The Effect of Neutrophil Elastase Inhibitor in Hepatectomy with Ischemia in Dogs

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Activated neutrophils play an important role in reperfusion injury following hepatic ischemia. Neutrophil elastase is a powerful proteolytic enzyme. We investigated the possibility that ONO-5046 - Na, which is a new recombinant-specific neutrophil elastase inhibitor, can reduce ischemia and reperfusion injury in the canine liver. Adult mongrel dogs (n = 19) were used in this experimental study. Seventy-five percent of the liver was resected after 90 min of vascular occlusion. The animals were divided into two groups. The ONO group (n = 8) was given ONO-5046 - Na dissolved in saline starting 30 min prior to clamping the hepatic inflow and continuing for 4 hr after reperfusion at a rate of 10 mg/kg. The nontreatment group (n = 11) received a saline solution for the same period. ALT and LDH levels were significantly lower (P < 0.05) in the ONO group than in the nontreatment group after reperfusion. Purine nucleoside phosphorylase and hyaluronic acid levels, which are markers of endothelial damage, were significantly lower (P < 0.05) in the ONO group than in the nontreatment group after reperfusion. Histologically, widely spread hepatocyte necrosis was found in dogs in the nontreatment group that died prematurely. Neutrophil infiltration of the sinusoids was less evident in the ONO group than in the nontreatment group. Neutrophil elastase inhibitor may prevent injuries of both endothelial and parenchymal cells in extended hepatectomy with vascular occlusion.

Key Words: ischemia-reperfusion; neutrophil elastase inhibitor; hepatectomy with vascular occlusion. © 1999 Academic Press

INTRODUCTION

Extended hepatectomy for large liver tumors has become feasible by the utilization of vascular occlusion techniques, such as Pringle's method or total hepatic vascular exclusion (THVE). However, vascular occlusion is accompanied by damage to the liver, due to ischemia-reperfusion injury [1]. Postoperative hepatic dysfunction due to ischemia-reperfusion injury is a serious problem in extended hepatectomy because of the small functional hepatic reserve. In such cases, the prevention of ischemia-reperfusion injury is quite critical.

Recent studies have suggested that ischemia-reperfusion injury of the liver is closely related to neutrophils. Activated neutrophils play an important role in reperfusion injury following hepatic ischemia through the generation of oxygen free radicals, the release of inflammatory mediators such as proteolytic enzymes and arachidonic acid metabolites, and the increased expression of adhesion molecules [2, 3]. Neutrophil elastase is one of these powerful proteolytic enzymes [4], which is normally limited by its own inhibitors in vivo [5]. As these inhibitors are deactivated by oxygen free radicals or chemical mediators released in conjunction with ischemia-reperfusion, neutrophil elastase induces endothelial injury and destroys its matrix [6–8]. N-[2-(4-methoxyphenyl)-3-nitrophenylamino]benzoyl-L-arginine ethyl ester (ONO-5046 - Na) has been produced as a recombinant-specific neutrophil elastase inhibitor [9]. We investigated the protective effect of ONO-5046 - Na in reducing ischemia-reperfusion injury in the canine liver.

MATERIALS AND METHODS

Animals. Twenty-five healthy adult mongrel dogs, weighing 9 to 15 kg, were used in this experimental study. All the animals were kept in accordance with the guidelines described in the National Institute of Health Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 85-23, revised 1985, Office of Science
and Health Reports). The animals had free access to water and standard pellet food, but were not fed for 12 h prior to the experiment.

Operative procedure. Following the subcutaneous administration of ketamine hydrochloride (2 mg/kg of body weight), anesthesia was induced with intravenous pentobarbital sodium (15 mg/kg). The animals were intubated and anesthesia was maintained with a mixture of nitrous oxide and oxygen in a ratio of 1:1. Using a respirator (MD-800; Senko Med. Co., Ltd., Tokyo), muscular relaxation was obtained with 0.1 mg/kg pancuronium bromide.

The procedure was performed under sterile conditions. A catheter was used to infuse Ringer's lactate solution during the experimental period at a rate of 10 mL/kg/h. The arterial blood pressure was monitored directly through a catheter introduced into the right femoral artery. The electrocardiogram, heart rate, and esophageal temperature were all monitored continuously throughout the procedure.

