections rehydrated. After being washed in PBS, the sections were microwaved three times in sodium citrate buffer, pH 6.0, for 5 min each before undergoing immunostaining. The sections were immersed in a 0.3% solution of hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. We performed immunohistochemical staining for HSP70 using the same antibody used for ELISA. After blocking non-specific protein binding using non-immune rabbit serum for 30 min at room temperature, we incubated the sections overnight at 4 °C in the primary antibody diluted 1:1000 in PBS, pH 7.4. The sections were incubated for 30 min at room temperature with Simple Stain Rat PO, universal immuno-peroxidase polymer for rat tissue (Nichirei, Tokyo, Japan). Color was developed by incubation of the slides for 2–3 min with 1% diaminobenzidine tetrachloride (DAB) in 0.05 M Tris-HCl, pH 7.6, containing hydrogen peroxide. Photography was performed with an Olympus microscope after the slides had been sealed with glass coverslips.

Morphological evaluation by hematoxylin and eosin staining

Formalin-fixed liver specimens were embedded in paraffin. Then, 4-μm-thick sections were cut and stained with hematoxylin and eosin. Histological assessment was performed in the livers subjected to 60 or 180 min of warm ischemia followed by 120 min of reperfusion, as well as in the control group. Photography was performed with an Olympus microscope (Olympus, Tokyo, Japan). The number of necrotic cells was counted in ten high-power (×400) fields. Morphological criteria such as increased eosinophilia, vacuolization, cell disruption, loss of architecture, and karyolysis were used to determine necrosis. We estimated the percentage of necrosis by evaluating the number of microscopic fields with necrosis compared with the entire histological section. This process is in contrast to apoptosis, which includes morphological features of cell shrinkage, chromatin condensation, and the formation of apoptotic bodies.

In situ localization of apoptotic cells: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Using 5-μg-thick prepared sections from which the paraffin had been removed, we performed terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) in livers subjected to 60 or 180 min of warm ischemia followed by 120 min of reperfusion, as well as in the control group, using an in situ cell death detection kit (Roche, Mannheim, Germany) and following the manufacturer's instructions. The number of apoptotic cells was counted in ten high-power (×400) fields. Apoptotic cells were identified by morphological criteria (cell shrinkage, chromatin condensation and margination, and the formation of apoptotic bodies).

Analysis of DNA fragmentation by agarose gel electrophoresis

In addition to histological evaluation, DNA fragmentation was detected by agarose gel electrophoresis. This assay detects DNA fragmentation induced by caspase-activated endonucleases during apoptosis as well as DNA fragmentation during oncotic necrosis. DNA fragmentation was analyzed in livers subjected to 60 or 180 min of warm ischemia followed by 120 min of reperfusion, as well as in the control group.

DNA was extracted from the frozen liver tissues utilizing the Apoptotic DNA ladder kit (Roche). Ten micrograms of DNA were subjected to electrophoresis on 1.5% agarose gel containing 0.05% ethidium bromide. The gel was visualized and photographed under ultraviolet transillumination.

Assay for caspase-3 activity

Caspase-3 activity has been implicated in apoptotic cell death in a number of models. Activation of caspases, especially the effector caspase-3, is considered essential for most apoptotic processes.

To address the relation of caspase-3 to ischemia-reperfusion injury, we also measured the proteolytic activity of caspase-3 in controls and livers subjected to 60 or 180 min of warm ischemia followed by reperfusion for 120 min ($n = 6$ for each group).

We measured caspase-3 activity using the CPP32/caspase-3 colorimetric protease assay kit (MBL) and following the manufacturer's instructions. Optimal density was measured at 405 nm in a microtiter plate reader.

Quantification of Bcl-2 and Bax by ELISA

In order to investigate the role of Bcl-2 and Bax in hepatic ischemia-reperfusion injury, we assessed Bcl-2 and Bax protein levels by ELISA in controls and livers subjected to 60 or 180 min of warm ischemia followed by 120 min of reperfusion ($n = 6$ for each group). The relative expression of Bcl-2 and Bax proteins and the Bcl-2/Bax ratio were calculated from the results of ELISA.

The same protein extracts as with the caspase-3 assay were used for analysis in the Bcl-2 and Bax ELISA. The absolute amounts of Bcl-2 and Bax in the samples were determined via BCA protein

reagent assay system, with BSA as the standard (nanograms Bcl-2 or Bax/milligram total protein). Diluents of the supernatant in carbonate buffer (50 mM, pH 9.6) were used for ELISA and protein assay.

Fifty microliters of samples and standards (sc-4096 and sc-4239, Santa Cruz Biotechnology, Santa Cruz, Calif.) were placed in wells of microtiter plates at 4 °C overnight, and the non-specific binding sites were saturated by PBS-1% BSA and the plate incubated at room temperature for 1 h. Fifty microliters of anti-Bcl-2 monoclonal antibody (sc-7382, Santa Cruz) and anti-Bax monoclonal antibody (sc-7480, Santa Cruz) diluted 1:1000 with PBS-BSA were added. After incubation of 1 h at room temperature, 50 μl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Lot 297, MBL), diluted 1:2000 with PBS-BSA, was added. After 1 h of incubation at room temperature, TMB substrate (KPL) was added. After a few minutes, the reaction was terminated with 1 N sulfuric acid. The colored reaction product was quantified immediately with a microplate reader (Model 550, Bio-Rad) at dual wavelengths of 450/655 nm. The Bcl-2 and Bax concentration was then determined by extrapolation from the standard curve to calculate relative expression. The relative expression of Bcl-2 and Bax proteins is the ratio of livers subjected to ischemia-reperfusion to controls, and the relative mean ratio of Bcl-2/Bax was determined.

