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The effect of neutrophil elastase inhibitor on acute tubular necrosis after renal ischemia-reperfusion injury

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Abstract. In renal transplantation, ischemia-reperfusion (I/R) injury is a major cause of renal dysfunction. Activated neutrophils are reported to be closely involved in I/R injury after renal transplantation. Neutrophil elastase, a protease released from activated neutrophils, damages tubular endothelial cells. We investigated the beneficial effect of neutrophil elastase inhibitor (ONO-5046.Na) on renal I/R injury in rats. The study was conducted using 10 male Lewis rats (270-320 g) that were intravenously administered ONO-5046.Na (30 mg/kg before ischemia and after reperfusion) (group A) and control rats (group B) in a 90-min renal warm I/R injury model. Neutrophil elastase expression was analyzed using immunohistochemical staining, and the degree of renal dysfunction was evaluated using H&E staining and blood biochemistry. Neutrophil elastase was detected in tubular endothelial cells. The necrotic area extended to and encompassed nearly all the ischemic kidney within 12 h after reperfusion. The necrotic area and the grade of neutrophil elastase staining were significantly reduced in group A compared to group B. Significant differences in blood urea nitrogen and serum creatinine levels were observed. Survival rates over a 14-day period were examined. No rats survived for more than 4 days in group B. However, 2 of the 10 rats (20%) in group A survived for a 14-day period. To conclude, ONO-5046.Na inhibits neutrophil elastase and reduces acute tubular necrosis. Thus, it is a potent therapeutic agent for the control of renal I/R injury in renal transplantation.

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Introduction

Ischemia-reperfusion (I/R), an invariable consequence of renal transplantation, is a pressing clinical problem. It begins at the onset of acute tubular necrosis (ATN), when transplantation takes a long ischemic interval due to the use of a kidney from a cardiac arrest donor. The longer the ischemic interval, the higher the incidence rate of ATN (1).

Recent studies of I/R injury have focused on the function of neutrophils, the mechanisms of the action of inflammatory cytokines, oxygen-free radicals, the coagulation system, vascular plugging, edema, and other complications (2).

Activated neutrophils have been shown to be involved in the development of I/R-induced renal injury (3). Neutrophil elastase, a protease released from activated neutrophils, damages tubular endothelial cells. Furthermore, neutrophil elastase enhances cytokine production, adhesion molecule expression and superoxide release (4), thus finally inducing ATN. The inhibition of neutrophil action is therefore essential for ATN prevention.

ONO-5046.Na (Ono Pharmaceuticals, Osaka, Japan) is a specific and competitive neutrophil elastase inhibitor. This inhibitor is clinically used to ameliorate acute respiratory distress syndrome. It is reported to have a positive effect on heart (5), lung (6-8), liver (9-11) and intestinal (12) I/R injury in experimental models; however, no study has addressed its effect on renal I/R injury.

We hypothesized that neutrophil elastase inhibition by ONO-5046.Na may significantly attenuate renal I/R injury. Using a rat model, the effect of ONO-5046.Na on renal I/R injury was investigated in order to prevent ATN.

Materials and methods

Ischemia-reperfusion model. Male Lewis rats (270-320 g) were used. The right kidney was harvested by laparotomy using pento-barbital sodium anesthesia, then the left renal artery and vein were clamped with a hemostasis clip for 90 min.

Table I. Effect of ONO-5046.Na on survival over a 14-day period.

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8-14
Group A (n=10)	10	10	10	9	6	4	4	3	2
Group B (n=10)	10	10	10	5	0	0	0	0	0

Survival (in days) over a 14-day period after an ischemic interval of 90 min. None of the 10 rats (0%) in group B survived more than 4 days. However, 2 of 10 rats (20%) in group A survived for a 14-day period.

Table II. Effect of ONO-5046.Na on blood biochemistry.

After reperfusion	Group A (n=5)	Group B (n=5)
BUN (mg/dl)		
6 h	46.2±3.96	50.2±5.16
12 h	77.9±4.98[a]	91.6±8.02[a]
sCre (mg/dl)		
6 h	1.78±0.14	1.92±0.13
12 h	2.46±0.09[a]	2.96±0.11[a]

The serum concentrations (means ± SD) of blood urea nitrogen (BUN) and serum creatinine (sCre) were measured at 6 and 12 h after reperfusion. A difference in blood biochemistry appeared ~2 h after reperfusion. Statistical procedures were performed using ANOVA; [a]P<0.05.

