Results

Experiment 1

Effects of Ischaemia on the TM of the endothelium. In the normal rat kidney, TM was stained strongly on the peritubular capillaries. At 5 min after reperfusion, TM expressed in a patchy pattern with only weak staining in some parts of the peritubular capillaries especially in the outer medulla of the kidney. At 18 h after reperfusion, the pattern of TM expression appeared irregular, and the intensity of the staining greatly diminished (Figure 1).

Experiment 2

Effects of RHS-TM on renal function and coagulation parameters. Rats which underwent renal I/R exhibited significant increases in levels of serum creatinine and BUN ($P < 0.01$ *vs* the sham group). RHS-TM significantly suppressed these increases ($P < 0.05$ *vs* the saline group). In the argatroban group, however, serum creatinine and BUN levels showed a tendency to be lower, but not statistically significant (Figure 2). Significant differences were not observed in PT or APTT among four groups (Figure 2).

Effects of RHS-TM on renal cortical microcirculation and tubules. By direct imaging of the renal cortex at 24 h after IRI using a CCD video microscope, information on renal blood flow and the condition of the renal tubules was obtained (Figure 3). The movement of the erythrocytes was observed in the cortical peritubular capillaries and RBC velocity in the cortex was determined by the analysis of the speed of the erythrocytes. Gross differences were observed between the saline-injected rats and normal rats. In the saline group, the renal cortical peritubular capillaries shrunk and blood flow was severely impeded as compared with normal rats; the speed was 0.15 ± 0.02 mm/s in the saline group and 0.59 ± 0.08 mm/s in normal rats, $P < 0.01$. Furthermore, most of the renal tubules were destroyed or obstructed by detached epithelial cells. In contrast, in RHS-TM-injected rats, the condition of the cortical capillaries was close to that of normal rats. Only a mild destruction of the tubules was observed, and RBC velocity in the cortex significantly improved: the speed was 0.35 ± 0.07 mm/s in the RHS-TM group ($P < 0.01$ *vs* the saline group). In contrast, among argatroban-treated rats, severe destruction and obstruction of the tubules was observed, and RBC velocity was not significantly different from the saline-treated rats (Figure 3).

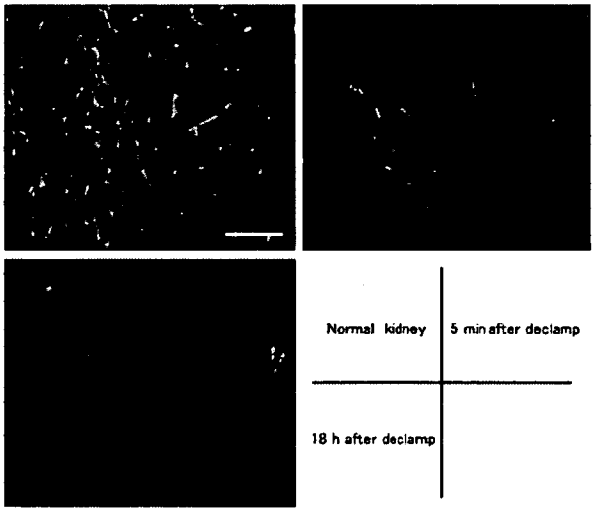

Fig. 1. The expression of TM in the kidney after ischaemia reperfusion. In the normal rat kidney, TM expressed in a linear pattern on the peritubular capillaries (upper left panel). At 5 min after declamping, the staining for TM became weaker (upper right panel). At 18 h after reperfusion, the intensity of TM expression further diminished (lower left panel). Bar, 100 μm.

Effects of RHS-TM on tubular injury. Examination of PAS stained kidney sections from the saline-treated rats showed that tubular injury occurred mainly in the outer medulla of the kidney. RHS-TM greatly ameliorated the tubular injury, while argatroban partially attenuated the tubular damage (Figure 4). The severity of the tubular damage, including tubular dilatation, degeneration and cast formation was scored. Treatment with RHS-TM resulted in significantly better scores than the saline group (2.2 ± 1.5 *vs* 4.4 ± 0.5, $P < 0.01$). In contrast, the argatroban-treated group (2.9 ± 1.2) failed to show significantly better scores than the saline-treated group (Figure 4).

Effects of RHS-TM on cellular infiltration. The numbers of macrophages decreased in both the RHS-TM and argatroban groups compared with the saline group (3.25 ± 1.04, 4.10 ± 0.56, 2.04, and 7.08 ± 1.71, respectively, $P < 0.01$) (Figure 5). RHS-TM administration also suppressed neutrophil infiltration when compared with saline (2.07 ± 0.64 and 5.83 ± 2.04, respectively, $P < 0.01$). In contrast, administration of argatroban (3.82 ± 1.61) did not make a significant difference (Figure 6).

Effects of RHS-TM on apoptosis in the kidney. The contribution of apoptosis to the renoprotective properties of RHS-TM was assessed by performing TUNEL assay on the kidney sections. In the saline-treated rats, TUNEL-positive cells were localized mainly in the tubules of outer medulla. RHS-TM significantly decreased the number of TUNEL-positive cells as compared with saline only (8.52 ± 4.6 and 26.4 ± 7.4, $P < 0.05$) while argatroban did not (20.26 ± 10.5) (Figure 6). Only a few TUNEL-positive cells were observed in the sham group.

Effects of RHS-TM on the expression of rat TM. Peritubular capillaries were strongly stained for rat TM in the sham group, whereas, only weak and patchy staining for rat TM was observed on the peritubular capillaries of the outer medulla at 24 h after reperfusion in the saline group—consistent with the results shown in Experiment 1. RHS-TM significantly

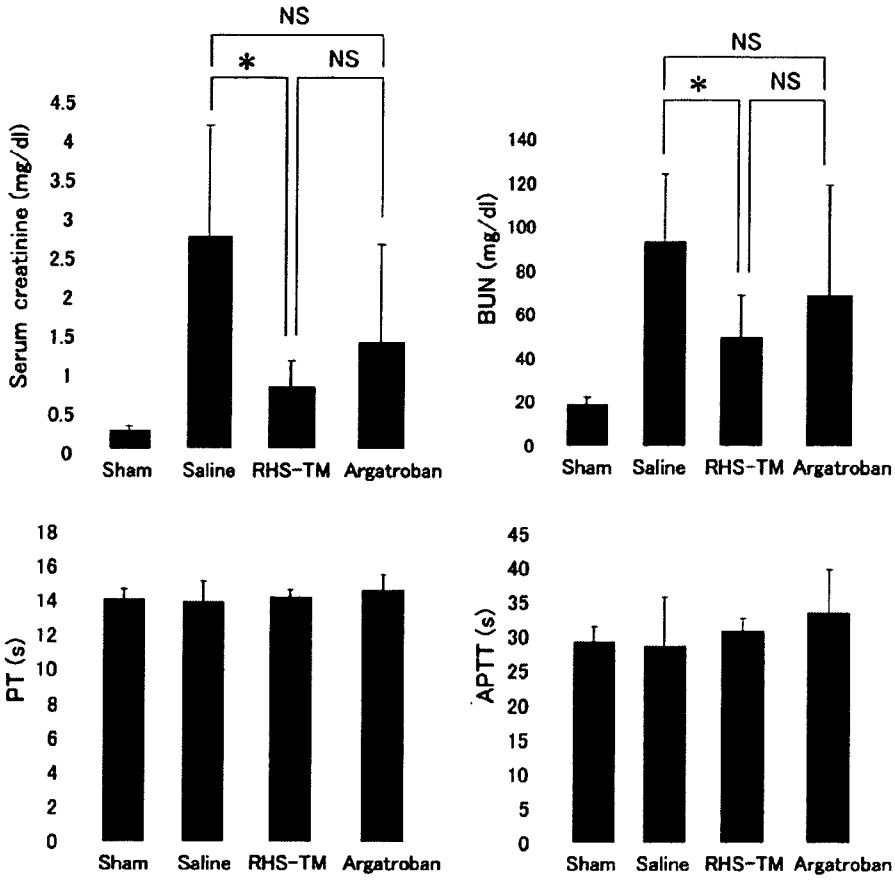

Fig. 2. Effects of RHS-TM on renal function and coagulation parameters. Bar graphs demonstrate the levels of serum creatinine (upper left), blood urea nitrogen(BUN)(upper right), prothrombin time (PT) (lower left), and activated partial thromboplastin time (APTT) (lower right). RHS-TM significantly suppressed the increases in levels of serum creatinine and BUN at 24 h after renal I/R. Argatroban also showed a tendency to improve the renal function, which, however, was not statistically significant. No significant differences were observed in the levels of PT or APTT among four groups. $^{*}P < 0.05$.

prevented the reduction of TM expression on the endothelium, while argatroban did not (Figure 7).

Experiment 3

Dose-dependent effects of RHS-TM. Rats given saline only exhibited significant increases in levels of serum creatinine at 24 h after renal I/R. RHS-TM significantly suppressed the increase when given in a dose of 0.25 mg/kg. A lower dose (0.025 mg/kg) of RHS-TM also showed a tendency to improve the renal function, which, however, was not statistically significant (Figure 8).

In vitro *experiment*

Effects of RHS-TM and argatroban on apoptosis in HUVECs. In order to study the endothelial cell damage by oxidative stress, HUVECs were incubated in culture medium containing 300 μM hydrogen peroxide and one of three different doses of RHS-TM (2, 10 or 50 μg/ml), or saline. A significant number of TUNEL-positive cells were observed in HUVECs treated with hydrogen peroxide and saline. RHS-TM attenuated the number of TUNEL-positive cells induced by hydrogen peroxide in a dose-dependent manner (Figure 9).

