Abstract

Background: The effects of Edaravone (Edr) on hepatic ischemia-reperfusion (I/R) injury and liver regeneration were examined in a pig hepatectomy model.

Methods: One hour of ischemia was induced by occluding the vessels and the bile duct of the right and median lobes. About a 40% left hepatectomy was performed after reperfusion. Six animals received Edr (3 mg/kg/h) intravenously and six control animals received saline just before reperfusion. Remnant liver volume, hemodynamics, and levels of AST, ALT, LDH, and LA were compared between the groups. Expression of TGF-β1 and IL-6 mRNA in hepatic tissues was examined using RT-PCR. Apoptosis and cell proliferation were demonstrated by TUNEL and Ki-67 staining, respectively.

Results: Serum AST, LDH, and LA levels were significantly lower at 3 hours and 1 week after perfusion in animals that had received Edr. In the Edr group, hepatic tissues showed a greater tendency for the expression of TGF-β1 mRNA to be inhibited at 1 week, although the difference was not significant. Also at 1 week in the Edr group, TUNEL-positive cells in the hepatic sinusoidal endothelium were significantly fewer, and Ki-67-positive cells were significantly more numerous.

Conclusion: We conclude that Edr reduces hepatic injury and supports tissue regeneration after I/R injury in this pig model.

Key words: Edaravone (Edr), hepatic ischemia-reperfusion injury (I/R), apoptosis, hepatocyte proliferation, hepatectomy, TGF-β, Ki-67, liver resection, pig

Introduction

Edaravone (Edr: 3-methyl-1-phenyl-2-pyrazolin-5-one), a potent free radical scavenger, has been shown to protect cardiomyocytes and the brain against ischemia/reperfusion (I/R) injury [1,2]. However, the mechanism responsible for this protective effect after I/R injury in the liver remains unknown [3]. We sought to define the effects of Edr after I/R in a porcine liver resection model. During hepatectomy, compression of the hepatoduodenal ligament (the Pringle method) is performed for blood flow control, but this can lead to
postoperative I/R injury that may adversely affect outcome [4]. Hepatic I/R damage is a result of multiple factors, including ischemia-induced hypothermia, cytokines, coagulopathy, and increased levels of cell adhesion molecules.

In addition, expression of transforming growth factor-β (TGF-β) has been demonstrated in a wide variety of diseases, and in normal cells and tissues, being most abundant in platelets and bone [5]. TGF-β1 exerts inhibitory effects on cell growth, but can also have a stimulatory effect on fibrosis and certain mesenchyme cells. Particularly in the context of the liver, TGF-β1 is a potent inducer of apoptosis in hepatocytes, and inhibits liver cell proliferation in vitro. It also plays a crucial role in terminating liver regeneration after partial hepatectomy in rats.

In the present study, we investigated the possible protective effect of Edr against hepatic injury and its effect on liver regeneration, particularly the suppression of hepatic cell apoptosis, after hepatic ischemia-reperfusion injury in a pig hepatectomy model.

Materials and Methods

Experimental groups: The study was performed using male pigs, weighing 23-26 kg (SEASCO, Saitama, Japan), in accordance with the Guidelines for the Care and Use of Laboratory Animals, Dokkyo University School of Medicine (Approval No. 0429). Two groups of animals were prepared: a group (n=6) in which Edr (supplied by Mitsubishi-Tanabe Pharma Co., Ltd., Osaka, Japan) was administered intravenously at a dose of 3 mg/kg/h, just at the commencement of reperfusion to completion of the liver resection, just from commencement of the clamp to 30 minutes. In a control group (n=6), physiological saline was administered intravenously at a dose of 3 mg/kg/h, just at the commencement of reperfusion to completion of the liver resection, just from commencement of the clamp to 30 minutes. In a control group (n=6), physiological saline was administered intravenously during the same period. The dose of Edr was the same as that employed clinically for treatment of brain infarction [6].

Surgical procedure: A chevron incision was performed under general anesthesia, and each of the branches of the portal vein, hepatic artery, and bile duct were carefully isolated and taped. Hemi-hepatic (approximately 60%) liver ischemia was induced by clamping the right and middle hepatic vessels and bile duct using vessel tapes, and was maintained for 60 min; the left portal branch was left patent to avoid bowel congestion. After declamping, the left portal vein and artery were ligated, and a left hemi-hepatectomy was performed (approximately 40%) (Figure 1). Liver transection was achieved by the crush-clamping method using Pean forceps. During liver transection, the exposed Glisson’s vessels were ligated with 2-0 or 3-0 silk. The hepatic vein was closed using continuous 4-0 Prolene sutures. During this procedure, hemodynamic parameters (systolic and diastolic arterial pressure, and heart rate) were monitored via a femoral arterial line. All pigs received Ringer solution during the procedure. After the operation, the pigs were cared for in the Dokkyo Medical University pig care unit and free oral intake was allowed after one postoperative day. No antibiotics were administered either orally or intravenously after surgery.

