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Caspase inhibition protects from liver injury following ischemia and reperfusion in rats

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Abstract Normothermic ischemia and reperfusion of the liver results in microcirculatory failure followed by necrosis and cell death. Recently, another type of cell death, apoptosis or programmed cell death, was found to be activated during the early phase of reperfusion after liver ischemia. Caspases are cysteine proteinases specifically involved in the initiation and execution phases of apoptosis. The aim of this study was to demonstrate that inhibition of apoptosis by a specific inhibitor of caspases might protect the liver against ischemia/reperfusion injury. Rats were divided into three groups: group 1, control, PBS administration; group 2, Z-Asp-cmk (Z-Asp-2,6-dichlorobenzoyl-oxymethylketone) treatment; group 3, sham-operated control animals. Z-Asp-cmk (0.5 mg Z-Asp-cmk dissolved in 300 µl PBS solution containing 1 % DMSO) was injected intravenously, 2 min prior to induction of 120 min ischemia. Survival rates were compared and serum activities of aspartate aminotransferases and alanine aminotransferases were assessed in the blood collected from the suprahepatic vena cava. Histology of the liver was assessed 6 h after the end of ischemia. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling method (TUNEL method) and by electrophoresis for analysis of DNA fragmentation. Caspase activity was determined by measuring hydrolysis of the CPP32-like substrate Ac-DEVD-pNA and absorption of paranitroaniline. Z-Asp-cmk treatment significantly increased 7-day survival (95 %) compared with that in nontreated rats (30 %, $P < 0.001$). Serum activities of aminotransferases and the extent of liver congestion and necrosis were significantly ($P < 0.001$) decreased after treatment with Z-Asp-cmk. TUNEL-positive cells were detected 3–6 h after reperfusion in the control group. In Z-Asp-cmk pretreated rats, a dramatic decrease in the number of TUNEL-positive cells was observed. Analysis of DNA fragmentation of freshly isolated hepatocytes confirmed these results. Caspase activity was increased 3–6 h after reperfusion in the control group, but significantly ($P < 0.001$) decreased after treatment with Z-Asp-cmk. These findings demonstrate that liver injury following ischemia and reperfusion can be prevented by inhibition of caspases. Caspase inhibitors may have important implications for therapy in liver disease and after liver transplantation.

Key words Normothermic liver ischemia · Apoptosis · Caspases · Rats

Introduction

Normothermic hepatic ischemia/reperfusion injury occurs during surgical resectioning, liver transplantation, and hemorrhagic shock [13]. The injury results in microcirculatory failure followed by necrosis and cell death [24]. Recently, another type of cell death, apoptosis or programmed cell death [14], was found to be activated during the early phase of reperfusion after liver ischemia in rat [20] and after liver transplantation in pig [21]. Caspases are cysteine proteinases specifically involved in the initiation and execution phases of apoptosis [4]. Several studies have demonstrated that inhibitors of this class of proteinases block essentially all forms of apoptosis in vitro [4, 10, 17, 18] and that caspase overexpression induces apoptosis in various cell lines [4, 9, 11]. The aim of this study was to demonstrate that inhibition of apoptosis by a specific inhibitor of caspases might protect the liver against ischemia/reperfusion injury.

Materials and methods

Animal preparation and hepatic ischemia procedure

All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals. Male Lewis Rats (LEW RTI[1]) weighing 250–300 g were purchased from the CNRS-CNSEAL (Orléans La Source, France). In each experiment there was less than 25 g difference between the animals. The rats, housed individually in Plexiglas cages, were allowed free access to food and water before, during, and after the ischemia. The animal rooms were windowless with temperature (22 ± 2 °C) and lighting controls (light on at 07.00 h and off at 21.00 h; 14 h light/10 h dark). All experiments were started at between 08.00 h and 11.00 h. A segmental normothermic ischemia of the liver was induced as previously described [2]. Briefly, the anterior abdominal wall was shaved and prepped with povidone-iodine (Betadine) solution. The abdomen was entered through a midline incision under ether anesthesia and ischemia induced by occluding the blood vessels including the bile duct to the median and left lateral lobes with an atraumatic vascular clamp. After 120 min warm ischemia the vascular clamp was released. This procedure was considered to render ischemia in 70% of liver tissue [19]. The abdomen was closed in two layers with 2–0 silk. Sham-operated animals underwent manipulation of the liver and mobilization of the relevant vessels, but had no clamp application. After the operation animals were kept in individual cages. Mortality rate and survival time were recorded every 6 h. Animals alive 7 days after the operation were considered as permanent survivors. Necropsy was performed on all animals to insure absence of surgically related complications.