Discussion

The present study demonstrated that the levels of TM expression in the kidney decreased after ischaemia and reperfusion. It is known that plasma concentrations of soluble TM are elevated in critically ill patients, such as those with disseminated intravascular coagulation or sepsis [9,10]. This is most likely due to endothelial damage with increased release of membrane-bound TM into the circulating blood. It has been reported that TNF-α causes the endothelial cells to release TM from their surface [28]. Moreover, it was demonstrated that soluble TM was released from the liver graft into circulation, and those levels correlated well with early liver damage observed among patients who received

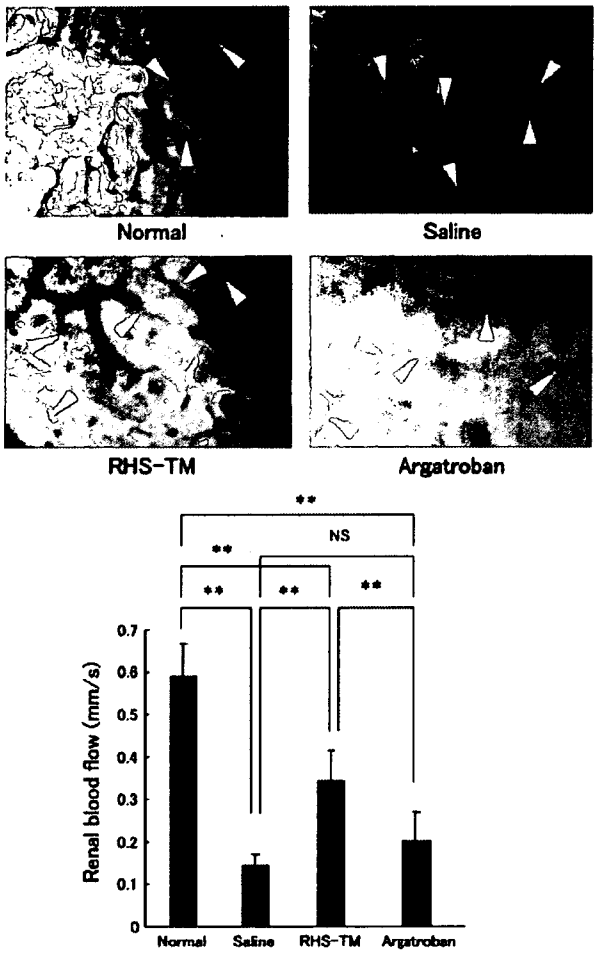

Fig. 3. Effects of RHS-TM on renal cortical microcirculation. Representative photographs show the images of the renal cortical microcirculation recorded using a CCD video microscope. In the saline group (upper right panel), the renal cortical peritubular capillaries shrunk as compared with normal rats (upper left panel). The condition of the cortical capillaries in the RHS-TM group (lower left panel) was close to that of normal rats. In contrast, the appearance of the peritubular capillaries of the argatroban group (lower right panel) was not different from that of the saline group. Graphic presentation demonstrates that RBC velocity was severely impeded after renal ischaemia and that RHS-TM significantly improved RBC velocity, while argatroban did not. Arrow heads indicate the vessels measured. $^{**}P < 0.01$. Bar, 100 μm.

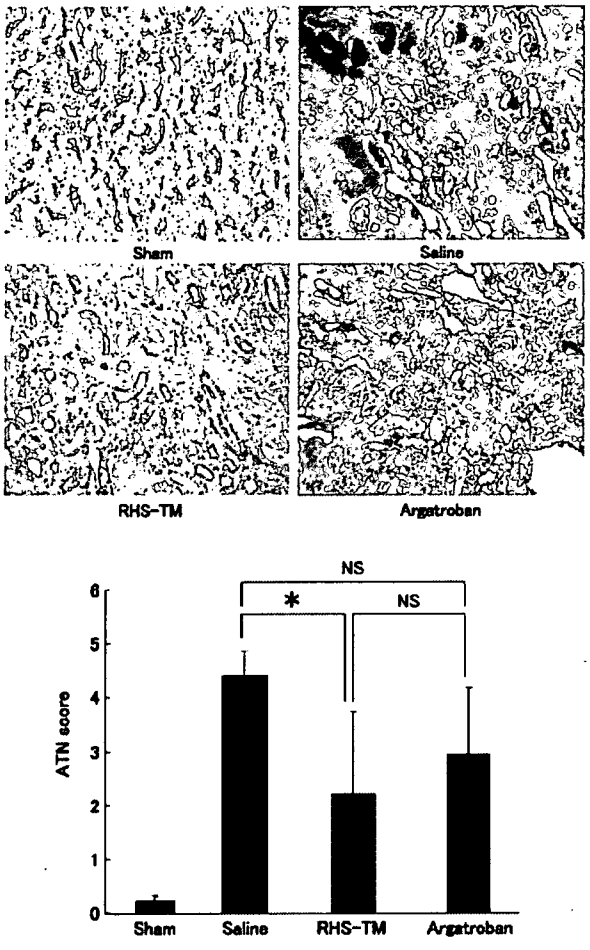

Fig. 4. Histology of the kidneys. Representative micrographs show renal histology (PAS). The sham-operated rats showed normal histology (upper left panel). The saline-treated rats (upper right panel) show tubular cast formation, tubular dilatation and tubular degeneration in the outer medulla of the kidney. Kidney sections from rats treated with RHS-TM (lower left panel) show only mild tubular damage. Argatroban partially attenuates the tubular damage (lower right panel). Graphic presentation demonstrates the scores indicating the severity of the tubular damage. The RHS-TM group showed significantly better scores than the saline group, while the argatroban group failed. $^{*}P < 0.05$. Bar, 100 μm.

liver transplant [11]. In our study as well, it is likely that the extracellular part of TM was released from the endothelial cells damaged by the ischaemia. Therefore, it would be reasonable to assume administration of RHS-TM ameliorates ischaemic renal damage.

We studied the therapeutic effects of RHS-TM using a rat model of I/R injury. The results clearly demonstrated that intrarenal administration of RHS-TM before reperfusion markedly ameliorated renal function and tubular damage. Renal cortical microcirculation was also significantly improved by RHS-TM, suggesting that endothelial cell dysfunction [22] may be attenuated by RHS-TM. The protocol, transient intrarenal administration of RHS-TM, was employed in this study in order to mimic the situation of kidney transplantation, where RHS-TM can be applied to the donor kidney during the harvesting period [29]. Because anti-coagulatory effects of RHS-TM may be harmful for the recipient during the operation, the protocol was made to fully prevent the systemic action of RHS-TM. This protocol may also implicate the possible application of RHS-TM to the kidney during warm ischaemic period in the major vascular surgery.

In the present study, argatroban, a direct thrombin inhibitor, was used as a control reagent. RHS-TM reduced macrophage and granulocyte infiltration, and suppressed apoptotic cell death in the kidney.

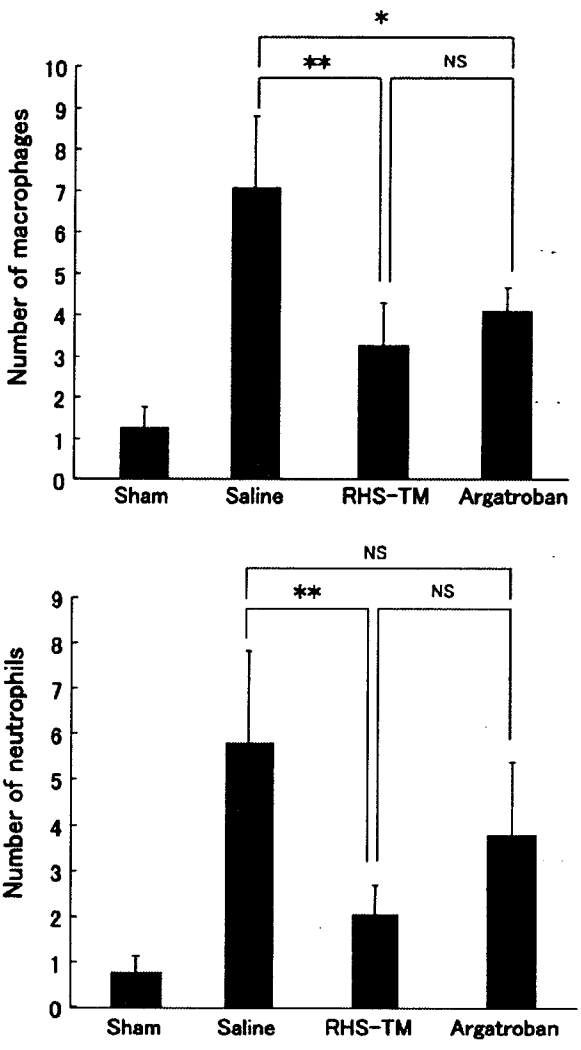

Fig. 5. Effects of RHS-TM on macrophage infiltration and neutrophil infiltration. Bar graphs demonstrate the number of macrophages (upper panel) and neutrophils (lower panel) in the outer medulla at 24 h after renal ischaemia. The numbers of macrophages decreased in both the RHS-TM and argatroban groups compared with the saline group. RHS-TM administration also decreased the number of neutrophils when compared with saline, while argatroban failed to show a significant difference. $^{*}P < 0.05$, $^{**}P < 0.01$.

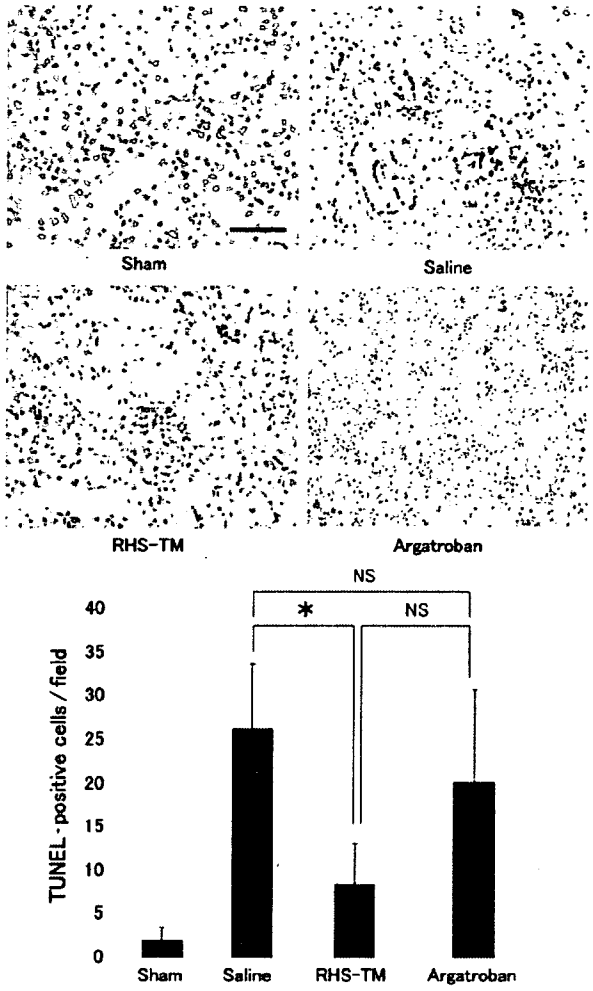

Fig. 6. Effects of RHS-TM on apoptosis in the kidney. Representative micrographs show apoptotic cells detected by TUNEL staining. TUNEL-positive cells were localized in the tubules of the outer medulla. Graphic presentation demonstrates the number of TUNEL-positive cells counted. RHS-TM significantly decreased the number of apoptotic cells as compared with saline only, while argatroban did not. $^{*}P < 0.05$. Bar, 100 μm.