Statistical analysis

All values are expressed as mean ± standard error (SE). Comparisons between groups were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. P values of below 0.05 were considered statistically significant.

Results

HSP70 expression after warm ischemia-reperfusion

HSP70 expression was significantly increased in livers subjected to various periods of warm ischemia at any time point after reperfusion, compared with that of controls ($P < 0.01$, Fig. 1). HSP70 expression was gradually increased when the duration of warm ischemia was increased up to 90 min and was significantly diminished in the livers subjected to 120 or 180 min of warm ischemia. These results were equally evident after 30, 60, and 120 min of reperfusion. When the duration of reperfusion was increased from 30 to 60 and 120 min, no significant increase in HSP70 expression was observed in livers subjected to various periods of warm ischemia.

Expression of HSP70 mRNA and apoptosis-related gene mRNA after warm ischemia-reperfusion

HSP70 mRNA expression was not detected in controls (Fig. 2). After 30 min of reperfusion, HSP70 mRNA was highly expressed in the livers subjected to 30 or 60 min of warm ischemia and was markedly suppressed in the livers subjected to 120 or 180 min of warm ischemia (Fig. 2a). Similar results were observed after 60 and 120 min of reperfusion. Maximal expression of HSP70 mRNA was observed in livers subjected to 30 or 60 min of warm ischemia and was markedly suppressed in the livers subjected to more than 120 min of warm ischemia (Fig. 2b, c). There was no obvious alteration in G3PDH mRNA expression at all times after reperfusion (Fig. 2).

Caspase-3, IAP-2, p53, and Bax mRNA was expressed equally at any time point after reperfusion. In contrast, Bcl-2 mRNA was detected in the livers subjected to fewer than 90 min of warm ischemia, but was markedly suppressed in livers subjected to more than 120 min of warm ischemia after 30, 60, and 120 min of reperfusion (Fig. 2).

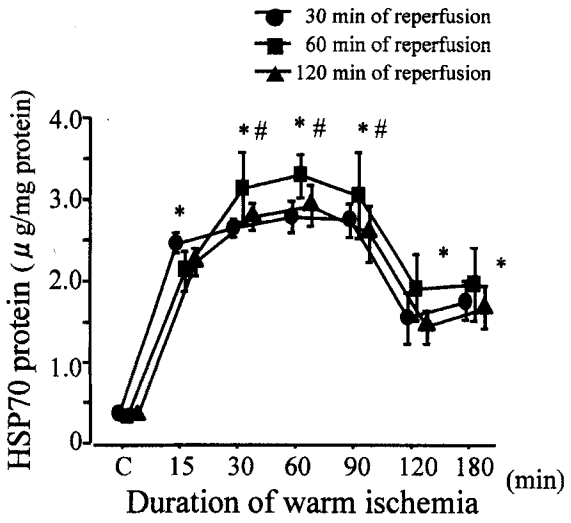

Fig. 1 Expression of HSP70 measured by ELISA in rat livers subjected to 15, 30, 60, 90, 120, or 180 min of warm ischemia followed by 30 (*circles*), 60 (*squares*), or 120 (*triangles*) minutes of reperfusion. The absolute amount of HSP70 is expressed in relation to total protein (micrograms HSP70/milligram total protein). Results are expressed as mean ± SE. After ischemia-reperfusion, HSP70 expression was significantly increased. It was augmented as the duration of warm ischemia increased up to 90 min and significantly diminished at more than 120 min of ischemia. These results were equally evident after 30, 60, and 120 min of reperfusion. No significant difference was observed in the levels of HSP70 between 30, 60, and 120 min of reperfusion. $*P < 0.01$ vs controls (C), $\#P < 0.05$ vs livers subjected to 120 or 180 min of warm ischemia

HSP70 immunohistochemistry

HSP70 was expressed in neither the nucleus nor cytoplasm of hepatocytes in controls (Fig. 3a). In the livers subjected to 60 min of warm ischemia, HSP70 expression was evident predominantly in the nucleus, but was also present in the cytoplasm (Fig. 3b). HSP70 was equally expressed in the periportal region and pericentral region in livers subjected to 60 min of warm ischemia. In the livers subjected to 180 min of warm ischemia,