The abdomen was opened using a median incision. The liver was isolated by dividing the ligaments and all the peritoneal attachments to the liver. A catheter was inserted via a mesenteric vein to monitor portal venous pressure. The catheter was manipulated into the right hepatic vein by hand, and the position of its tip was confirmed by ultrasound. The common bile duct, portal vein, and hepatic artery were isolated. Our evaluation was limited to reperfusion injury due to hepatic inflow occlusion alone, excluding the effect of ischemic congestion. We adopted the following surgical procedure according to the model of MacKenzie et al. [18]. Clamping the portal pedicles of the caudate and right lateral lobes for 60 min induced partial ischemia of the liver and allowed mesenteric portal intransposition via patent portal vein. Following reperfusion, the nonischemic lobes of the liver (right central, quadratus, left central, left lateral, and quadri- lobe lobes) were resected immediately. This resulted in resection of 73 ± 3% of the liver [11], which is considered to be equivalent to an extended hepatectomy with vascular occlusion of the remnant liver without portal congestion. The abdominal wall was closed in two layers. After recovery from anesthesia, the animals were returned to their cages.

Experimental design. The animals were divided into two groups. The ONO group (n = 8) received ONO-5046·Na dissolved in a physiologic saline solution intravenously at a rate of 10 mg/kg/h, starting 30 min prior to clamping the hepatic inflow and continuing until 4 h after reperfusion. The nontreatment group (n = 11) received a physiologic saline solution intravenously over the same period.

Sample collection and assessments. Hepatic venous blood was collected, and the serum was stored at −80 °C until used for biochemical analyses. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), purine nucleoside phosphorylase (PNP), hyaluronic acid, and neutrophil elastase levels were measured. ALT, AST, and LDH were measured with an autoanalyzer with a UV assay (Hitachi 756-OH, Tokyo). PNP activity was measured using the method of Kawasaki et al. [12]. Hyaluronic acid was measured using a sandwich binding protein assay (Chugai Pharmaceutical Co., Ltd., Tokyo). Neutrophil elastase activity was determined by using a spectrophotometer to measure proportional p-nitroaniline levels (Sigma Chemical Co., St. Louis, MO).

Histopathological study of the liver. Liver specimens were collected for histological examination from the right lateral lobe pre- and postreperfusion in other dogs that underwent the same surgical procedure (n = 3 in each group). Specimens were fixed with 10% Formalin, embedded in paraffin, sliced into 3-μm-thick sections, and mounted. One of the sections was stained with hematoxylin and eosin and another was stained using the modified AO-D-chlorosce- etate esterase technique of Yam et al. [13] to count the number of neutrophils. Neutrophils in the sinusoids of the portal area were counted in 15 high-power fields (×400) under a light microscope.

Statistical analysis. The results are expressed as the mean ± standard error of the mean. Statistical significance was determined with an analysis of variance using Student's unpaired t test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

The animals weighed 12.0 ± 1.2 kg in the ONO group and 11.2 ± 2.4 kg in the nontreatment group. Before hepatic ischemia, the mean arterial pressures (MAP) in the ONO and nontreatment groups were 140 ± 12 and 145 ± 18 mm Hg, respectively. There were no significant changes in the general or hemodynamic status of the animals with the administration of ONO-5046·Na. During hepatic ischemia, the MAP in the ONO and nontreatment groups were 135 ± 20 and 142 ± 16 mm Hg, respectively. We quantified intestinal congestion by measuring the mean portal venous pressure (m-PVp). The m-PVp values were isoechischemia and during ischemia in the ONO group were 10.9 ± 1.2 and 10.2 ± 1.2 mm Hg, respectively, while the respective values in the nontreatment group were 10.2 ± 0.9 and 11.1 ± 1.4 mm Hg. There was no intestinal congestion in either group. Immediately after reperfusion, MAP decreased to 163 ± 38 mm Hg and m-PVp increased to 17.2 ± 5.2 mm Hg in the nontreatment group. The changes were most pronounced in the nontreatment dogs that died within 12 h of reperfusion. In the ONO group, MAP was 129 ± 25 mm Hg and m-PVp was 12.2 ± 4.2 mm Hg. Six dogs in the nontreatment group developed shock (MAP < 60 mm Hg) within 120 min of reperfusion and never recovered. In contrast, only one dog in the ONO group developed similar hemodynamic changes.