Inhibitors and substrates

Stock IL-1β converting-enzyme inhibitor III (Z-Asp-cmk) [8, 16] was dissolved in 100% DMSO to a final concentration of 50 mg/ml. Z-Asp-cmk, acetyl-DEVD-pNA, and biotinyl-DEVD-CHO were purchased from Alexis Biochemicals.

Experimental groups

Rats were divided into three groups. In group 1 (control group, $n = 20$) animals were injected intravenously, via the dorsal penile vein, with 300 µl PBS solution, 2 min prior to induction of 120 min ischemia. Animals of group 2 ($n = 20$) were injected intravenously, via the dorsal penile vein, with 0.5 mg Z-Asp-cmk dissolved in 300 µl PBS solution containing 1% DMSO, 2 min prior to induction of 120 min ischemia. Group 3 were sham-operated control animals ($n = 5$).

Measurement of aminotransferases

Another set of animals was prepared as previously described [7] for measurement of aminotransferases. In groups 1 and 2 (10 rats for each group), 6 h after the end of ischemia, blood samples for measurement of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were obtained via the indwelling venous line and quantitated using standard clinical automated analysis. Blood sampling at identical times was performed in five sham-operated control animals.

Histological studies

Liver tissue was taken 6 h after the end of ischemia in groups 1 and 2 (10 rats of each group) and in five sham-operated control rats. Specimens were fixed in 10% formalin and stained with hematoxylin and eosin for light microscopical examination. The extent of sinusoidal congestion and liver necrosis was semiquantitatively assessed in 45 samples of median and left lateral lobes and scored as follows: congestion: none = 0, minimal = 1, mild = 2, moderate = 3, severe = 4; liver necrosis: none = 0, single-cell necrosis = 1, up to 30% lobular necrosis = 2, up to 60% lobular necrosis = 3, more than 60% lobular necrosis = 4. Blind analysis was carried out for all histological studies. To detect apoptotic cells, liver tissue was taken at 0, 3, 6, and 12 h after the end of ischemia. Three rats per group were used for each time span. Livers were excised and tissues were immediately cryopreserved. Six-micrometer sections were prepared for the terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling method (TUNEL method) of Gavrieli et al. [12], with minor modifications. The morphology of hepatocytes in situ was also examined on paraffin-embedded tissue section using both TUNEL and propidium iodide labeling. Two to three hundred hepatocytes on sections were examined and the number of TUNEL-positive hepatocytes per 100 hepatocytes was calculated. To avoid potential error in statistical sampling, fields were randomly selected. Histological examination was performed by one of the authors in a blind manner. A negative control was included in each experiment by performing the same procedure without terminal transferase.

Isolation of hepatocytes

To detect apoptotic cells, livers were digested with collagenase at 0, 3, 6, and 12 h after the end of ischemia. Three rats per group were used for each time span studied. Briefly, hepatocytes were isolated from rats with collagenase (Sigma type IV, Sigma Chemical, St. Louis, Mo.) by the perfusion method [22], modified as described [1].

DNA fragmentation

Freshly isolated hepatocytes from nonischemic and ischemic liver lobes were prepared from untreated or Z-Asp-cmk-treated rats. DNA fragmentation was assessed as described previously [15].

Caspase assay

After isolation of hepatocytes, cytosols were prepared at 4 °C and immediately assayed for enzymatic activity. Caspase activity was determined by the measuring hydrolysis of the CPP32-like substrate Ac-DEVD-pNA and absorption of paranitroaniline [15].

Statistical analysis

Significance in mortality results was assessed using the χ^2 test. Results were expressed as mean ± SEM. The comparison for statistical significance was performed according to the Kruskal-Wallis test for serum activities of aminotransferases and histological parameters. Statistical significance was set at $P < 0.05$.

Results

Survival rate

Preoperative treatment with Z-Asp-cmk significantly improved the 7-day survival rate following 120 min warm liver ischemia (95%), compared to the control group (30%, $P < 0.001$). Autopsy of the rats showed severe necrosis of the liver in all cases. In sham-operated control animals, the 7-day survival was 100%.