Argatroban significantly suppressed the number of macrophages, but not granulocytes. It also showed a tendency to ameliorate renal function; this however was not statistically significant. It is known that coagulation and inflammation enhance each other, leading to tissue damage [20]. The moderate therapeutic effects of argatroban can be explained by its anti-coagulatory properties. The results of the present study suggest that RHS-TM has some effect beyond that of an anti-coagulant.

There are several possible pathways by which RHS-TM exerts its renoprotective properties. First, RHS-TM can suppress inflammation by inhibiting the activation of proteinase-activated receptor-1 (PAR-1, one of the thrombin receptors). Thrombin is known to promote inflammatory reactions by activating PAR-1, which is reported to be involved in the various processes of renal injury [30,31]. Although histology did not show thrombus formation in the control kidneys after I/R injury, it is possible that a small amount of thrombin was generated locally in the renal microcapillaries, resulting in the activation of PAR-1. In the present study, the number of macrophages decreased when either RHS-TM or argatroban was administered, suggesting that the effect of RHS-TM on macrophage infiltration was attributable to its direct anti-thrombin action. Nevertheless, the fact that argatroban failed to ameliorate renal function demonstrates that the inhibition of PAR-1 alone was not

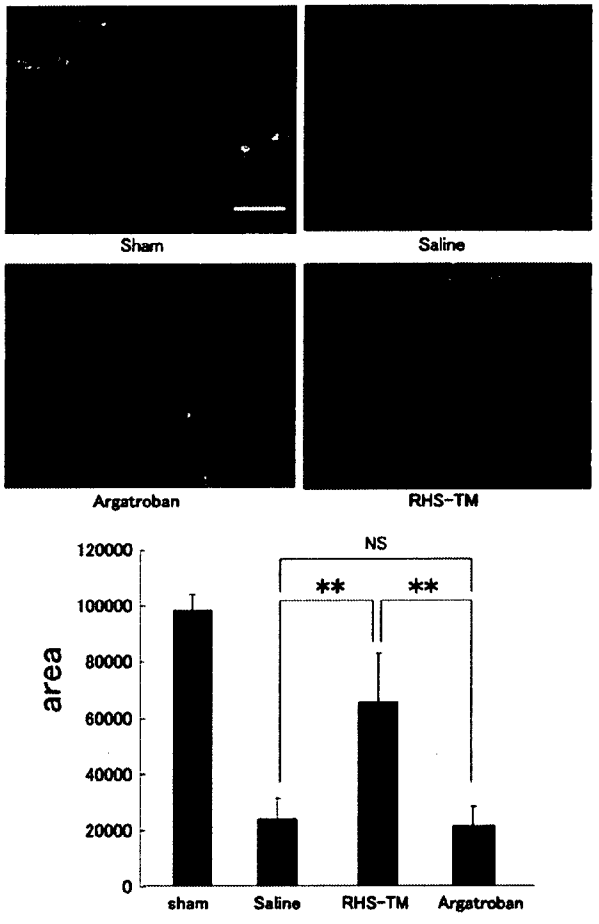

Fig. 7. Effects of RHS-TM on the expression of rat TM. Representative micrographs show the immmunostaining for rat TM. The graph demonstrates that the area positively stained for rat TM. RHS-TM prevented the reduction of TM expression at 24 h after I/R injury, while argatroban did not. $^{**}P < 0.01$. Bar, 100 μm.

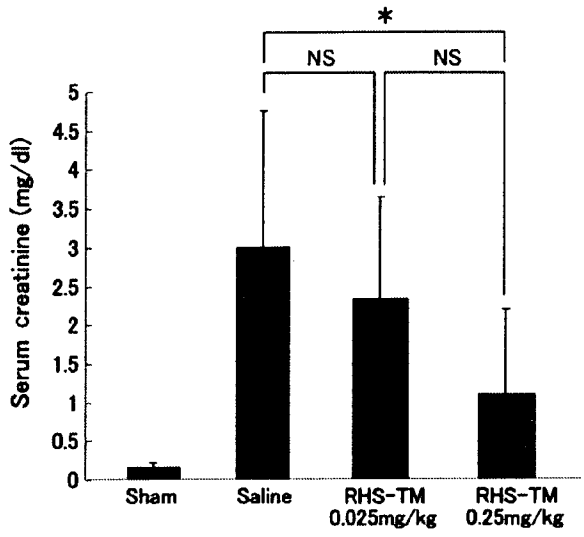

Fig. 8. Dose-dependent effects of RHS-TM on renal function. The graph demonstrates that 0.25 mg/kg of RHS-TM significantly suppressed the elevation of the serum creatinine after I/R renal injury, while 0.025 mg/kg showed only a moderate effect. $^{*}P < 0.05$.

sufficient to effectively suppress I/R injury. Secondly, TM can strongly enhance thrombin's ability to activate protein C (ikeguchi 42–50). Activated protein C is known to be a potent anti-inflammatory molecule. In fact, previous studies demonstrated that activated protein C attenuates I/R injury of the liver [32] and the kidney [33], and that heparin did not show renoprotection [33]. Still, it is not likely that RHS-TM acted through protein C in the present study, since RHS-TM can efficiently activate human protein C but not protein C of rat origin [34]. Thirdly, RHS-TM may exert its anti-inflammatory effects by activating proCPR (or TAFI). In a previous study, we demonstrated that RHS-TM suppresses leucocyte/macrophage infiltration in the glomeruli of rats with thrombotic glomerulonephritis [20]. When RHS-TM binds to thrombin, the complex potently activates proCPR to CPR, which degrades the anaphylatoxins C3a and C5a by cleaving their terminal arginine residues [35]. Again, it is not very likely that RHS-TM enhanced the action of anaphylatoxins via activating proCPR in this study since thrombus formation or complement deposition was not evident in the glomeruli after I/R injury, whereas massive thrombosis and strong C3 deposition were observed in the glomeruli of rats with thrombotic glomerulonephritis as shown in our previous study [20].

Recently, more direct anti-inflammatory effects of TM have also been demonstrated. RHS-TM is composed of three domains: an N-terminal lectin-like domain, an EGF-like domain consisting of six EGF-like repeats and a Ser/Thr-rich domain [8,36]. An EGF-like domain is critical for the anti-coagulant cofactor activities of TM (i.e. inhibition of thrombin and promotion of APC formation) [8,37]. It was shown that the N-terminal lectin-like domain of TM confers protection from inflammatory cell mediated tissue damage by suppressing adhesion molecule expression via NF-κB and mitogen-activated protein kinase pathways [38]. Moreover, Abeyama *et al.* [39] reported that the N-terminal lectin-like domain of TM acts against inflammation by sequestering high-mobility group-B1 protein. Therefore, the anti-inflammatory property of RHS-TM may be attributable to these direct effects of TM.

Immunostaining for rat TM showed that RHS-TM attenuated the reduction in the level of rat TM at 24 h after I/R injury, suggesting that RHS-TM may have exerted renoprotection by limiting endothelial cell injury. In order to study the effects of RHS-TM on the endothelial cells, apoptotic cell death was examined by *in vitro* experiments using HUVECs. TUNEL assay demonstrated that hydrogen peroxide induced a substantial degree of apoptosis, and the number of apoptotic cells was significantly attenuated by

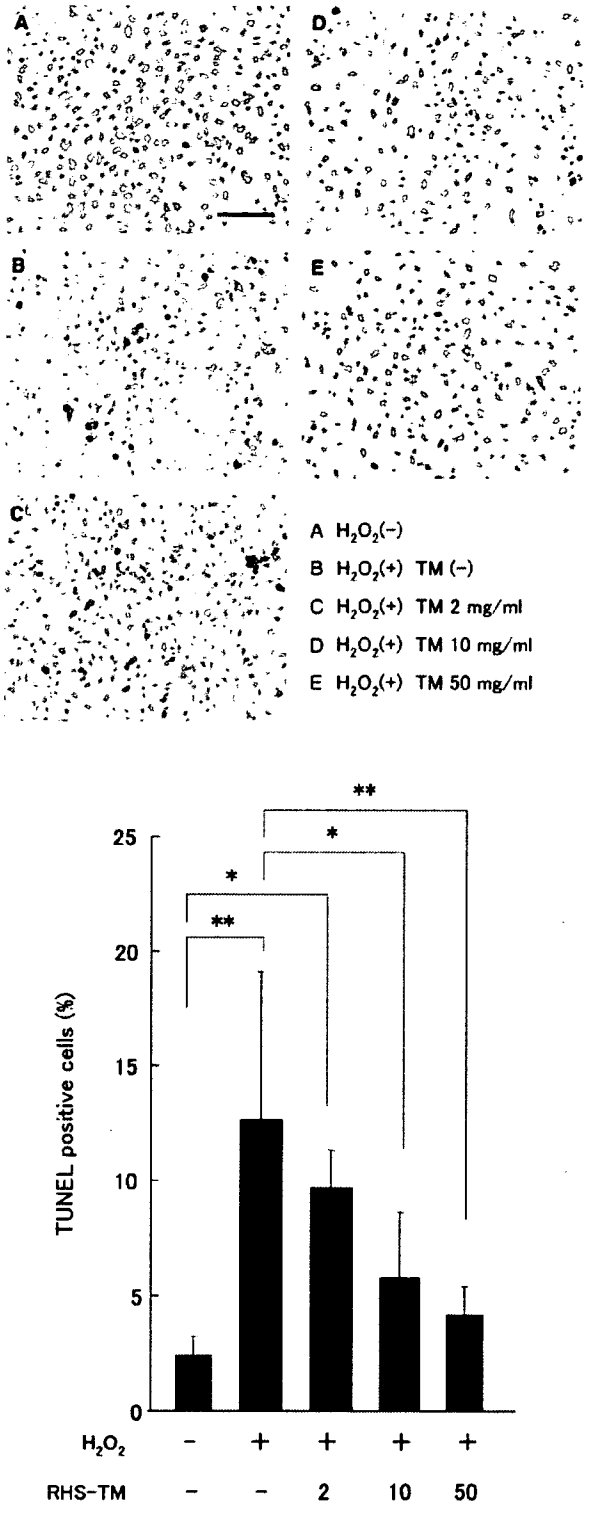

Fig. 9. Effects of RHS-TM and argatroban on apoptosis in HUVECs. Representative micrographs show TUNEL staining of HUVECs (A-E). The graph demonstrates that RHS-TM reduced the number of TUNEL positive cells induced by hydrogen peroxide in a dose-dependent manner. $^{*}P < 0.05$, $^{**}P < 0.01$. Bar, 100 μm.

incubation with RHS-TM. Conway *et al.* [38] reported that the soluble lectin-like domain of TM protects vascular endothelial cells from serum deprivation–induced cell death. The result was consistent with our data. Conway and Abeyama also showed that the lectin-like domain of TM suppresses NF-κB activation [39]. NF-κB activation is shown to play a role in the process of apoptosis induced by reactive oxygen intermediates [40]. Therefore, suppression of NF-κB could be one of the mechanisms by which RHS-TM protected endothelial cells from apoptosis. However, other studies showed that certain kinds of lectins inhibit apoptotic cell death via different pathways [41,42]. Further studies will be needed to explore the anti-apoptotic effects of RHS-TM.