The clip was subsequently removed to permit reperfusion. ONO-5046.Na (30 mg/kg) was delivered intravenously before ischemia and after reperfusion to prevent the activation of neutrophils in the inferior vena cava. The abdomen was closed during I/R. The left kidneys were reperfused, and the rats sacrificed at 0, 1.5, 3, 5, 12 and 24 h after reperfusion. The kidneys were harvested for hematoxylin and eosin (H&E) staining and immunohistochemistry. As indicators of tubular endothelial cell dysfunction, blood urea nitrogen (BUN) and serum creatinine (sCre) were measured 6 and 12 h after reperfusion using a portable clinical analyser (I-STAT200; Fuso, Japan). Samples of ischemic and non-ischemic kidney tissue were fixed in 10% buffered formalin for 24 h for immunohistochemical staining.

Reagents. Sodium N-[2-[4-(2,2-dimethylpropionyloxy) phenylsulfonylamino]-benzoyl] amino acetate tetrahydrate (ONO-5046.Na; $C_{20}H_{21}N_2O_7SNa \cdot 4H_20$; mol wt 528.51) was purchased from ONO Pharmaceutical Company, Osaka, Japan. ONO-5046.Na is a potent, specific, and intravenously-administered neutrophil elastase inhibitor.

Immunohistochemical staining. Immunohistochemical staining was performed using a Vectastain avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA) as previously described (13). Primary antibodies against goat neutrophil elastase (Santa Cruz Biotechnology Inc., CA, USA) were diluted 1:30 with 1% bovine serum albumin in phosphate-buffered saline and allowed to react with the sample for 1 h at room temperature. Similar staining with non-immune goat serum was performed as a negative control.

Analysis of acute tubular necrosis. To quantify the acute tubular necrosis (ATN), the results of immunostaining were graded on a scale of 0 to 3 by two observers in a blinded manner and evaluated as no necrosis, mild, moderate or severe. Necrosis, capillary congestion, interstitial edema, cast, destruction, and flat and extended areas of tubular epithelial cells were also evaluated. To quantify ATN, the same two pathologists performed assessments throughout the study.

Statistical analysis. Results were expressed as the means ± standard deviation (SD). Survival rates were calculated using the Kaplan-Meier method. Differences in blood biochemistry were assessed by the Wilcoxon signed-rank test. Other statistical procedures were performed using the analysis of variance (ANOVA) (14).

Results

Effect of ON-5046.Na on survival rates. In group B, 10/10 rats survived days 1 and 2, 5/10 day 3 and 0/10 day 4. In group A, 10/10 survived days 1 and 2, 9/10 day 3, 6/10 day 4, 4/10 day 5, 3/10 day 7, and 2/10 survived a 14-day period (Table I).

Effect of ONO-5046.Na on blood biochemistry. The serum concentrations of BUN and sCre were measured at 6 h after reperfusion (BUN: A, 46.2±3.96; B, 50.2±5.16 mg/dl; and sCre: A, 1.78±0.14; B, 1.92±0.13 mg/dl) and at 12 h after reperfusion (BUN: A, 77.9±4.98; B, 91.6±8.02 mg/dl (P<0.05); and sCre: A, 2.46±0.09; B, 2.96±0.11 mg/dl (P<0.05) (Table II). A difference in blood biochemistry was apparent ~12 h after reperfusion.

H&E staining in the kidney. H&E staining showed normal architecture of the kidney before ischemia. In group B, tissue damage began 1.5 h after reperfusion. At 3 h after reperfusion, the internal spaces of the tubular epithelial cells were expanded, and slight destruction was apparent (Fig. 1A). At 12 h after reperfusion, necrosis extended throughout the ischemic kidney and approximately all tubular epithelial cells were destroyed (Fig. 1B).

However, in group A all tissue damage was very slight. At 3 h after reperfusion, the necrotic and destroyed areas of the tubular epithelial cells were barely visible with H&E staining (Fig. 1C). At 12 h after reperfusion, necrotic areas showing slight destruction, capillary congestion and cast in the tubular epithelial cells were limited (Fig. 1D).