AST and ALT serum levels

In groups 1 and 2, serum AST and ALT levels were increased 6 h after the end of the ischemic period. However, the release of liver enzyme was significantly lower in animals treated with Z-Asp-cmk (AST: 4.099 ± 1.461 IU/l and ALT: 4.522 ± 1006 IU/l) compared with hose of the control group (AST: 12.144 ± 2.543 IU/l and ALT: 13.032 ± 2.607 IU/l, $P < 0.001$). In sham-operated control animals, the aminotransferase serum levels were 193 ± 41 IU/l for AST and 211 ± 20 IU/l for ALT.

Histological studies

Immediately after occlusion of the hepatic vessels, the anterior lobes became pale. After the clamp was released, the liver turned dark and rapidly regained its normal color. The degree of liver necrosis and of congestion 6 h after reperfusion was significantly lower in the Z-Asp-cmk-treated group than in the control group (scores 1.8 ± 0.6 vs 3.6 ± 0.6 and 1.3 ± 0.4 vs 3.5 ± 0.9 in groups 2 and 1, respectively, $P < 0.001$). No histological lesions were observed in the sham-operated control animals. The antiapoptotic effect of Z-Asp-cmk was confirmed by the TUNEL assay, which detects DNA fragmentation in situ. Six hours after reperfusion numerous hepatocytes from ischemic liver lobes were TUNEL-positive, whereas virtually no TUNEL-positive liver cells were observed in the nonischemic liver lobes. Preoperative treatment with Z-Asp-cmk decreased the number of TUNEL-positive cells (more than 40% of the cells were TUNEL-positive 3–6 h after liver reperfusion in untreated rats, versus 2–3% in Z-Asp-cmk-treated animals).

DNA fragmentation

In untreated rats no evidence of apoptosis was observed in freshly isolated hepatocytes prepared from nonischemic liver lobes, whereas massive internucleosomal DNA fragmentation was found to occur in cells from ischemic liver lobes after a 3-h reperfusion period. The caspase inhibitor had no effect of its own on nonischemic liver lobes, but fully protected cells derived from ischemic liver lobes from apoptosis. Identical results were obtained after a 6-h reperfusion period.

Caspase assay

In untreated rats, caspase activity increased by eight- to ten-fold in hepatocytes prepared from ischemic liver lobes 3 h (1.49 ± 0.09 nmol/min × mg) to 6 h (1.87 ± 0.15 nmol/min × mg) after liver reperfusion. Preoperative treatment of rats with Z-Asp-cmk abrogated caspase activity 3 h (0.27 ± 0.19 nmol/min × mg) to 6 h (0.43 ± 0.11 nmol/min × mg) after liver reperfusion.

Discussion

This study showed that Z-Asp-cmk treatment improved survival after normothermic liver ischemia in vivo. This result was associated with a reduction of liver injury, a decrease of aminotransferase levels, and an inhibition of hepatocyte apoptosis. The protective effect of Z-Asp-cmk can be totally accounted for by caspase inhibition since this inhibitor was found to abolish ischemia-mediated activation of caspases, as determined in an enzymatic assay. The precise mechanisms of ischemia/reperfusion injury remain obscure, even if hepatic cell death in our model is probably multifactorial. Several studies have shown that this type of cell death may be induced in rat liver by ischemia [14, 20, 21]. It has been

suggested that it represents a transient response to acute ischemia [23]; it plays a role in ischemia/reperfusion injury and is triggered by mild hypoxia, reactive oxygen intermediates, and cytokines produced by Kupffer cells and neutrophils [3, 5, 6]. All these signals are thought to converge in the activation of caspases, "the executioners of apoptosis" [4]. It is now well established that caspase activation is a prerequisite for many forms of cell death [4, 9, 11] and that blockade of caspases in various cell lines and some animal models is generally sufficient to inhibit apoptosis induced by different stimuli [4, 10, 17, 18]. The pivotal role of caspases in the regulation of apoptosis is likely to explain why Z-Asp-cmk is so efficient in protecting rats from lethal normothermic ischemia/reperfusion in the present study.

In conclusion, these findings have demonstrated that liver injury following ischemia and reperfusion can be prevented by inhibition of caspases. Liver apoptosis is negligible in physiological conditions, because hepatocyte turnover is very slow, but it plays an important role in many pathological conditions including liver ischemia. Caspase inhibitors may have important implications in therapy of liver disease and after liver transplantation.

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