In conclusion, the results of our study provide evidence that the transient intrarenal administration of RHS-TM during the warm ischaemic period attenuates renal injury. The findings may have therapeutic implications for the preservation of transplanted kidneys or in preventing acute renal failure after major vascular surgery.

Acknowledgements. The authors thank excellent technical assistance to Ms Asano N, Sawa Y and to Mr Suzuki N. This study was partly supported by a Research Grant for Research on Human Genome, Tissue Engineering Food Biotechnology from the Ministry of Health, Labor and Welfare (H17-regeneration-010) (awarded to S.M.).

Conflict of interest statement. None declared.

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Received for publication: 22.8.06
Accepted in revised form: 24.7.07

International Journal of Urology (2007) 14, 219–225 doi: 10.1111/j.1442-2042.2007.01689.x

Effects of olprinone, a phosphodiesterase III inhibitor, on ischemic acute renal failure

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Objective: Renal ischemic reperfusion injury (IRI) is unavoidable and is still one of the major problems in renal transplantation. The aim of this study was to investigate the effects of olprinone, a phosphodiesterase III inhibitor, on renal IRI.

Methods: After a right nephrectomy, renal IRI was induced in rats. Olprinone was given in two different ways: sustained systemic administration and transient local administration to the kidney. Control rats were treated with saline. Using a magnifying endoscope, the renal blood flow speed was measured at 23 h after reperfusion. Then, blood samples were collected, and kidney specimens were taken for histological study. In order to study the mechanism, we performed *in vitro* experiments, using human proximal renal tubular cells (HK-2) incubated with tumor necrosis factor (TNF)-α along with olprinone or saline, and interleukin (IL)-8 was measured in the culture supernatant.

Results: In the saline group, the blood flow speed (BFS) was greatly reduced compared to that in normal kidneys. In both olprinone-treated groups, BFS of the renal microcirculation significantly increased, tubular damage and macrophage infiltration attenuated, and renal function greatly improved. Olprinone inhibited the increase in the IL-8 levels resulting from the incubation of HK-2 with TNF-α.

Conclusions: Our study successfully demonstrates that olprinone has renoprotective properties when applied locally as well as systemically. The results suggest that olprinone might be clinically useful in renal transplantation for the donor kidney, the recipient, and even in treating acute renal failure.

Key words: acute renal failure, ischemia reperfusion, olprinone, renal microcirculation, renal transplantation.

Introduction

Ischemic reperfusion injury (IRI) of the kidney occurs when renal perfusion is reduced during shock. It is also a significant complication of vascular surgery of the aorta or kidney.[1,2] IRI injury is unavoidable in renal transplantation, and occurs during organ retrieval and storage. Ischemia reperfusion injury is closely associated with increased acute rejection episodes and late allograft failure.[3,4] These problems are almost always observed in cadaveric renal allograft that has been subjected to warm or cold ischemia, then reperfusion itself. The unsatisfactory results from such transplants hamper efforts to enlarge the donor pool through the use of marginal donor organs.[5] It is known that even kidney grafts from brain-dead organ donors suffer more frequently from acute rejection episodes than those from more ideal living sources.[6] Brain death leads to an increased expression of cytokines, adhesion molecules and a higher rate of leukocyte infiltration.[6,7] These changes are consequences of a massive catecholamine release followed by subsequent hypotension and ischemia. One approach to suppress renal IRI is to treat the recipient with renoprotective drugs pre and post operation. Another potential therapeutic approach is to improve organ quality prior to transplantation by the treatment of the donor or donor kidney with various reagents before organ harvesting.[7]

Ischemia reperfusion injury of the kidney involves a complex and interrelated sequence of events that result in tubular damage, inflammation associated with the release of cytokines, and eventual death of renal cells.[8] Although reperfusion is essential for the survival of ischemic tissue, reperfusion itself causes additional injury.[9] Ischemia alone results in only a moderate increase in microvascular permeability, while the majority of leakage occurs because of reperfusion.[10] The reperfusion injury is attributed to calcium overload, neutrophil infiltration and the generation of reactive oxygen species (ROS).[8,9] Ischemia in the proximal tubular cells causes generation and release of tumor necrosis factor (TNF)-α. TNF-α increases the phosphodiesterase (PDE) activity in the endothelial cells and decreases intracellular cyclic ester adenosine monophosphate (cAMP).[11,12] Intracellular cAMP suppresses endothelial permeability and is considered to be a primary mediator of microvascular permeability.[10]

Endothelial cell dysfunction is the primary cause of the no-reflow phenomenon in postischemic kidneys.[13] Endothelial cells in the renal vasculature undergo an early swelling, leading to a narrowing of the lumen. Both the endothelium of renal microvessels and the tubular epithelium show a loss of polarity in ischemic kidneys.[13] Living and dead cells slough into the tubular lumen, contributing to cast formation. The casts then cause increased intratubular pressure and a reduced glomerular filtration rate. Loss of the epithelial–cell barrier and of the tight junctions between viable cells can result in back-leakage of the glomerular filtrate, further reducing the effective glomerular filtration rate.[14]

Olprinone (1,2-dihydro-6-methyl-2-oxo-5-[imidazo{1,2-a}pyridin-6-yl]-3-pyridine carbonitrile hydrochloride monohydrate, E-1020) is a PDE-III inhibitor developed in Japan (Eisai, Tokyo, Japan) and is clinically used for patients with heart failure. Olprinone has many properties. It was originally developed as a cardiotonic agent, having positive inotropic and vasodilator actions. It improves myocardial mechanical efficiency[15] via elevation of intracellular cAMP levels in both cardiomyocytes and vascular smooth muscle cells. It also increases myocardial contractility and reduces vascular resistance, leading to an improvement of hemodynamic status.[16] Moreover,

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Received 29 June 2006; accepted 14 September 2006.

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olprinone augments cerebral blood flow by its direct vasodilatory effect on the cerebral arteries. The cerebrovascular reactivity to olprinone is markedly observed especially in patients with impaired cerebral circulation.[17] Olprinone inhibits vascular contractility by decreasing cytosolic Ca^{2+} levels and the Ca^{2+} sensitivity of the contractile elements. These effects may be mediated by an increase in cyclic AMP content.[18] In addition, olprinone has anti-inflammatory actions at therapeutic concentrations clinically used for heart failure.[19] PDE-III inhibitors have a diuretic effect in patients with chronic heart failure who retain normal renal function, but do not in those with concomitant renal failure.[20] Finally, olprinone inhibits both von Willebrand factor mediated and fibrinogen mediated platelet aggregation.[21]

The present study was performed in order to determine the effects of olprinone on renal microcirculation, histological change, and renal function using a rat model of IRI. Two different protocols were applied in our study. Sustained systemic administration of olprinone was employed in order to study the effects when applied to the recipient and transient local administration to the kidney was employed in order to study the effect of olprinone when applied to the donor kidney during the harvesting period. The latter protocol may also suggest the possible application of olprinone to the kidney during the warm ischemic period in major vascular surgery. Furthermore, an *in vitro* experiment was performed to investigate the possible mechanisms for its renoprotective properties.

Materials and methods

Animals

Male Sprague–Dawley (SD) rats weighing 350–400 g were purchased from Chubu Kagaku Shizai (Nagoya, Japan) and were allowed free access to food and water. The experiments were performed according to the Animal Experimentation Guidelines of Nagoya University Graduate School of Medicine (Nagoya, Japan).

Experimental protocols

Two different protocols were applied to the total of 26 rats. One was sustained systemic administration and the other was transient local administration to the kidney.

Protocol A: Systemic administration

Fourteen rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (25 mg/kg), right nephrectomy was performed via right flank incision, and mini-osmotic pumps (MOP) (8.0 ± 1.0 μL/h, duration 1 day, and reservoir volume of 200 μL) were implanted subcutaneously. Seven rats (systemic saline group) were implanted with MOP filled with 200 μL saline and the other seven rats were implanted with MOP filled with 200 μL olprinone 0.3 μg/kg/m (Eisai). Thirty minutes later, the left renal vessels were exposed via left flank incision, and were clamped for 45 min using a 20-mm microaneurysm clip with a closing force of 156 g (Mizuho Ikakogyo, Nagoya, Japan). After removing the clamp, the muscle and skin were sutured separately. At 23 h after the reperfusion, the blood flow in cortical peritubular capillaries (CTC) was observed by charge-coupled device (CCD) video microscope and rats were killed at 24 h. Blood and kidney samples were collected for the study (Fig. 1a).