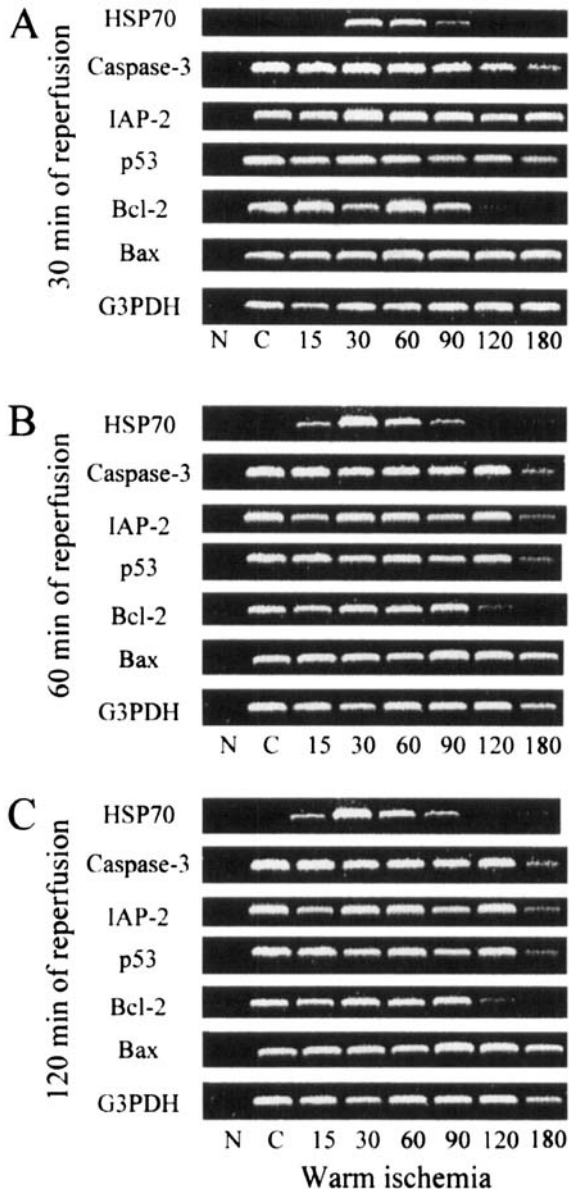

Fig. 2a–c Identification of HSP70, caspase-3, inhibitor of apoptosis protein-2 (*IAP-2*), p53, Bcl-2, and Bax mRNA by RT-PCR analysis in rat livers subjected to 15, 30, 60, 90, 120, or 180 min of warm ischemia followed by 30, 60, or 120 min of reperfusion. G3PDH was used as an internal control in this system. **a** At 30 min after reperfusion, no expression of HSP70 mRNA was detected in control livers. HSP70 mRNA was highly expressed in livers subjected to 30 or 60 min of warm ischemia, but markedly suppressed in livers subjected to 120 or 180 min of warm ischemia. Caspase-3, IAP-2, p53, and Bax mRNA were equally expressed in control livers and livers subjected to warm ischemia at all time points. Bcl-2 mRNA was suppressed in livers subjected to 120 or 180 min of warm ischemia. **b**, **c** At 60 and 120 min of reperfusion, similar results as with 30 min of reperfusion were observed (*N* no template, *C* controls)

HSP70 was faintly expressed in both nuclei and cytoplasm, and expression was markedly diminished compared with that of 60 min of warm ischemia (Fig. 3c).

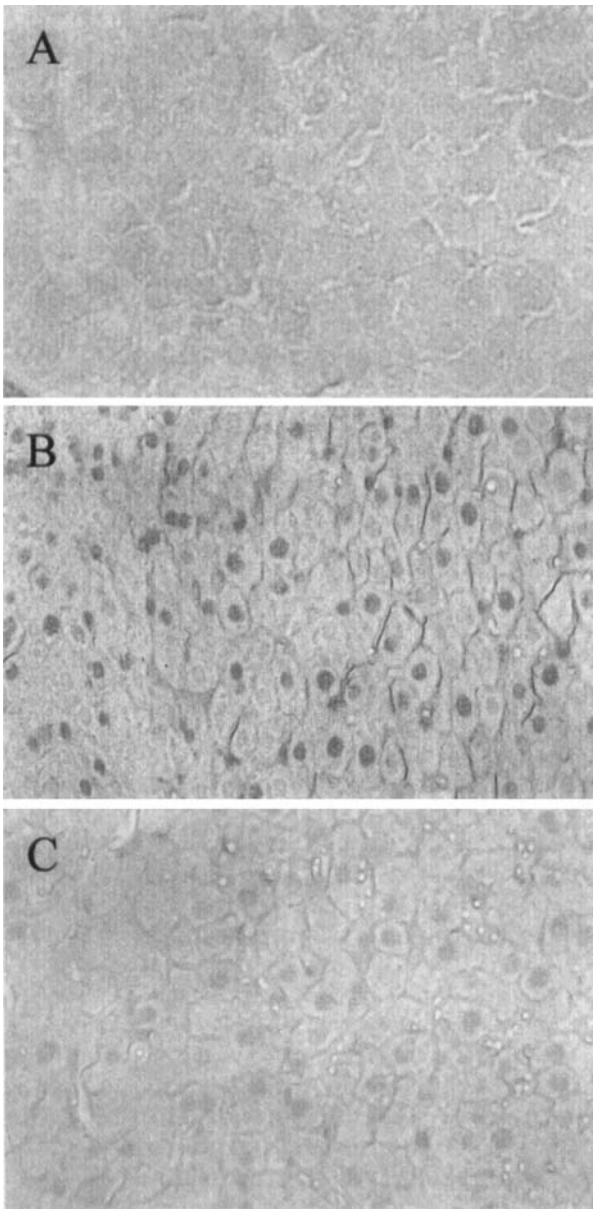

Fig. 3a–c Immunohistochemical identification of HSP70 in controls and livers subjected to 60 or 180 min of warm ischemia followed by 120 min of reperfusion. **a** No expression was seen in controls. **b** High expression of HSP70 was observed in the nuclei in livers subjected to 60 min of warm ischemia. **c** Expression of HSP70 was faintly detected in the nuclei in livers subjected to 180 min of warm ischemia (original magnification ×400)

Hematoxylin and eosin staining

Almost normal architecture was observed in the controls. The sinusoids appeared normal and there was no infiltration by inflammatory cells (Fig. 4a). In livers subjected to 60 min of warm ischemia followed by 120 min of reperfusion, there was a mild infiltrate of neutrophils and mononuclear inflammatory cells in

sinusoids and perivascular space. Hepatocytes were mildly swollen with scattered foci of early necrosis. Sinusoids were focally congested with erythrocytes unrelated to the necrotic area (Fig. 4b). In the livers subjected to 180 min of warm ischemia followed by 120 min of reperfusion, sinusoids were congested with numerous erythrocytes throughout the section. Hepatocytes were characterized by patchy areas of coagulative necrosis (Fig. 4c).