Serial changes in markers of parenchymal cell injury. The ALT levels were significantly (P < 0.05) lower immediately and 30 and 120 min after reperfu- sion in the ONO group. The respective ALT levels in the ONO and nontreatment groups were 95 ± 14 and 237 ± 53 IU/L immediately after reperfusion, 159 ± 15 and 258 ± 77 IU/L 30 min after reperfusion, and 214 ± 32 and 506 ± 108 IU/L 120 min after reperfusion (Fig. 1). The LDH levels were significantly (P < 0.05) lower immediately after reperfusion in the ONO group and were 321 ± 21 and 745 ± 183 IU/L in the ONO and nontreatment groups, respectively (Fig. 2). The AST levels were lower in the ONO group than in the nontreatment group, but the difference was not significant (data not shown).

Serial changes in markers of endothelial cell injury. PNPs were significantly different (P < 0.05) immediately after reperfusion in the ONO group (7.8 ± 1.4 U/L) compared with the nontreatment group (23.5 ± 2.7 U/L) and tended to remain lower for 24 h after reperfusion (Fig. 3). The hyaluronic acid levels were significantly (P < 0.05) lower at all times after reperfusion in the ONO group. The respective hyalu- ronic acid levels in the ONO and nontreatment groups were 43 ± 9 and 165 ± 56 μg/mL immediately after reperfusion, 88 ± 12 and 306 ± 66 μg/mL 30 min after reperfusion, and 72 ± 13 and 216 ± 54 μg/mL 120 min after reperfusion (Fig. 4).
Serial changes in neutrophil elastase activity. The neutrophil elastase activity increased after reperfusion in both groups. The neutrophil elastase activities immediately and 30 and 120 min after reperfusion were 17.5 ± 5.3, 19.2 ± 6.3, and 12.3 ± 6.6 ng/mL in the ONO group and 23.8 ± 10.6, 29.2 ± 16.3, 29.6 ± 15.6 ng/mL in the nontreatment group, respectively.

Histopathological examination. Hemorrhage and congestion of the portal area were observed in the nontreatment group 120 min after reperfusion. These conditions progressed to hepatocyte necrosis 6 h after reperfusion. Massive neutrophil infiltration and microthrombi in the sinusoids of the portal area were observed in the liver specimens, especially in the dogs that died within 24 h. On the other hand, minimal microscopic changes were observed 120 min after reperfusion in the ONO group (Fig. 5). With chloroaceta esterase staining, neutrophil sequestration was observed as time went by. The amount of neutrophil infiltration in the sinusoids preischemia and 30, 60, and 120 min after reperfusion was 2.6 ± 0.8, 11.1 ± 3.3, 16.2 ± 4.3, and 26.3 ± 14.1 in the ONO group, and 3.1 ± 0.9, 22.9 ± 10.3, 34.7 ± 9.8, and 47.8 ± 14.8 in the nontreatment group, respectively. The neutrophil infiltration in the ONO group was less evident than in the nontreatment group, but the difference between the groups was not significant. Figure 6 shows the amount of neutrophil infiltration in each group 120 min after reperfusion (Fig. 6).

Survival rate. All the surviving dogs were observed for 3 days following reperfusion. The 12-h survival rate after reperfusion in the ONO and nontreatment groups was 75 and 45.5%, respectively. The 24-h survival rate after reperfusion in the ONO and nontreatment groups was 62.5 and 36.4%, respectively (Table 1). An autopsy was performed in all the dogs that died prematurely and all the surviving dogs were sacrificed 3 days after the operation. The livers of the surviving dogs were viable based on the clinical data and histopathological findings. The histopathological findings suggested that the primary cause of all the premature deaths was hepatic failure. All the nontreatment dogs that died within 12 h of reperfusion had serosanguinous ascites, intestinal congestion, pulmonary edema, and pleural effusion at autopsy. Only one dog in the ONO group had similar findings.