Protocol B: Local administration

A right nephrectomy was performed via flank incision on 12 rats. Seven days later, rats were anesthetized and the abdominal cavity was opened via midline incision. (Because many rats died when right nephrectomy was performed on the same day as ischemia, local administration and reperfusion, we waited for 7 days until rats had totally recovered from the initial surgical stress.) The aorta was clamped above and below the left renal artery. The left renal vein was closed by a string and a hole was made in the vein wall using a 23-G needle. A 24-G cannula was placed in the aorta between the two clamps, and 2 mL saline or 0.2 mg/kg olprinone dissolved in 2 mL saline was injected slowly into the left kidney, making the blood in the kidney go out through the hole in the vein, until the olprinone started to come out. The olprinone or saline which came out of the vein hole was completely absorbed using a sponge on a stick. The two aorta clamps were taken off soon after clamping the renal artery and vein with a 20-mm microaneurysm clip, leaving olprinone or saline trapped inside the kidney. After 45 min of ischemic period, another clamp was placed at the proximal site of the renal vein and the original clamp at the renal artery and vein was removed. The olprinone or saline coming out of the vein hole was again completely wiped away, and the hole was closed by being pressed for

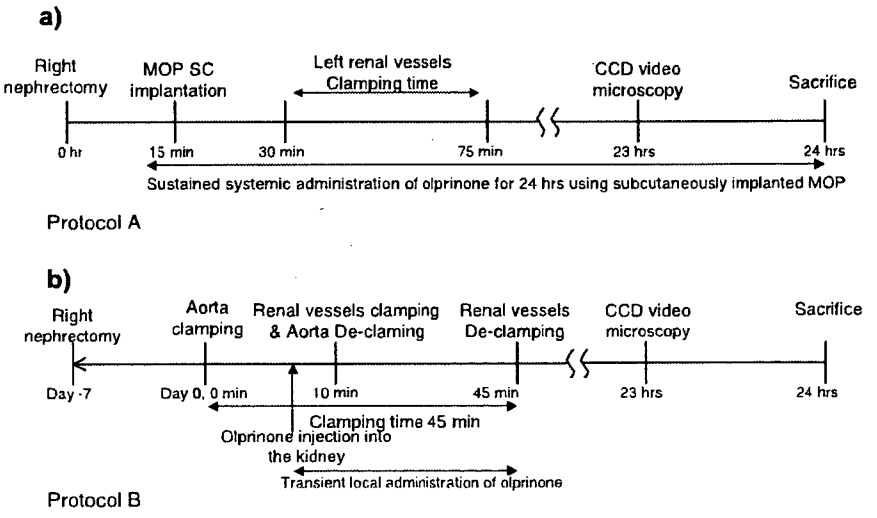

Fig. 1 (a) Experimental protocol A for sustained systemic administration of olprinone using mini-osmotic pumps (MOP). (b) Protocol B for transient local administration.

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5 min. The blood flow was observed at 23 h and blood and kidney samples were collected for the study at 24 h (Fig. 1b). The blood flow was observed in four normal rats as well. All the procedures were performed on a heated table (37°C) to maintain the body temperature.

CCD video microscope

An intravital CCD video microscope which has a pencil lens probe with a tip diameter of 1 mm was used in this study. The probe has a magnification of ×520 and spatial resolution of 0.86 μm, permitting identification of individual erythrocytes.[13] This device enabled us to get a direct image of microcirculation in various organs in humans as well as organs in large and small animals, including pigs, rabbits, rats and mice. The blood flow in renal CTC was observed, and the images were recorded using the freeze-frame mode. The speed of the blood cells in individual segments of the CTC was analyzed using a point tracking method and motion detection program.[22]

Histology and immunohistochemistry

The kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 2 μm. They were stained with periodic acid-Schiff (PAS) by standard methods. Immunostaining was performed using another part of the kidney, which was frozen in O.C.T. compound (Miles, Montana, USA). Sections (2 μm thick) were fixed in acetone. The primary antibody employed was monoclonal Ab against ED-1 (antirat macrophages/monocytes; Serotec, Raleigh, NC, USA), and the secondary antibodies were fluorescein conjugates of goat antimouse immunoglobulin (Ig)G antibody (Zymed Laboratories, San Francisco, CA, USA).

Histological examinations were performed and the degrees of the tubular damage, including cell necrosis, cast formation, and tubular dilatation and degeneration (loss of brush border and detachment of tubular epithelial cells), were scored. The changes were reviewed in at least 10 non-overlapping fields (×200) for each slide by two independent observers in blind fashion. Acute tubular necrosis (ATN) scores were then determined for each rat as follows: 0, none; 1, ≤ 10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, > 76% of the tubules damaged.[23] Interstitial macrophage infiltration was assessed by counting ED-1 positive cells in 10 non-overlapping fields (×400) of the outer medulla, and expressed per field. The morphological analysis was carried out using a Zeiss microscope (Oberkochen, Germany).

Measurement of blood urea nitrogen (BUN) and serum creatinine

Twenty-four hours after reperfusion injury, blood samples were taken from the aorta and serum was collected. BUN and serum creatinine levels were measured using Daiya Auto UN or Daiya Auto Crea Kit (Daiya Shiyaku, Tokyo, Japan).

Measurement of hemodynamic changes

Additional experiments were performed in order to study the effects of olprinone on hemodynamics. Olprinone was administered systemically using MOP as described in protocol A. The systolic blood pressure (SBP) was measured using a Softron bp-98 A (Softron, Tokyo, Japan) before and soon after the implantation of MOP, and again at 1, 2, 3, 4 and 23 h intervals. The measurement was performed three times at each time point and the mean value was obtained.

In vitro experiments

In vitro experiments were performed to study the anti-inflammatory and anti-apoptotic properties of olprinone. Human proximal renal tubular cells, HK-2, were incubated in three types of culture medium: (i) serum-free culture medium as a control; (ii) serum-free culture medium containing 0.1 ng/mL of TNF-α; and (iii) serum-free culture medium containing 0.1 ng/mL TNF-α and 3 μg/mL olprinone. After 46 h incubation, the levels of interleukin (IL)-8 in the culture supernatant were measured using Medgenix EASIA kit (BioSource, Fleunes, Belgium). Three dishes were used for each type of treatment.

Statistical analyses

The levels of BUN and serum creatinine, ATN scores and renal blood flow speed of the four groups were analyzed by two-way analysis of variance (ANOVA). When statistical difference was indicated by ANOVA, further analysis was performed using the Mann–Whitney *U*-test to determine the difference between any pair of groups. Statistical analyses were performed using Statview for Windows v. 5.0. A significant difference was defined as $P < 0.05$.

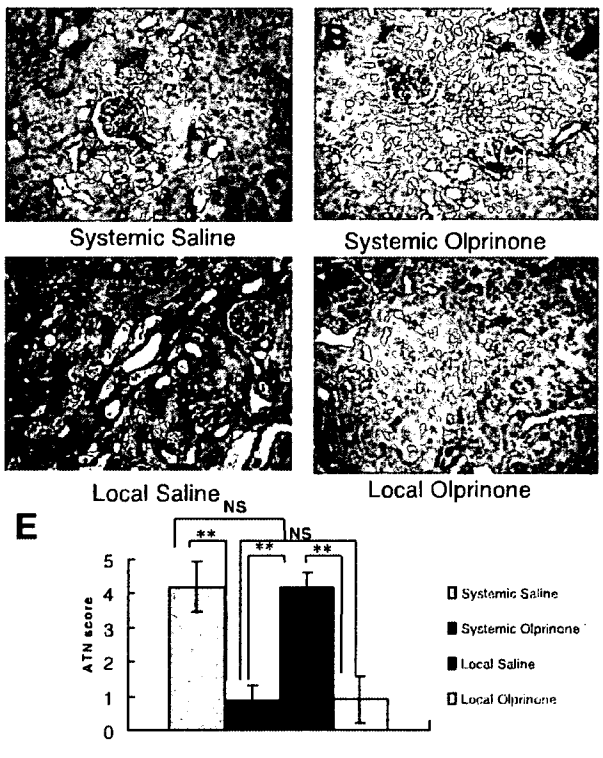

Fig. 2 Effects of olprinone on tubular damage induced by renal ischemic reperfusion injury (IRI). Representative histological findings of the kidneys taken at 24 h: (a) systemic saline group; (b) systemic olprinone group; (c) local saline group; and (d) local olprinone group. (e) Acute tubular necrosis (ATN) scores of four groups. Data are expressed as mean ± SD values. NS: not significant, $^{**}P < 0.01$. Bar, 100 μm.

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Results

Effects of olprinone on renal blood flow and renal tubules

By direct imaging of the renal cortex at 23 h after IRI using CCD video microscopy, information on renal blood flow and the condition of renal tubules was obtained. The movement of blood cells in cortical peritubular capillaries was recorded and cortical blood flow speed was determined by an analysis of the speed of blood cells. There were gross differences between the saline groups and the olprinone groups. In both the systemic and the local saline groups, the renal arterioles and cortical peritubular capillaries shrunk and blood flow was severely hampered. The speed was 0.12 ± 0.04 mm/s in the controls (the systemic saline group), but 0.59 ± 0.07 mm/s in normal rats ($P < 0.0001$). Furthermore, most of the renal tubules were destroyed or obstructed by detached cells (epithelial casts) in the saline groups. In contrast, in the systemic olprinone group, the condition of the cortical capillaries did not look different from those in normal rats and the cortical blood flow speed was greatly improved to 0.39 ± 0.08 mm/s ($P = 0.0001$ vs saline MOP). In the local olprinone group, only a few destroyed tubules and epithelial casts were observed, and the blood flow speed was significantly faster (0.27 ± 0.09 mm/s) than in normal rats ($P = 0.0002$), but was slower than the systemic olprinone group ($P = 0.0007$) (Fig. 2).

Effects of olprinone on renal histology

Examination of PAS-stained kidney sections from the saline-treated rats showed that tubular injury occurred mainly in the outer medulla of the kidney. The severity of the tubular damage was scored, including cell necrosis, cast formation, and tubular dilatation and degeneration (loss of brush border and detachment of tubular epithelial cells). Olprinone administration resulted in significantly better scores than saline groups (systemic saline 4.1 ± 0.7 vs systemic olprinone 0.88 ± 0.4, $P < 0.0001$ and local olprinone 4.2 ± 0.4 vs local saline 0.9 ± 0.6, $P < 0.0001$) (Fig. 3).