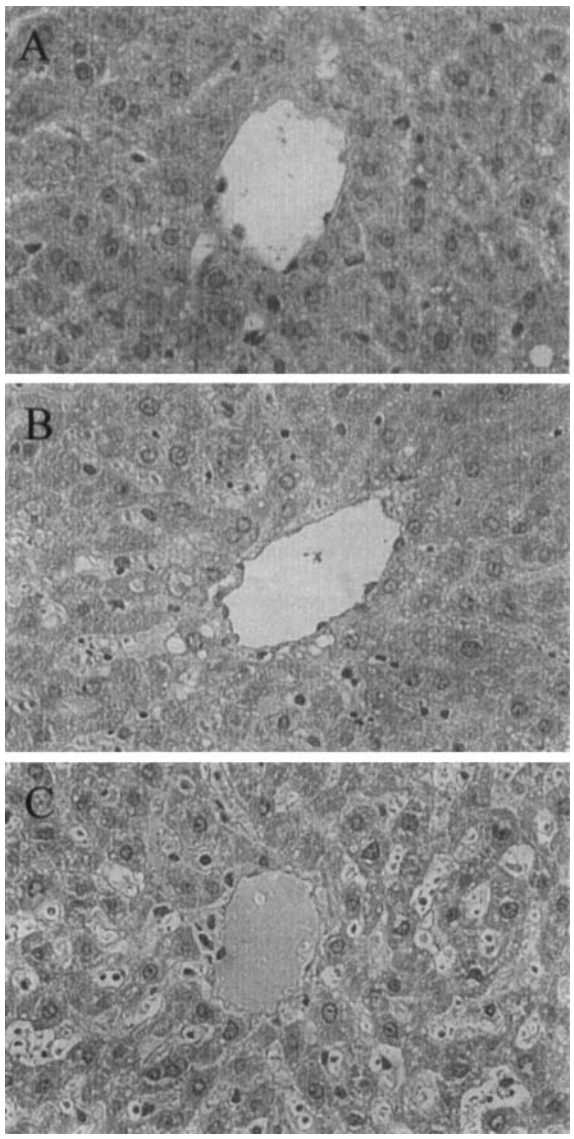

Fig. 4a–c Histology of controls and livers subjected to 60 or 180 min of warm ischemia followed by 120 min of reperfusion. **a** Almost normal architecture was observed in controls. **b** Hepatocytes were mildly swollen with scattered foci of early necrosis in the livers subjected to 60 min of warm ischemia. Sinusoids were focally congested with erythrocytes unrelated to the necrotic area. **c** Sinusoids were congested with numerous erythrocytes in livers subjected to 180 min of warm ischemia. Hepatocytes were characterized by patchy areas of coagulative necrosis (H & E; original magnification ×400)

Morphological criteria such as increased eosinophilia, vacuolization, cell disruption, loss of architecture, and karyolysis were used to determine necrosis. The evaluation of necrosis in post-ischemic livers showed a progressive increase in the number of necrotic cells dependent on the length of warm ischemia periods after 120 min of reperfusion (Fig. 6a). In controls, approximately $0.2 \pm 0.1\%$ of all cells were necrotic. At 120 min after reperfusion, $6.2 \pm 0.8\%$ and $23.2 \pm 1.1\%$, respectively, of all cells were necrotic in the livers subjected to 60 or 180 min of warm ischemia, and the number of necrotic cells was significantly increased compared with that of controls ($P < 0.0001$).

TUNEL staining

Using the TUNEL assay and applying morphological criteria (cell shrinkage, chromatin condensation, and the formation of apoptotic bodies), we counted apoptotic cells in ten random high-power (×400) fields. In controls, approximately $0.3 \pm 0.1\%$ of all cells were apoptotic (Fig. 5a, Fig. 6b). In the livers subjected to 60 min of warm ischemia and 120 min of reperfusion, $2.9 \pm 0.3\%$ of all cells were apoptotic, and the proportion of apoptotic cells was significantly increased compared with that of controls ($P < 0.0001$, Fig. 5b, Fig. 6b). In the livers subjected to 180 min of warm ischemia and 120 min of reperfusion, $7.4 \pm 0.2\%$ of all cells were apoptotic, and the proportion of apoptotic cells was significantly higher compared with that of both controls and livers subjected to 60 min of warm ischemia ($P < 0.0001$, Fig. 5b, Fig. 6b). Furthermore, most of the apoptotic cells were hepatocytes.

DNA laddering

No DNA laddering could be detected in the controls. In the livers subjected to 60 min of warm ischemia, DNA fragments were faintly detected (Fig. 7). They were more pronounced in the livers subjected to 180 min of warm ischemia, and the highest expression detected was approximately 500 bp.