DISCUSSION

The utility of hepatic vascular exclusion in reducing blood loss during an extended hepatectomy has been well documented. This procedure, however, is associ-
TABLE 1

<table>
<thead>
<tr>
<th>Time after reperfusion (hr)</th>
<th>ONO group</th>
<th>Non-treatment group</th>
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<tbody>
<tr>
<td>4</td>
<td>81.9 (9.2)</td>
<td>87.5 (7.8)</td>
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<td>6</td>
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PNP is a marker of endothelial cell injury [35]. Normally, endothelial cells rapidly take up hyaluronic acid, so elevated hyaluronic acid levels also reveal endo-
thelial cell injury [36, 37]. Endothelial cells are ex-
posed to intestinal endotoxins, chemical mediators re-
leased from other organs, and activated neutrophils more
directly than the parenchymal cells [38]. In ad-
dition, relative portal hypertension, which is caused by
the sudden reduction of the vascular area in an ex-

ated with ischemia-reperfusion injury of the liver. The
function of the residual liver is critical for survival in
spite of its reduced volume. Therefore, it is important to
reperfusion injury in an extended hepatec-
tomy with vascular occlusion. In recent studies, several
researchers have shown that neutrophils play an im-
portant role in reperfusion injury of the liver [2, 3].
Neutrophils are activated by chemical mediators, released
as a result of the small intestinal congestion associated
with portal clamping [14] and by the sinusoidal endo-
thelial cells of the liver, especially Kupffer’s cells [3].
Activated neutrophils release cytokines, which stimu-
late other neutrophils [15]. At the same time, the ap-
pearance of neutrophil chemotactic factors, such as the
interleukin-8 family, causes activated neutrophils to
accumulate in the reperfused liver [16]. After this ap-
pereession of adhesion molecules on the surface of the
endothelial cells [17], activated neutrophils can easily
migrate to the endothelial cells, roll toward the en-
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dental cell injury, which is caused by increased
endothelial cell permeability and increased
endothelial cell damage [39]. This results in a
severe liver ischemia/reperfusion and resec-
tion model. The half-life of the agent is 6.6 ± 0.3 min at
a dose of 10 mg/kg, so we administered ONO-5046:
Na continuously for 4 h after reperfusion.
In warm liver ischemia, TNFα in the rat liver and endothelial in the dog liver are elevated during isch-
emia [29, 30]. In the canine small bowel warm ischemic
model, myeloperoxidase activity in the tissue is ele-
vated, although TNFα is decreased in the cold isch-
emic model [31]. We speculated that immunostaining,
cytokines and mediators were induced and activated
neutrophils are directly in the portal circulation
and TNFα mRNA [33]. Therefore, ONO-5046:Na given
beginning 30 min prior to clamping the hepatic inflow
might produce significant effects on the neutrophil cell

Several protease inhibitors have been developed in
order to prevent neutrophil-elastase-induced tissue in-
jury [21, 22]. However, there have been few clinical
studies in which these neutrophil elastase inhibitors
have a significant effect. One reason is that these in-
hibitors are readily inactivated by oxidation with the
superoxide produced by activated neutrophils. The
clinical dosage of these inhibitors administered in vivo
is not sufficient to augment the effects of natural pro-
tease inhibitors of the airway neutrophil elastase [23].
Table 1: Survival Rate after Extended Hepatocetomy

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dition, relative portal hypertension, which is caused by
the sudden reduction of the vascular area in an ex-

aped hepatectomy to microcirculatory improvement and
endothelial cell injury [39]. The persistent bile duct
sustains the function of paren-

chymal cells.

The survival of the ONO animals declined after dis-
continuing the agent. After reperfusion, neutrophil-
dominant tissue injury is believed to continue for at
least 24 h [2, 3, 39]. Therefore, in our opinion, in
clinical applications the agent should be continued for
at least 24 h after reperfusion. Long-term intravenous
infusion of ONO-5046:Na with no major side effects
was reported in a preliminary study [28, 35]. Yasuyama
et al. [28] reported that a combination of ONO-5046:Na
and anticoagulant factors was more effective in reduc-
ing reperfusion injury than ONO-5046:Na alone in the
rat liver. In addition to neutrophil-activated injury, re-
perfusion injury of the liver is associated with com-
ponents of neutrophils and the inflammatory cyto-

kine network, the arachidonic acid cascade, and platelet-derived microaggregation [28]. The combined
administration of ONO-5046:Na with antagonists of the
other pathways may improve clinical survival.

In conclusion, neutrophil elastase is one of the key
mediators of reperfusion injury in the liver. Ischemia-
reperfusion injury of the sinusoidal endothelial cells
and parenchymal cells was reduced by administration
of the neutrophil elastase inhibitor ONO-5046:Na.

The administration of a neutrophil elastase inhibitor
may reduce ischemia-reperfusion injury in hepatec-
tomy with vascular occlusion and may have a clinical
role in hepatic surgery involving ischemia-reperfusion,
including liver transplantation.

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