Measurements and sampling protocol: Blood samples were obtained from the arterial line immediately after laparotomy, at 5 and 180 minutes after reperfusion, and at 1 week after surgery. The levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and lactic acid (LA) were evaluated. Samples of hepatic tissue were obtained from the right lobe at laparotomy, immediately after reperfusion, and at 1 week after surgery, and were subjected to TUNEL and Ki-67 staining, and determination of IL-6 and TGF-β1 mRNA expression. Serum levels of AST, ALT, LDH, and LA were measured using standard clinical methods on an automated analyzer (Model 7170, Hitachi, Inc., Tokyo, Japan).
Total liver weight was calculated on the basis of previous experimental data from 30 pigs (data not shown). We calculated the average percentage of total liver weight to body weight (2.64%), estimated the total liver weight from preoperative body weight, and calculated the residual liver weight by subtracting the weight of the resected liver from the estimated total liver weight. The percentage increase in liver weight was calculated as: Estimated remnant liver weight immediately after resection / Remnant liver weight at one week after resection x 100.

**Histological examination:** Samples of liver tissue were obtained at the time of laparotomy, from the remnant liver just after hepatectomy, and at 1 week after surgery, fixed with 10% formalin for 24 h, and embedded in paraffin. Sections 3 μm thick were stained by the in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method using an apoptosis in situ detection kit (Wako Pure Chemical, Inc., Osaka, Japan) in accordance with the manufacturer’s instructions. The mean numbers of apoptotic cells per 10 high-power fields were calculated and compared between the two groups.

**Immunohistochemistry for Ki-67:** Four-micrometer-thick sections from paraffin-embedded tissue blocks were placed on slides. The sections were deparaffinized, rehydrated through a graded ethanol series at room temperature, and then pretreated with 0.3% H2O2 in methanol for 20 min at room temperature to quench the endogenous peroxidase activity. The sections were then placed in 0.01 mol/l citrate buffer (pH 7.2) and were subjected to microwave heating (95°C) for 40 min to facilitate antigen retrieval. They were then incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 20 min, followed by anti-mouse Ki-67 antibody (clone TEC-3; DAKO Japan, Kyoto, Japan; dilution 1 : 200) for 1 h. Thereafter, the sections were incubated with biotinylated secondary antibody (DAKO Japan) at room temperature for 30 min, washed with PBS, and treated with peroxidase-conjugated streptavidin (DAKO Japan) for 30 min. Finally, the sections were incubated in 3,3′- diaminobenzidine tetrahydrochloride with 0.05% H2O2 for 3 min, and then counterstained with Carazzi hematoxylin. Three hundred epithelial cells were counted in four different visual fields (magnification x200) on each section. The Ki-67 labeling index was calculated as the percentage of cells showing positive staining.

**Quantitative real-time PCR:** Immediately after surgery, a 30-mg sample of the excised liver tissue was snap-frozen in liquid nitrogen and kept at −80°C until extraction of total RNA using a Nucleospin II kit (Macherey-Nagel, Germany). Reverse transcription reactions were performed using a SuperScript III First-strand Synthesis System for t-PCR (Invitrogen, Carlsbad, CA). Briefly, 1 μg of total RNA, oligo dT primer, and dNTPs were incubated at 65°C for 5 min, and then 10 μl of cDNA synthesis mixture was added and incubated at 50°C for 50 min. The reaction was terminated by adding 1 μl of RNase H and was incubated at 37°C for 20 min. Real-time PCR was performed with an ABI Prism 7700 sequence detector (Applied Biosystems, Warrington, UK). The PCR reaction was carried out in a final volume of 1 μl cDNA and 2 μl 10x SYBR Green (Applied Biosystems) using 40 cycles at 95°C for 30 s and at 60°C for 30 s. The specific primers were designed using Primer 3 software (http://frodo.wi.edu/cgi-bin/ primer3/primer3_www.cgi) and were synthesized by Sigma Genosys (Hokkaido, Japan). The sequences of each primer were as follows: GAPDH: sense 5′-CCA CCC AGA AGA CTG TGG AT-3′, anti-sense 5′-AAG CCC CAG TTC CAA TTC TT-3′; and IL-6, TGF-β1: sense 5′-CCC CTG TCC ATC CCT TTA TT-3′, anti-sense 5′-AAG CCC CAG TTC CAA TTC TT-3′. For each PCR run, a standard curve was constructed from serial dilutions of cDNA from the PANCI cell line. The levels of expression of IL-6 and TGF-β1 were calculated using the formula: relative expression (t) = (copy number of IL-6 and TGF-β1 number / copy number of GAPDH) x 100. For non-template reactions and standard cDNA dilutions from PANCI cells, liver samples were assayed in triplicate. The average and standard deviation were calculated, and the t-value was determined from the averages.