Caspase-3 activity assay

Results of the caspase-3 activity assay were consistent with the data obtained with in situ TUNEL staining and DNA laddering (Fig. 8). In controls, caspase-3 activity was 0.056 ± 0.001. At 120 min of reperfusion, caspase-3 activity in the livers subjected to 60 or 180 min of warm ischemia was 0.084 ± 0.003 and 0.110 ± 0.025,

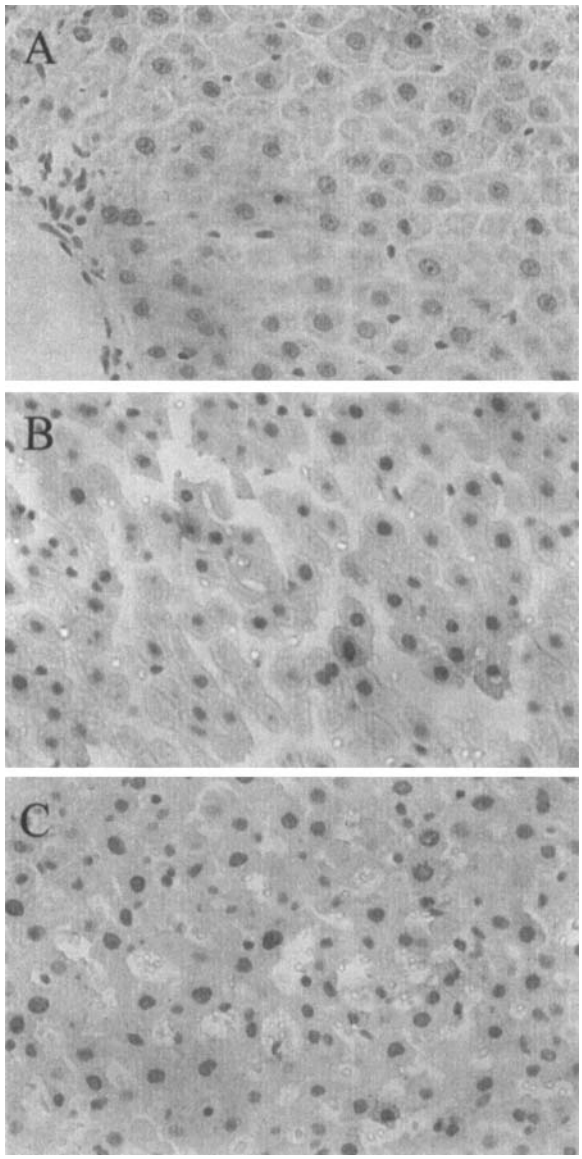

Fig. 5a–c In situ TUNEL staining protein in controls and livers subjected to 60 or 120 min of warm ischemia followed by 120 min of reperfusion. **a** Few positive cells were seen in control livers. **b** Apoptotic cells were occasionally present in the livers subjected to 60 min of warm ischemia. **c** An increasing number of hepatocytes were identified as having undergone apoptosis in livers subjected to 180 min of warm ischemia (original magnification ×400)

respectively. In livers subjected to 60 min of warm ischemia, caspase-3 activity was significantly augmented compared with that of controls ($P < 0.05$) and increased by 50% above that of controls. In the livers subjected to 180 min of warm ischemia, caspase-3 activity was significantly increased compared with that of the controls and 60 min of warm ischemia ($P < 0.001$ and $P < 0.05$, respectively) and increased by 96% above that of controls and by 31% above that of the livers subjected to 60 min of warm ischemia.

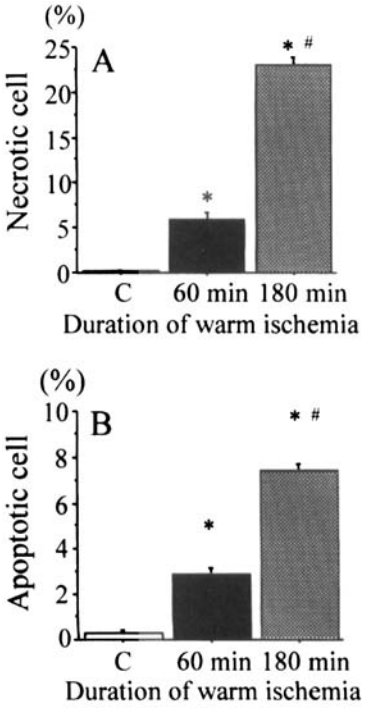

Fig. 6a,b Evaluation of necrotic cells in H & E staining and apoptotic cells in TUNEL assay with morphological criteria. Results are expressed as mean ± SE. **a** Evaluation of necrosis in post-ischemic livers showed a progressive increase in number of necrotic cells dependent on the length of warm ischemia periods after 120 min of reperfusion. In controls (*C*), approximately $0.2 \pm 0.1\%$ of all cells were necrotic (*open bar*). At 120 min after reperfusion, 6.2 ± 0.8 and $23.2 \pm 1.1\%$ of all cells were necrotic in the livers subjected to 60 (*filled bar*) or 180 (*shaded bar*) minutes of warm ischemia, respectively. **b** In controls, approximately $0.3 \pm 0.1\%$ of all cells were apoptotic (*open bar*). In the livers subjected to 60 min of warm ischemia and 120 min of reperfusion, $2.9 \pm 0.3\%$ of all cells were apoptotic, and the proportion of apoptotic cells was significantly increased compared with that of controls (*filled bar*) ($P < 0.0001$). In the livers subjected to 180 min of warm ischemia and 120 min of reperfusion, $7.4 \pm 0.2\%$ of all cells were apoptotic (*shaded bar*). $*P < 0.0001$ vs controls, $\#P < 0.0001$ vs 60 min of warm ischemia

Expression of Bcl-2 and Bax protein after ischemia-reperfusion

The relative expressions of Bcl-2 protein in the livers subjected to 60 or 180 min of warm ischemia were $119.1 \pm 12.5\%$ and $63.3 \pm 7.1\%$, respectively (Fig. 9, open bars). The relative expression of Bcl-2 protein in the livers subjected to 60 min of warm ischemia was significantly increased compared with that of the livers subjected to 180 min of warm ischemia ($P < 0.05$).