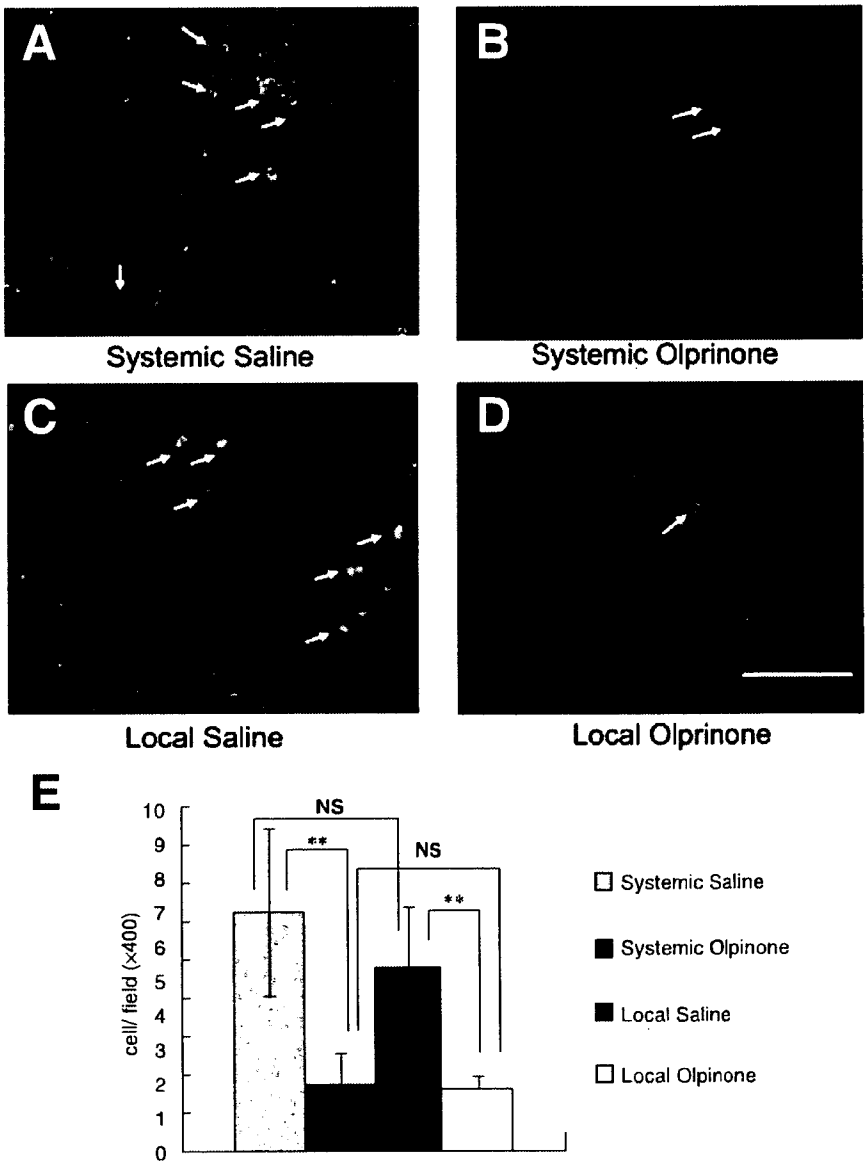

Fig. 3 Effects of olprinone on macrophage infiltration caused by renal IRI. Representative histological findings of the kidneys taken at 24 h: (a) systemic saline group; (b) systemic olprinone group; (c) local saline group; and (d) local olprinone group. (e) Macrophage infiltration in four groups. Data are expressed as mean ± SD values of number of macrophages counted in 10 non-overlapping fields (×400) of each renal specimen. NS: not significant, $^{**}P < 0.01$. Bar, 100 μm.

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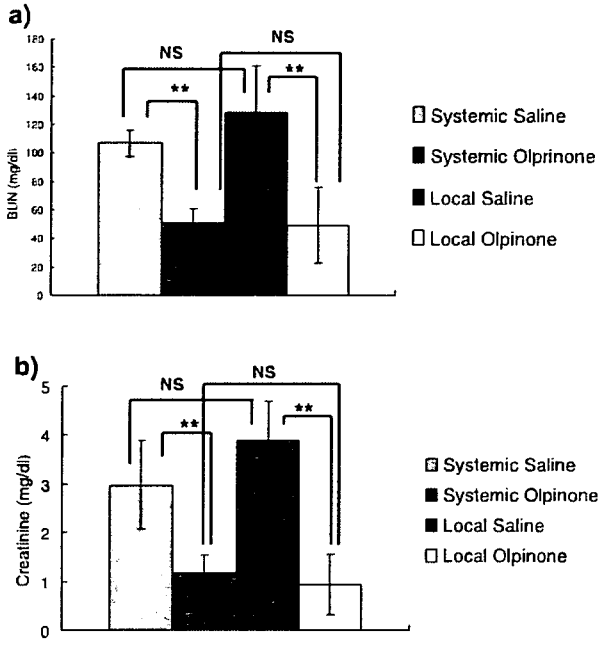

Fig. 4 Effects of olprinone on renal function. IRI increased urea nitrogen (BUN) and creatinine levels at 24 h after IRI. Olprinone suppressed these increases when administered locally as well as systemically. (a) Levels of BUN. (b) Serum levels of creatinine. Data are expressed as mean ± SD values of all experiments of each group. NS: not significant, $^{**}P < 0.01$.

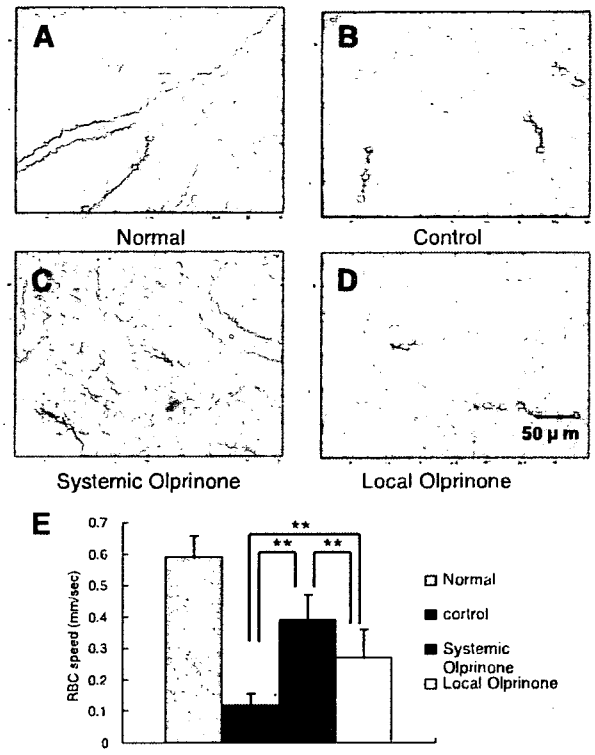

Fig. 5 Effects of olprinone on renal microcirculation. Representative images taken by charge-coupled device (CCD) video microscope: (a) normal rats; (b) saline group; (c) systemic olprinone group; and (d) local olprinone group. In the saline group (b), renal arterioles shrunk, and tubular lumens were blocked by epithelial casts. In the systemic olprinone group (c), renal arterioles were dilated, and few tubules were blocked by epithelial casts. In the local olprinone group (d), the diameter of renal arterioles was also larger than control group, and just a few tubules were blocked by epithelial casts. (e) Red blood cells (RBC) speed in all groups. Data are expressed as mean ± SD. $^{**}P < 0.01$. Bar, 50 μm.

The numbers of macrophages were determined by counting the ED-1-positive cells. Macrophage infiltration was significantly suppressed in olprinone groups compared to saline groups (systemic saline 6.95 ± 2.4 vs systemic olprinone 1.94 ± 0.89, $P = 0.0001$ and local saline 5.35 ± 1.7 vs local olprinone 1.76 ± 0.42, $P = 0.0067$) (Fig. 4).

Effects of olprinone on renal function

Rats which underwent renal IRI exhibited significant increases in levels of BUN and serum creatinine. Olprinone treatment significantly suppressed these increases. BUN was 95.2 ± 9.4 mg/dL in the systemic saline group and 51 ± 10.4 mg/dL in the systemic olprinone group ($P < 0.0001$), and 129 ± 32 mg/dL in the local saline group and 49 ± 26 mg/dL in the local olprinone ($P = 0.0008$). Serum creatinine was 2.98 ± 0.9 mg/dL in the systemic saline group and 1.17 ± 0.3 mg/dL in the systemic olprinone group ($P = 0.0004$), and 3.8 ± 0.8 mg/dL in the local saline group and 0.95 ± 0.6 mg/dL in the local olprinone group ($P < 0.0001$) (Fig. 5).

Effects of olprinone on blood pressure

Systolic blood pressures were 127.5 ± 17 mmHg and 126.6 ± 17 mmHg before and soon after the implantation of MOP, respectively. Then, SBP were 121.3 ± 9.8 mmHg at 1 h, 126.5 ± 3.7 mmHg at 2 h, 126.5 ± 2.8 mmHg at 3 h, 128 ± 9 mmHg at 4 h, and 135.4 ± 21 mmHg at 23 h. No significant change in the hemodynamic status resulted from the dosage of olprinone administered in the present study (Fig. 6).

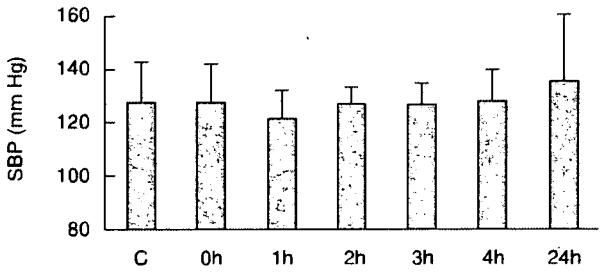

Fig. 6 Effects of systemic administration of olprinone on blood pressure. The systolic blood pressure (SBP) was measured using a softron bp-98A. No significant change was observed in the hemodynamics during the administration of olprinone using MOP. Data are expressed as mean ± SD. C, control, before MOP implantation. 0 h: the time soon after MOP implantation.

Effects of olprinone on IL-8

In order to study whether olprinone has direct anti-inflammatory effects or not, HK-2 cells were incubated for 46 h under three different

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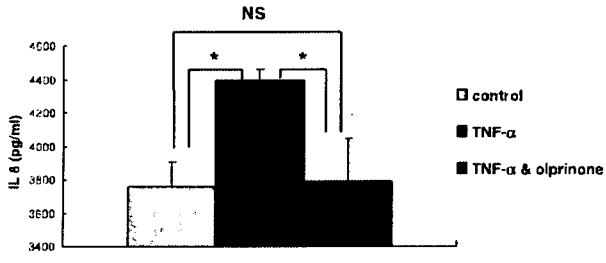

Fig. 7 Effect of olprinone on IL-8 release from HK-2 stimulated with tumor necrosis factor (TNF)-α. Human proximal tubular cells, HK-2, were incubated for 46 h in a culture medium containing 0.1 ng/mL of TNF-α with or without 3 μg/mL of olprinone. Adding TNF-α to the culture media increased IL-8 levels (4400 ± 58.6 pg/mL) compared to the serum-free media alone (control) (3760 ± 145.7 pg/mL) ($P < 0.05$). Olprinone suppressed the increase of IL-8 to a level (3790 ± 250.5 pg/mL) similar to that of the control ($P < 0.05$).

conditions. Adding TNF-α to the culture media increased IL-8 levels (4400 ± 58.6 pg/mL) compared to the serum-free media alone (3760 ± 145.7 pg/mL; $P < 0.05$). Olprinone suppressed the increase of IL-8 by TNF-α to the level (3790 ± 250.5 pg/mL) similar to that in the control ($P < 0.05$) (Fig. 7).