**Statistical analysis**

All values are expressed as means ± S.D. Parameters were evaluated using a Student’s t test or paired t test. Differences between the two groups were evaluated using analysis of variance and were considered to be significant at P <0.05.
Results

There were no significant inter-group differences in body weight, amount of intraoperative hemorrhage, liver resection time, resected liver weight or proportion of liver volume resected (Table 1). There were also no significant inter-group differences in hemodynamics (Fig. 2 a-c), heart rate, or systolic and diastolic blood pressure.

Serum chemistry showed that the levels of AST and LDH (Fig. 3 a and b) were significantly lower at 1 week after surgery in the animals that had received Edr relative to the controls (P=0.022, P=0.009). Also, the level of LA was significantly lower at 3 h after reperfusion in pigs that had received Edr (Fig. 3 c, P=0.003).

<table>
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<tr>
<th>Table 1. Characteristics of pigs undergoing hepatectomy with and without Edr treatment.</th>
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<td><strong>Body weight (before operation)</strong></td>
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<td><strong>Body weight (after operation)</strong></td>
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Figure 2. (a) Systolic blood pressure, (b) diastolic blood pressure, and (c) heart. There were no significant inter-group differences in the three parameters.

Figure 3a. The aspartate aminotransferase (AST).

Figure 3b. Lactate dehydrogenase (LDH) levels in animals administered Edr were significantly lower at 1 week after reperfusion relative to the controls (P=0.022, P=0.009).

Figure 3c. The lactic acid (LA) level was significantly lower at 180 minutes after reperfusion in the Edr-treated pigs (P=0.003).

At 1 week after surgery, the proportionate increase in weight of the remnant liver was slightly increased in the Edr group relative to the control group, although the difference was not significant (P=0.668, Fig. 4).

In pig hepatic tissues, expression of TGF-β1 mRNA...
in the control group was significantly increased at 1 week after surgery, compared with the respective value at the start of laparotomy (Fig. 5, \( P=0.033 \)). Although this value did not differ significantly from that in pigs treated with Edr, it showed a tendency to be decreased at this time point. There was no significant inter-group difference in the expression of IL-6 mRNA at 1 week after surgery (Figure 6, \( P=0.672 \)).

TUNEL staining at 1 week after heptectomy revealed fewer TUNEL-positive cells in animals treated with Edr relative to the controls (Fig. 7a-d), and the difference was statistically significant \((48.5\pm13.4 \text{ vs. } 84.7\pm18.8 \text{ cells/10 fields}, \text{Fig. 7e, } P=0.02)\). However, the number of TUNEL-positive cells did not differ significantly between the groups immediately after perfusion \((55.5\pm19.2 \text{ vs. } 52.0\pm13.8 \text{ cells/10 fields}, P=0.778)\).

At 1 week after surgery, Ki-67-positive cells were detected more frequently in the Edr-treated animals, and the difference was statistically significant relative to the controls (Fig. 8a-d and e, \(41.5\pm25.5\% \text{ vs. } 12.2\pm6.1\%, P=0.035\)).
Figure 8. (a-d) Immunostaining of Ki-67. The number of Ki-67-positive cells in liver tissue was greater in Edr-treated pigs than in control pigs at 1 week after surgery. (e) The difference between the two groups at one week was statistically significant (P=0.035).

Discussion

In 1908, James Pringle first reported a hepatectomy procedure in which the control of hemorrhage was achieved by blocking the blood supply to the liver in cases of traumatic liver damage. Since then, the so-called Pringle maneuver has been performed for management of hemorrhage during hepatectomy [4]. However, this method has a risk of inducing abnormally high hepatic enzyme levels, icterus, hyperammonemia, lactacidemia, and intractable accumulation of pleural and peritoneal effusion postoperatively. Furthermore, its effect on liver regeneration after liver resection is still unclear. Despite the reduction of intraoperative hemorrhage achieved by the Pringle method, it may have an influence on outcome when the ischemic time is long, or in cases of hepatectomy for liver diseases, such as hepatic cirrhosis and fatty liver [7]. A factor that has a strong influence on outcome in this setting is ischemia-reperfusion injury, for which various countermeasures have been proposed. These include modified surgical methods, such as the intermittent Pringle method, partial hepatic pedicle clamping, and ischemic pre-conditioning, as well as administration of steroids, prostaglandin E1 or erythropoietin [7-14]. However, few studies have assessed the effectiveness of these approaches, which may be better clarified using animals that are similar in size to humans. In general, the process of I/R injury consists of multiple steps. Hypoxia due to cessation of the blood supply impairs oxidative phosphorylation in mitochondria, leading to profound cellular damage [15]. Furthermore, reperfusion exacerbates cellular damage by producing reactive oxygen species, activating pro-inflammatory cytokines, such as IL-10 and TNF-α [16], and up-regulating cell adhesion molecules, such as P-, E-, and L-selectins [17,18], resulting in tissue destruction.