The relative expression of Bax protein in the livers subjected to 60 or 180 min of warm ischemia was $58.7 \pm 8.4\%$ and $64.5 \pm 7.1\%$, respectively (Fig. 9, filled bars). The relative expression of Bax protein in livers subjected to 60 min of warm ischemia was significantly diminished compared with that of controls ($P < 0.05$).

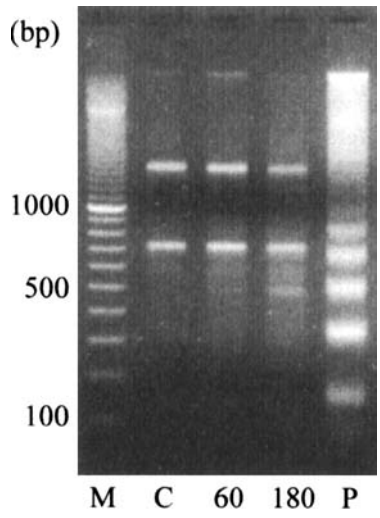

Fig. 7 DNA fragmentation analysis on agarose gel electrophoresis in controls and livers subjected to 60 or 180 min of warm ischemia followed by 120 min of reperfusion. No laddering was observed in control livers (*C*). Ladders were modestly detected in livers subjected to 60 min of warm ischemia. Ladders were evident in the livers subjected to 180 min of warm ischemia. Molecular weight (*M*) was marked to the left of the gel; positive control (*P*) is shown to the right of the gel

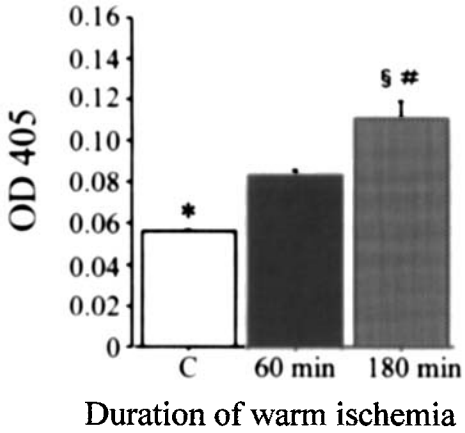

Fig. 8 Analysis of caspase-3/CPP32 proteolytic activity in controls (*C*) (*open bar*) and livers subjected to 60 (*filled bar*) or 180 (*shaded bar*) minutes of warm ischemia followed by 120 min of reperfusion. Caspase-3 activity is expressed as absorbance at 405 nm. Results are expressed as mean ± SE. As the duration of warm ischemia increased, caspase-3 activity was increased. $*P<0.05$ vs controls, $\S P<0.01$ vs controls, $\#P<0.05$ vs livers subjected to 60 min of warm ischemia

The ratio of Bcl-2/Bax in the livers subjected to 60 or 180 min of warm ischemia was 2.28 ± 0.40 and 0.99 ± 0.07, respectively (Fig. 9, bottom). The ratio of Bcl-2/Bax in the livers subjected to 60 min of warm ischemia was markedly increased compared with that of both the controls and the livers subjected to 180 min of warm ischemia ($P<0.01$).

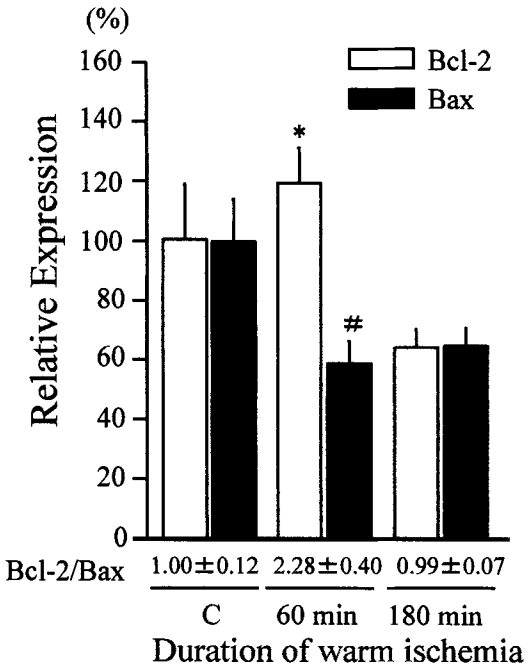

Fig. 9 The relative expression of Bcl-2 and Bax proteins and relative mean ratio of Bcl-2/Bax in controls (*C*) and livers subjected to 60 or 180 min of warm ischemia followed by 120 min of reperfusion. Expression of Bcl-2 (*open bars*) and Bax (*filled bars*) proteins was calculated from the result of ELISA, and the relative mean ratio of Bcl-2/Bax was determined (*bottom*). Results are expressed as mean ± SE. The ratio of Bcl-2/Bax was significantly increased in the livers subjected to 60 min of warm ischemia compared with that of controls and 180 min of warm ischemia. $*P<0.05$ vs livers subjected to 180 min of warm ischemia, $\#P<0.05$ vs controls

Discussion

The objective of this study was to identify markers such as HSP70 to predict the viability of livers subjected to warm ischemia-reperfusion injury. This should be useful in deciding whether organs from NHBDs can be used for transplantation.