Discussion

The present study was performed to examine the effect of olprinone on the kidney using a rat model of IRI. Direct vision using an intravital CCD video microscope demonstrated that the decrease in renal blood flow induced in response to ischemia and/or reperfusion was greatly improved by olprinone, whether via sustained systemic administration or transient local administration. Histological examination showed that tubular injury, including tubular cell necrosis, cast formation, tubular dilatation and regeneration, was suppressed in olprinone treated groups. Olprinone significantly improved renal function in rats which underwent renal IRI.

These renoprotective properties of olprinone could be mediated by several mechanisms. First, it is well known that olprinone has vasodilatory action. Olprinone inhibits PDE-III, the enzyme which is responsible for the degradation of cAMP, leading to an increase in cAMP. This increase in turn attenuated vascular contractility by decreasing cytosolic Ca^{2+} levels and suppressing the Ca^{2+} sensitivity of contractile elements. We reproved the vasodilator action of olprinone by direct observation of renal microcirculation using an intravital CCD video microscope. The renal arterioles and cortical peritubular capillaries were dilated in the olprinone-treated groups, while they were shrunk in the saline-treated groups. In spite of the fact that olprinone has vasodilatory action, no considerable hemodynamic changes were observed during the systemic administration of olprinone. Olprinone may protect endothelial cells in the renal vasculature, maintaining the integrity of the endothelial layers, resulting in the better preservation of the tubular epithelium.

The second possible mechanism by which olprinone may protect the kidney is as an anti-inflammatory one. We found that the number of macrophages detected by ED-1 staining had significantly decreased in the olprinone-treated groups. Also, *in vitro* experiments showed that olprinone reduced the level of IL-8 released from HK-2, human proximal tubular epithelial cells in response to TNF-α. We chose TNF-α because it is known to play a major role in the development of IRI. Zager *et al.*[11] demonstrated that ischemic injury in proximal tubular cells causes the generation and release of TNF-α, which leads to the induction of various cytokines including IL-8, and results in inflammation and renal injury. TNF-α is known to increase endothelial PDE activity and to decrease intracellular cAMP.[12] Olprinone inhibits PDE-III, which results in an increase in cAMP. The elevation of endothelial cell cAMP levels inhibits NF-κB activation by targeting p38 mitogen activated protein kinases (MAPK).[24] cAMP also activates protein kinase A, which inhibits NF-κB.[25] The activation of NF-κB leads to the transcription of various proinflammatory molecules including IL-8. IL-8 is one of the key proinflammatory chemokines responsible for recruiting and activating neutrophils, T cells and monocytes to sites of inflammation.[26] Therefore, we assume that olprinone inhibited IL-8, blocked the inflammation cascade and ameliorated the consequent renal injury. The present observation may suggest that olprinone acts not only on the endothelial cells but also on the tubular epithelia cells, suppressing the release of inflammatory cytokines and resulting in less tubular damage.

Mizutani *et al.* demonstrated that olprinone reduced I/R-induced increases in serum levels of BUN and creatinine, improved histology, and increased the renal tissue and plasma level of cAMP.[27] Our data is consistent with these results, offering three additional findings. First, we proved that transient local administration of olprinone was as effective as sustained systemic administration applied for 24 h by subcutaneously implanted MOP. The method of local transient administration, which was employed in our study, is clinically applicable for renal transplantation during the periods of both kidney harvesting from cadaver donors and vascular anastomoses. Second, we proved by direct vision using an intravital CCD video microscope that olprinone improved renal microcirculation, and lessened the epithelial cast formation. In contrast, laser Doppler devices do not measure absolute blood flow, and the results are usually expressed as changes relative to a variable baseline.[28] The third novel finding in our study is the anti-inflammatory action of olprinone. Mizutani *et al.* demonstrated in rats that olprinone decreased the level of cytokine-induced neutrophils chemoattractant (CINC), the rat equivalent of human IL-8, secreted after renal IRI. However, it is possible that olprinone suppressed the CINC secretion indirectly by increasing renal blood flow and thus attenuating tubular damage. In the present study, we proved that olprinone suppressed IL-8 release from the cultured proximal tubular cells when stimulated with TNF-α, suggesting a direct anti-inflammatory action.

In conclusion, the present study showed that olprinone has renoprotective properties by both sustained systemic administration and transient local administration. Anti-inflammatory action of olprinone was also demonstrated. The results suggest that olprinone would be clinically useful in renal transplantation for both recipient by sustained systemic administration and donor *in vivo* or donor kidney *in vitro* during harvesting period using systemic or local administration, respectively. And it would be useful even in treating or preventing acute renal failure in patients undergoing major vascular surgery.

Acknowledgments

The authors thank Ms N Asano, Y Sawa and Mr N Suzuki for their excellent technical assistance. This study was partly supported by a Research Grant for Research on Human Genome, Tissue Engineering Food Biotechnology from the Ministry of Health, Labor and Welfare (H17-regeneration-010) (awarded to SM).

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Original Paper

nephron Clinical Practice

Nephron Clin Pract 915
DOI: 10.1159/000XXXXXX

Received: April 17, 2007
Accepted: November 26, 2007
Published online: ■■■

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High Mobility Group Box Chromosomal Protein 1 in Patients with Renal Diseases

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Key Words
Vasculitis · Inflammation · IgA nephropathy · Cytokines · Crescentic glomerulonephritis · Henoch-Schönlein purpura · Antineutrophil cytoplasmic antibody

Abstract
Background/Aim: The high mobility group box chromosomal protein 1 (HMGB1), a nuclear DNA-binding protein, has recently been recognized as a new proinflammatory cytokine. The purpose of this study was to examine the significance of HMGB1 in patients with renal diseases. ***Methods:*** HMGB1 concentrations in sera were measured by enzyme-linked immunosorbent assay, and antibodies against HMGB1 were examined by Western blotting in patients who underwent renal biopsies and in healthy controls. Immunohistochemistry for HMGB1 was also performed. ***Results:*** Serum HMGB1 was more likely to be positive in patients who underwent renal biopsies as compared with the controls. Patients with anti-neutrophil cytoplasmic antibody-related glomerulonephritis (ANCA-GN) and those with Henoch-Schönlein purpura nephritis showed a significantly higher tendency to be HMGB1 positive. The presence of anti-HMGB1 antibody was not associated with the presence of serum HMGB1. Immunohistochemistry revealed that HMGB1 was expressed in mononuclear cells in the interstitium or in the glomeruli of some patients with ANCA-GN or IgA nephropathy (IgAN). Subanalysis demonstrated that among patients with IgAN, those who had crescent formation showed a higher tendency to be HMGB1 positive than those who did not. ***Conclusions:*** HMGB1 was expressed in the sera of patients with renal diseases who underwent renal biopsies, especially among those who had vasculitis including ANCA-GN, Henoch-Schönlein purpura nephritis, and IgAN with glomerular crescents.
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Introduction

The high mobility group box chromosomal protein 1 (HMGB1; previously called HMG1) was originally discovered as a nuclear DNA-binding protein. It is a ubiquitous protein present in eukaryotic cells with highly conserved amino acid sequences among species [1–3]. As a nuclear protein, HMGB1 stabilizes the nucleosomal structure, enables the binding of transcription factors to their cognate DNA sequences, and facilitates gene transcription [4–7].

HMGB1 is also expressed in the extracellular space. Relatively recent studies have demonstrated that it functions as a potent proinflammatory mediator [8–12].

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1660–2110/08/0000–0000$24.50/0

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HMGB1 has been shown to be elevated in the blood of patients with septic shock [8] or hemorrhagic shock [13]. High levels of HMGB1 were also detected in the synovial fluid of rheumatoid arthritis patients [14]. Moreover, Hatada et al. [15] reported that patients with disseminated intravascular coagulation showed high plasma levels of HMGB1. They also demonstrated that HMGB1 plasma levels correlated well with the severity of the disease. Experiments have revealed that the extracellular form of HMGB1 mediates the induction of delayed endotoxin lethality [8], acute lung injury [16], joint inflammation in rheumatoid arthritis [17], and ischemic liver damage [18, 19].

It has also been shown that anti-HMGB1 antibodies are elevated in some patients with rheumatoid arthritis [20], ulcerative colitis [21], or autoimmune hepatitis [22]. The pathological role of these antibodies is unclear. However, since HMGB1 exists only in the intracellular space under normal conditions, the presence of anti-HMGB1 antibodies may suggest that HMGB1 has at some time existed in the extracellular space.

To date, extensive studies have been performed on the role of HMGB1. However, no concrete information has been available concerning the relationship between HMGB1 (or anti-HMGB1 antibodies) and renal diseases. In our unpublished preliminary study, patients with antineutrophil cytoplasmic antibody-related glomerulonephritis (ANCA-GN), Henoch-Schönlein purpura nephritis (HSPN), and IgA nephropathy (IgAN) tended to be positive for serum HMGB1, while patients with diabetic nephropathy (2 out of 38 were positive) or chronic renal failure (1 out of 9 was positive) did not. Therefore, in the present study, we focused on patients who underwent renal biopsy and tested for the presence of serum HMGB1 as well as for the antibodies that accompanies it.

Patients and Methods

Patients and Biopsy Samples

Patients who underwent renal biopsy at Nagoya University Hospital and affiliated hospitals between June 2004 and March 2006 were eligible for the study, and those who agreed to participate in the present study were registered. As controls, 49 healthy volunteers were also enrolled.

In patients with a normal renal function, the primary indications for renal biopsy were urinary protein levels >100 mg/dl or >1 g/day and microhematuria associated with proteinuria (>0.5 g/day). In patients with rapidly progressive renal insufficiency, the indication for renal biopsy was no obvious renal atrophy. In diabetic patients, renal biopsy was performed especially when the patient was suspected of having other renal diseases in addition to diabetic nephropathy. Such conditions included hematuria, proteinuria (>1 g/day) without diabetic retinopathy, and rapid onset of nephritic syndrome. In certain situations, patients who did not meet the aforementioned criteria underwent renal biopsy, but these patients were not eligible for this study.

The following hospitals participated in this study: Anjou Kosei Hospital, Kainan Hospital, Kakegawa City Hospital, Nishio City Hospital, Masuko Memorial Hospital, Fukuroi City Hospital, Nagoya Daiichi Red Cross Hospital, Nakatsugawa City Hospital, Chuburosai Hospital, Tokai-Chuo Hospital, Tousei Hospital, Handa City Hospital, Nagoya Kyoritsu Hospital, Nagoya Medical Center, and Nagoya University Hospital. Patients with other major diseases, including liver cirrhosis, shock, inflammatory bowel diseases, and malignancy, were excluded. Controls were those who showed normal serum creatinine levels and were negative for urinary protein. Peripheral blood samples and 24-hour urine samples were taken at the time of renal biopsies after obtaining informed consent. Blood was centrifuged at 1,500 *g* for 10 min, and the supernatant was used as serum.