Edr has potent hydroxy radical scavenging activity [19]. In various experimental models, Edr has been reported to protect organs such as the brain [20], heart [21], and liver [22,23] from free-radical-mediated injuries. Several studies have investigated the effect of Edr on I/R injury in the liver. Edr was found to improve portal flow and reduce the levels of hepatic enzymes, as well as decrease the malondialdehyde concentration [24]. Edr also decreased the degree of oxidative damage to mitochondria in the liver [23]. Although these results suggested that Edr might be useful for treatment of hepatic I/R injury, they indicated the effectiveness of Edr only in the acute phase of I/R injury, and its impact on liver regeneration after liver resection still remained unclear. Therefore, we investigated the protective effect of Edr in late-phase I/R liver injury at 1 week after partial hepatectomy, and examined whether Edr could partially suppress the up-regulation of cytokines, such as TGF-β, and increase the proliferation of hepatocytes, in terms of Ki-67 expression.

The serum levels of AST, LDH, and LA are the most objective parameters of damage to hepatic parenchymal cells, and clearly reflect the level of hepatic ischemia-reperfusion injury [25]. In the present study, serum levels of AST and LDH were significantly reduced at 1 week after hepatectomy in pigs that had received Edr, suggesting that Edr has the potential to inhibit hepatic injury.

TGF-β is a key mediator involved in the progression of liver disease, exerting cytotoxic effects, such as
induction of apoptosis and aggravation of microcirculation disorders, due to induction of expression of cell adhesion molecules. There are three major isoforms of TGF-β: TGF-β1, 2 and 3. These three isoforms are encoded by a distinct gene, and are expressed in a tissue-specific manner. TGF-β1 is expressed in endothelial, hematopoietic, and connective tissue cells, TGF-β2 in epithelial and neural cells, and TGF-β3 in mesenchymal cells. However, when expressed in the liver, their biological properties are very nearly identical [26]. TGF-β plays a major role in liver fibrosis by activating hepatic stellate cells (HSCs) (Ito cells, fat-storing cells) and Kupffer cells, the major cellular types involved in liver fibrogenesis. HSCs are activated after liver stress such as liver resection or I/R injury, and the activated HSCs produce extracellular matrix components as well as secreting many proinflammatory cytokines (TNF-alpha and TGF-β) [27]. TGF-β has been shown to have an antiproliferative action after partial liver resection in rats; when administered at the time of liver resection, it clearly delayed hepatocyte proliferation [28]. TGF-β is also a potent inducer of apoptosis in hepatocytes and several hepatoma cell lines in vivo [29-31]. In the present study, we demonstrated that Edr suppressed TGF-β1 expression in liver tissue, and exerted an anti-apoptotic effect in liver cells.

On the other hand, Ki-67 antigen is a prototypic cell cycle-related nuclear protein expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2, and M phase), but is absent in resting (G0) cells. Immunostaining for Ki-67 is useful for demonstrating the cell growth fraction in several tissues [32]. Therefore, we utilized Ki-67 expression to monitor cell proliferation in the remnant pig liver, and found that Edr increased the expression of Ki-67 in hepatocytes. Our present results suggest that Edr has a protective effect against I/R injury, and is able to suppress liver fibrosis and accelerate liver regeneration.

In conclusion, our results show that Edr has a protective effect against hepatic injury caused by reperfusion after hepatectomy. Although the exact mechanism responsible remains unclear, Edr inhibited the expression of TGF-β1, and thereby it may decrease hepatic injury, fibrosis, and apoptosis. Moreover, Edr is able to increase hepatocyte proliferation. Although the most appropriate dose, timing, and duration of Edr treatment in humans remain unclear, the present results obtained in large animals indicate that Edr may be clinically applicable for preventing I/R injury, particularly in hepatectomy.

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Supporting Information
None

Conflict of Interest statement
The authors have no conflicts of interest to declare.

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