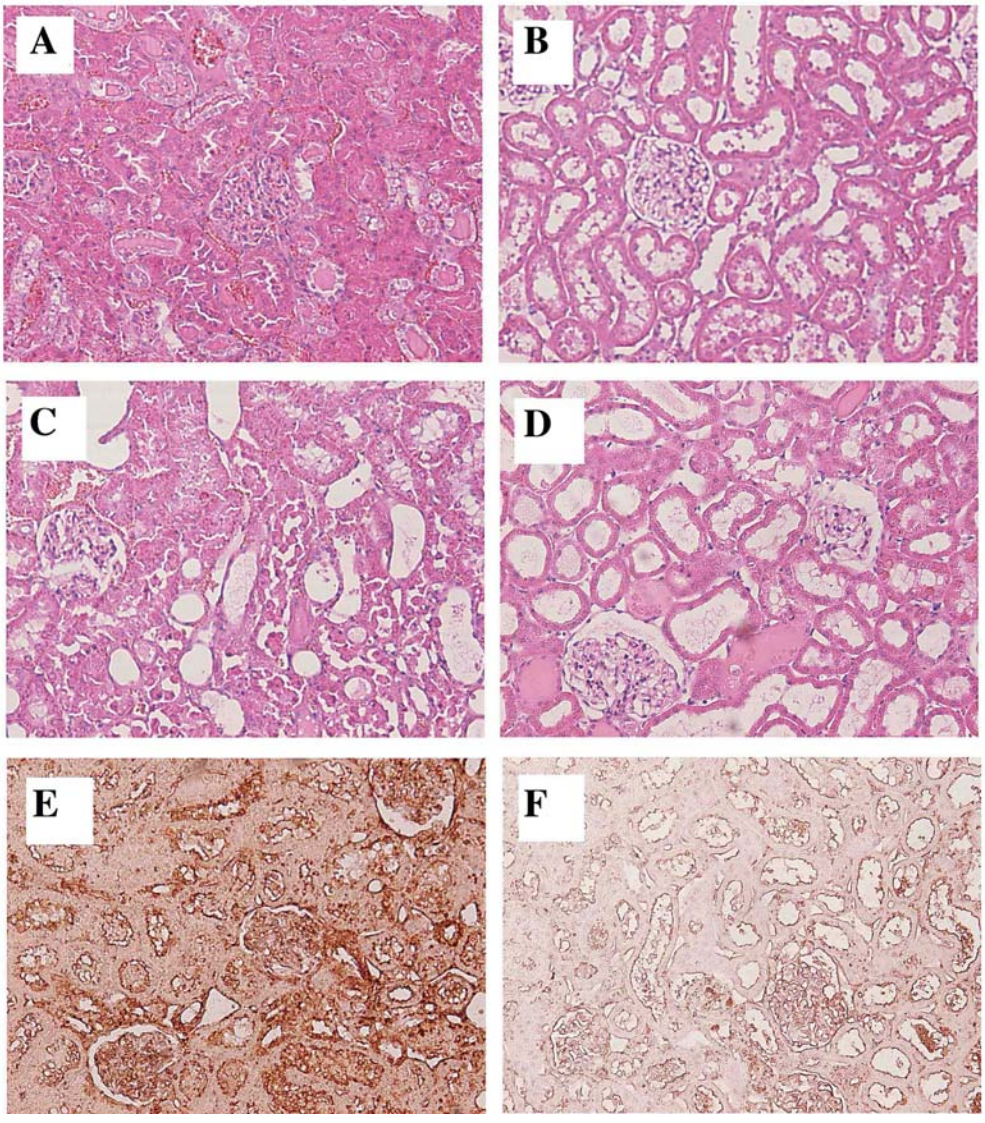

Figure 1. H&E and immunohistochemical staining in the kidney. In group B, neutrophil elastase staining was most intense in the endothelial cells only 1.5-3 h after reperfusion (E). At 3 h after reperfusion, the internal spaces of the tubular epithelial cells were expanded and slight destruction was apparent (A). At 12 h after reperfusion, necrosis extended throughout the ischemic kidney, and approximately all tubular epithelial cells were destroyed (B). However, in group A neutrophil elastase staining was very weak in the endothelial cells only 1.5-3 h after reperfusion (F). At 3 h after reperfusion, the necrotic and destructive areas of tubular epithelial cells were barely visible (C). At 12 h after reperfusion, necrotic areas showing slight destruction, capillary congestion and cast in the tubular epithelial cells were limited (D).

Immunohistochemical staining in the kidney. In group B, neutrophil elastase staining was negative in the tubular epithelial cells of the normal kidney, whereas slight staining of the endothelial cells was apparent 1.5-3 h after reperfusion. Neutrophil elastase staining was most intense in the endothelial cells only (Fig. 1E). Five hours after reperfusion, neutrophil elastase staining was weak. As of 12 h after reperfusion, neutrophil elastase staining was nearly at normal levels. However, in group A, neutrophil elastase staining was very weak in all tissues, and was very weak in the endothelial cells only 1.5-3 h after reperfusion (Fig. 1F).