Ischemia-reperfusion injury is responsible for primary liver dysfunction and failure after transplantation [7]. Cold preservation causes injury mainly to sinusoidal endothelial cells (SECs), whereas warm ischemia affects hepatocytes and endothelial cells [12].

Hepatic injury caused by ischemia has typically been described as coagulative necrosis. In recent years, an increasing number of publications suggested that SECs and hepatocytes undergo apoptosis during reperfusion after cold or warm ischemia [19]. According to these studies, 40–60% of hepatocytes undergo apoptosis during reperfusion. However, a recent report suggested that oncotic necrosis appears to be the principal mechanism of cell death for both SECs and hepatocytes.

In this study, we evaluated cellular injury caused after ischemia-reperfusion by hematoxylin and eosin (H & E) staining, TUNEL assay, DNA laddering, and caspase-3 activity. At 120 min of reperfusion, necrotic cells were 6.1% and 23.2% in the livers subjected to 60 and 180 min of warm ischemia, respectively. Thus, prolongation of warm ischemia periods before reperfusion facilitates necrotic cell death. We used TUNEL assay, DNA laddering, and caspase-3 enzyme activity to evaluate apoptotic cell death. However, TUNEL assay and DNA ladders detect DNA strand breaks, which occur during apoptosis and necrosis. Therefore, we used the TUNEL assay in combination with morphological criteria, i.e., cell shrinkage, chromatin condensation and margination, and apoptotic bodies, as the gold standard for identification of apoptosis. The number of apoptotic cells was significantly increased after ischemia-reperfusion. At 120 min of reperfusion, 2.9% and 7.4% of all cells were identified as having undergone apoptosis in the livers subjected to 60 and 180 min of warm ischemia, respectively. Caspase-3 activity was consistent with the results of TUNEL assay and DNA fragmentation, and a minor increase in caspase-3 activity could be detected. Thus, prolongation of warm ischemia periods before reperfusion also facilitates apoptotic cell death. Although necrosis of hepatocytes appears to be the principal mechanism of cell death, apoptosis may also be related to the process of cellular injury induced by warm ischemia-reperfusion in the liver.

In livers subjected to 60 min of warm ischemia, the proportion of apoptotic and necrotic cells was 2.9% and 6.1% of all cells, respectively. This means 68% of all dead hepatocytes died from oncotic necrosis. In the livers subjected to 180 min of warm ischemia, the proportion of apoptotic and necrotic cells was 7.4% and 23.2% of all cells, respectively. This means 76% of all dead hepatocytes died from oncotic necrosis. Therefore, prolongation of warm ischemia periods dominantly facilitates oncotic necrosis after reperfusion, whereas apoptosis can be induced by warm ischemia-reperfusion.

The expression of stress protein has two important aspects in clinical fields. One is their cytoprotective nature against noxious conditions, and the other is as a marker of cellular injury [1]. Accumulation of HSP70 mRNA reflects the severity of ischemia-reperfusion injury in the liver and may reflect the shortage in the HSP70 pool for adaptation against injurious conditions at real time rather than the amount of HSP70. Accumulation of HSP70 represents the consequences during a relatively long period [1].

In our previous study, no expression of HSP70 mRNA was detected in the livers subjected to ischemic conditions only (i.e., ischemia without reperfusion; data not shown). In this study, during the very early reperfusion period (within 120 min), the expression of HSP70 mRNA was augmented according to the prolongation of ischemia periods and reached a peak after 30 or 60 min of warm ischemia. The expression of HSP70 mRNA was diminished in the livers subjected to 90 min of warm ischemia and was markedly suppressed in livers with more than 120 min of warm ischemia, where severe cellular injury was observed. The expression of HSP70 mRNA was not affected by the duration of reperfusion during the very early reperfusion period, but, rather, was influenced by the duration of warm ischemia before reperfusion. Therefore, the expression of HSP70 mRNA was induced by reperfusion after warm ischemia and was presumed to relate closely to the duration of warm ischemia. In the livers subjected to more than 120 min of ischemia, many hepatocytes had become markedly injured, falling into necrosis or apoptosis. These hepatocytes may have had a markedly impaired ability to produce HSP70 mRNA against lethal ischemia-reperfusion stress. Although in previous studies [1] it has been suggested that the accumulation of HSP70 mRNA reflects the severity of ischemia-reperfusion injury, the expression of HSP70 mRNA was markedly diminished in the livers with severe cellular injury, in our present study. Therefore, these results suggest that the expression of HSP70 mRNA may be more specific and useful as a marker that reflects cellular injury caused by warm ischemia-reperfusion.

Previous studies have reported that expression of a stress-inducible form of HSP70 (HSP72) has been observed after a period of ischemia-reperfusion in the model of segmental hepatic ischemia [9, 11, 20]. In most reports, expression of HSP70 was detected at least 3 h after reperfusion, mostly 24 h or more, following brief warm ischemia from 15 to 60 min [9, 11, 20]. There are few reports using models of hepatic warm ischemia-reperfusion with long warm ischemia periods and brief reperfusion periods as performed in this study. In our present study, for the purpose of evaluating the viability of livers subjected to warm ischemia-reperfusion, we also made models predicted to have lost viability. Although we did not measure biochemical parameters such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) or other parameters related to liver function, these parameters have been generally shown to relate to histological assessment of necrosis in this model. Similarly to HSP70 mRNA, the expression of HSP70 was not affected by the duration of reperfusion during the very early reperfusion period (within 120 min), but was influenced by the duration of warm ischemia before reperfusion. Only a little HSP70 expression was shown in controls. Expression of inducible-form HSP70 was augmented as duration of warm ischemia increased up to 90min and significantly diminished in the livers subjected to more than 120 min of warm ischemia. The trend of HSP70 expression after warm ischemia-reperfusion seems to be slightly delayed

from that of HSP70 mRNA expression. Therefore, HSP70 mRNA may be a more sensitive marker of viability than HSP70 in livers subjected to warm ischemia-reperfusion. HSP70 expression is observed only if exposure time to warm ischemia is not too long. Cellular injury becomes irreversible, and there is impaired protein synthesis and progressive degradation of many cytoplasmic proteins including HSP70 in livers subjected to longer durations of warm ischemia.