Statistical analysis of acute tubular necrosis score. At 1 h after reperfusion, ATN scores were significantly increased [A, 0.88±0.57; B, 2.23±0.74 (P<0.01)]. At 24 h after reperfusion, the scores were highest [A, 2.21±0.75; B, 3.44±0.60 (P<0.01)]. All group B scores were significantly higher than the group A scores (Table III).

Table III. Statistical analysis of acute tubular necrosis (ATN) score.

After reperfusion	Group A (n=30)	Group B (n=30)
1 h	0.88±0.57[a]	2.23±0.74
3 h	1.04±0.45[a]	2.35±0.44
5 h	2.34±0.86[a]	3.12±0.74
12 h	2.72±0.52[a]	3.15±0.49
24 h	2.21±0.75[a]	3.44±0.60

ATN score (0-3) was determined for coded sections by two observers in a blinded manner: 0, no destruction; 3+, maximum destruction. All group B scores were significantly higher than the group A scores. Statistical procedures were performed using ANOVA; [a]P<0.01.

Discussion

In renal transplantation, I/R injury clinically affects renal function due to the ischemic interval being longer than in nephron-sparing surgery for accidental kidney tumors and extra-corporeal surgery. Some degree of renal I/R injury is inevitable in renal transplantation, and is one cause of graft dysfunction. It may also induce serious graft failure. The problem occurs at the onset of ATN, when transplantation requires a long ischemic interval as a consequence of using a kidney from a cardiac arrest donor. It has been reported that less extensive I/R injury results in decreased ATN after transplantation. A shorter period of I/R after transplantation correlates with better renal function and an increased post-operative survival rate. The priority in renal transplantation, and the principal clinical goal of our research team, is the prevention of I/R injury and the reduction of ATN (1).

Recent studies have suggested a significant relationship between I/R injury and neutrophils (3-12). Neutrophils release various substances that cause tissue damage, including oxgen-free radicals, neutrophil elastase and arachidonic acid (9). Activated neutrophils play a key role in I/R injury through the generation of cytokines and free radicals, the elastase of proteolytic enzymes and the expression of adhesion molecules. During the ischemic period, infiltration of neutrophils into inflammatory lesions is stimulated by the release of neutrophil elastase, a powerful protease considered to be the primary cause of neutrophil cell toxicity (15). During the reperfusion period, the release of superoxide radicals and chemical mediators induced by anoxia-reoxygenation deactivates these inhibitors, and neutrophil elastase destroys the cell matrix. This tissue destruction activates the cytokine network, and stimulation of the inflammatory cycle promotes further neutrophil accumulation and the release of more neutrophil elastase (9).

Several reports have demonstrated that neutrophil elastase inhibitor ameliorates heart, lung, liver and intestinal I/R injury in experimental models. Ueno *et al* demonstrated that ONO-5046.Na has a strong protective effect against I/R injury in canine heart transplantation (5), Ishikawa *et al* that it improves lung function in rabbit lung I/R injury (6), Tomizawa *et al* that it prevents lung I/R injury in dog lung transplantation (7), and Soejima *et al* that it attenuates liver I/R injury in rat liver transplantation (9). Tomizawa *et al* also demonstrated that treatment with ONO-5046.Na has a protective effect on hepatectomy with ischemia in a dog model (10), and Takayama *et al* showed that it attenuates lung injury after rat intestinal I/R injury (12). However, there have been no studies addressing renal I/R injury.

In this study, neutrophil elastase expression was detected in a rat model with renal I/R injury. Neutrophil elastase expression was most intense in the endothelial cells only 1-3 h after I/R injury; 12-24 h after I/R injury, maximum tissue damage was observed. Intravenously-administered ONO-5046.Na was also shown to reduce the degree of neutrophil elastase expression, BUN and sCre, and the ATN score in a rat 90-min renal warm I/R injury model.

In conclusion, ONO-5046.Na inhibited neutrophil elastase expression in endothelial cells, ameliorating damage following renal I/R injury and reducing ATN. Intravenously-administered ONO-5046.Na, which has been shown to attenuate I/R injury of the heart, lung, liver and intestine, improved survival rates associated with renal I/R injury. No previous study has addressed renal I/R injury, thus our findings are the first to indicate that administration of ONO-5046.Na may have a new clinical application in renal transplantation.

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