In our model of hepatic warm ischemia-reperfusion, high expression of HSP70 and mRNA was observed in the livers with modest cellular injury, but was markedly suppressed in livers with severe cellular injury. The expression of HSP70 and mRNA was detected in the short time of 30–120 min after reperfusion. While the lesion associated with the whole transplantation process from NHBDs is more complex, ischemia-reperfusion injury is one of the important mechanisms of primary dysfunction of donor livers after transplantation. Although evaluating the viability of livers from NHBDs before transplantation was impossible in our present study, predicting primary dysfunction of the donor liver may be possible by performing liver biopsy within the short time of 30–120 min after reperfusion during surgery by detecting HSP70 expression. However, the methods that were used to measure HSP70 and mRNA are so time-consuming that further development is required.

Soon after heat shock treatment, a significant portion of HSP70 was found to accumulate within the nucleus, in particular, the nucleolus [34]. Previous studies have demonstrated significant alteration in nucleolar integrity after heat shock and accompanying inhibition of ribosome biogenesis events [29]. With time of recovery from stress, and as the nucleoli regain both their normal morphology and function, HSP70 appears to exit the organelle and accumulates to relatively higher levels within the cytoplasm [36]. In our present observation examining the intracellular localization of HSP70 in the liver subjected to warm ischemia-reperfusion, HSP70 was highly expressed particularly within the nucleus in livers subjected to 60 min of ischemia and was diminished in livers subjected to 180 min of warm ischemia. These results were consistent with the result of ELISA for HSP70. No expression was observed in controls. Therefore, the inducible form of HSP70 may migrate into the nucleus soon after reperfusion and still remain in the nucleus after 120 min of reperfusion.

HSP70 can prevent apoptosis that is induced by a variety of initiators, although the precise nature of their involvement is not known [28]. HSP70 has been suggested to rescue cells from apoptosis at a late stage in the overall process [4]. HSP70-mediated cytoprotective function involves the inhibition of caspase activation [15, 24]. Heat-induced apoptosis correlates with an increase in stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) activity. HSP70 can prevent stress-induced apoptosis by affecting both upstream signal pathway (SAPK/JNK activation) and downstream effector events (caspase-3-mediated proteolysis). In this study, caspase-3 activity was significantly suppressed in the livers subjected to 60 min of warm ischemia compared with the livers subjected to 180 min of warm ischemia, but no evident difference was shown in caspase-3 mRNA expression. Therefore, similarly to previous reports, HSP70 may be one of the factors that inhibit activation of caspase-3.

Apoptosis is governed by a number of regulating genes mediated by apoptotic signals. Members of the Bcl-2 family that act as inhibitors of apoptosis include Bcl-2 and Bcl-X_L, and those that act as promoters of apoptosis include Bax, Bad, and Bcl-X_S. It has been suggested that protein–protein interactions between Bcl-2 family members play a major role in controlling the apoptotic process [2, 37]. Bax may form homodimers to accelerate cell death, or heterodimers with Bcl-2 and Bcl-X_L to inhibit cell death. Therefore, a change in the ratio of Bcl-2 and Bax protein expression may attenuate the anti-apoptotic effect of Bcl-2 in reducing post-ischemic apoptosis [2]. Bcl-2 acts upstream of the caspase by preventing its activation [32]. Prior heat stress markedly increases the interaction between HSP70 and the anti-apoptotic signal protein Bcl-2 [35]. Furthermore, overexpression of Bcl-2 by transfection of this gene into hepatocytes with an adenovirus protected against warm ischemia-reperfusion injury [3]. In this study, the ratio of Bcl-2/Bax and the expression of Bcl-2 mRNA were markedly diminished in the livers subjected to 180 min of warm ischemia followed by 120 min of reperfusion, compared with the controls and livers subjected to 60 min of warm ischemia followed by 120 min of reperfusion. In livers with severe hepatocellular injury, relative decrease of Bcl-2 protein induced by the suppression of Bcl-2 mRNA expression could be related to the degree of apoptosis. In the livers with modest cellular injury, the increase in Bcl-2/Bax ratio may inhibit apoptosis by suppressing the activation of caspase-3. Therefore, the Bcl-2 family can be another useful marker to evaluate viability in the liver subjected to warm ischemia-reperfusion.

In conclusion, in the model of hepatic warm ischemia-reperfusion, high expression of HSP70 and mRNA was observed in livers with modest cellular injury, but was markedly suppressed in livers with severe cellular injury. Although necrosis of hepatocytes appears to be the principal mechanism of cell death, apoptosis may also be related to the process of cellular injury induced by warm ischemia-reperfusion in the liver. Expression of HSP70 and mRNA can be an effective marker of viability in the liver subjected to warm ischemia-reperfusion

injury. The Bcl-2 family can also be another useful marker to evaluate viability in liver subjected to warm ischemia-reperfusion.

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