Study Protocols

The levels of HMGB1 were measured in serum of all study participants. The patients who underwent renal biopsies were divided into nine groups according to their diagnosis, as shown in table 1. The ratios of patients who were positive for serum HMGB1 were determined for each group, and the prevalence was compared with that of the controls. The levels of serum HMGB1 in each group of patients were also compared with those of the controls. The relationship between the expression of HMGB1 and the pathological or clinical parameters was assessed. The parameters investigated were pathological diagnosis, age, gender, serum creatinine, CRP, IL-1β, IL-6, TNF-α, urinary protein, and the presence or absence of anti-HMGB1 antibodies in the sera. IL-1β, IL-6, and TNF-α were measured using ELISA kits (Quantikine HS human IL-1β immunoassay, Quanti Glo human IL-6 Immunoassay 2nd generation, and Quanti Glo human TNF-α immunoassay 2nd generation, respectively; R & D Systems, Minneapolis, Minn., USA). Anti-HMGB1 antibody was detected by Western blotting. Other clinical data were gathered from our patient and pathology databases and reviews of medical records. The protocols were approved by the Nagoya University Ethical Committee.

Renal Histology

All renal biopsies were processed for light microscopy, immunofluorescence, and electron microscopy according to standard techniques. For each case, glass slides stained with hematoxylin and eosin, periodic acid-Schiff, Masson's trichrome, and periodic acid-methenamine-silver were reviewed. Immunofluorescence was performed on 2-μm cryostat sections by use of a panel of FITC-conjugated rabbit anti-human antibodies to IgG, IgM, IgA, C3, C1q, and fibrinogen (Dako Corp., Carpinteria, Calif., USA).

Determination of Serum HMGB1 Levels

The HMGB1 concentrations in sera were measured by ELISA at Shino-Test Corp. (Tokyo, Japan) as described [23]. Validated interassay and intra-assay coefficients of variation were <10%, and the limit of detection of this ELISA system was 0.3 ng/ml [24]. Therefore, a level of 0.3 ng/ml or more was defined as positive HMGB1.

Table 1. Prevalence and levels of serum HMGB1

Diagnosis	Number of patients	Number of patients with positive HMGB1 (%)	p vs. controls	average	HMGB1, ng/ml	
					min./median/max.	p vs. controls
ANCA-GN	22	13 (59.1)	<0.001	2.741	0/0.6/17.5	<0.001
HSPN	8	6 (75.0)	0.006	3.213	0/0.8/15.9	<0.001
IgAN	62	22 (35.5)	0.076	2.152	0/0/66.1	0.054
MN	41	14 (34.1)	0.207	1.610	0/0/19	0.100
FSGS	16	6 (37.5)	0.569	0.994	0/0/4.8	0.251
DN	9	3 (33.3)	1.355	2.400	0/0/19.5	0.214
SLE	24	5 (20.8)	4.870	3.383	0/0/39.6	2.780
MCD	27	4 (14.8)	7.368	1.833	0/0/42.2	7.256
Others	49	11 (22.4)	2.859	0.580	0/0/8.7	1.393
Total	258	85 (32.9)	0.034	1.645	0/0/66.1	0.034
Controls	49	6 (12.2)		0.208	0/0/3.2	

ANCA-GN = Anti-neutrophil cytoplasmic antibody-related glomerulonephritis; HSPN = Henoch-Schönlein purpura nephritis; IgAN = IgA nephritis; MN = membranous nephritis; FSGS = focal segmental glomerulosclerosis; DN = diabetic nephropathy; SLE = systemic lupus erythematosus nephritis; MCD = minimal change disease; others = other diseases (crescentic glomerulonephritis, mesangial proliferative glomerulonephritis, minor glomerular abnormality, benign glomerulosclerosis, tubulointerstitial nephritis, endocapillary proliferative glomerulonephritis).

Differences were considered to be significant at $p < 0.05$ (after Bonferroni correction).

Detection of Antibodies against HMGB1

The presence or absence of antibodies against HMGB1 in the sera was determined by Western blotting as described [24]. The serum samples, diluted 50-fold in PBS, were added to each lane of the membrane. Sera which were known to be anti-HMGB1 positive or anti-HMGB1 negative were used as controls.

Immunohistochemistry

Paraffin-embedded tissues were serially sectioned at 6 μm and soaked in a 0.3% H_2O_2 solution for 30 min. To study the localization of HMGB1, the sections were incubated with affinity-purified anti-HMGB1 polyclonal antibody (anti-peptide sequence KPDAAKKGVVKAEK) [24]. To visualize the presence of macrophages, the sections were boiled in sodium citrate buffer (10 mM, pH 6.0) for 30 min and incubated with a mouse anti-human CD68 monoclonal antibody (Dako). After washing, the slides were incubated with biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (Histofine; Nichirei Corp., Tokyo, Japan) and were then further incubated with peroxidase-conjugated streptavidin (Histofine). The slides were incubated with diaminobenzidine substrate (Vector Laboratories, Burlingame, Calif., USA) and counterstained with methyl green solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan).

Statistical Analyses

StatView version 5.0 (SAS Institute, Cary, N.C., USA) was used. For comparison of the data between two groups, the χ^2 test for categorical variables and the Mann-Whitney U test for quantitative variables were used. The patients were categorized into nine different groups according to their pathological diagnosis. The Bonferroni correction was applied for multiple comparisons of HMGB1 in serum. In the present study, HMGB1 in the serum of the control group was compared with that of the nine different groups and with that of all patients as one group. Since multiple ($n = 10$) comparisons were done, the Bonferroni correction was applied to each p value [25]. For comparison of clinical data among multiple groups, ANOVA and Fisher's PLSD (protected least significant difference) method were used. The associations of the presence of HMGB1 in the sera and the pathological or clinical parameters were analyzed by the univariate logistic regression method. Data are expressed as mean ± SD. Differences were considered significant at $p < 0.05$.

Results

Serum HMGB1 and Renal Diseases

During the study period, 321 patients underwent renal biopsy at Nagoya University Hospital and affiliated hospitals, and 298 were eligible for the study. Out of the 298 patients, 258 (86.6%) were included in the study after consenting. The distribution of the patients with each pathological diagnosis is shown in table 1. Forty-nine healthy volunteers served as controls after written informed consent was obtained.

Out of the 258 patients, 85 (32.9%) were positive for serum HMGB1. In contrast, only 4 out of the 49 controls (12.2%) were positive. The levels of HMGB1 were also

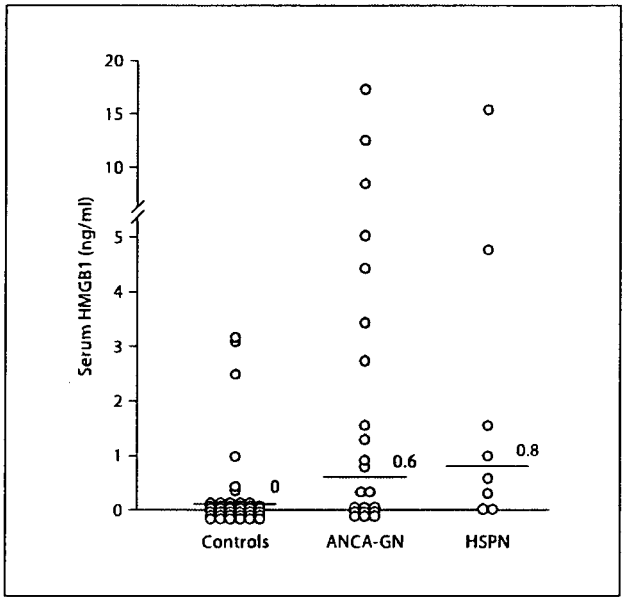

Fig. 1. Serum HMGB1 levels in controls and in patients with ANCA-GN and HSPN. Patients with ANCA-GN or those with HSPN showed significantly higher levels of HMGB1 in the sera than the controls ($p < 0.0001$). Horizontal bars indicate the median; open circles represent patients with negative serum HMGB1 and closed circles those with positive serum HMGB1.

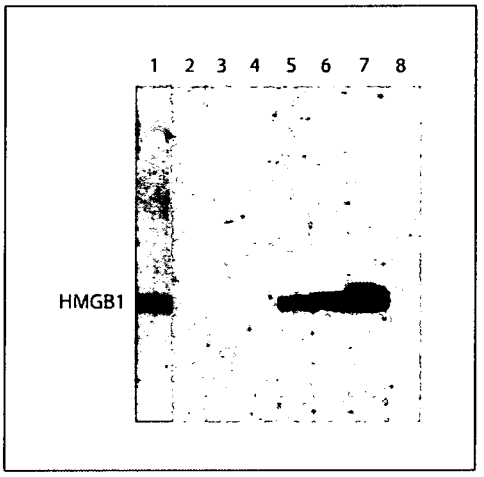

Fig. 2. Detection of anti-HMGB1 antibodies in serum. The presence or absence of anti-HMGB1 antibodies in serum was determined by Western immunoblotting. Lane 1: positive control serum; lane 2: negative control serum; lanes 3 and 4: sera from ANCA-GN patients with positive serum HMGB1; lane 5: serum from a membranous nephritis patient with positive serum HMGB1; lane 6: serum from a focal segmental glomerulosclerosis patient with negative serum HMGB1; lane 7: serum from a systemic lupus erythematosus patient with negative serum HMGB1, and lane 8: serum from an IgAN patient with positive serum HMGB1.

higher among those who underwent renal biopsies than among the controls ($p = 0.0034$; table 1). The ratios of the patients who were positive for serum HMGB1 and the levels of serum HMGB1 were determined for each kidney disease, and these were compared with those of the controls. Only among the patients with ANCA-GN or HSPN was there a >50% prevalence of HMGB1-positive serum. Patients with ANCA-GN and those with HSPN showed a significantly higher prevalence of positive HMGB1 as compared with the controls ($p < 0.0001$ and $p = 0.0006$, respectively, vs. controls). The serum levels of HMGB1 were significantly higher in the patients with ANCA-GN or HSPN in comparison with the controls (minimum/median/maximum in ng/ml 0/0.6/17.5, 0/0.8/15.9, and 0/0/3.2, respectively, $p<0.0001$ vs. controls; table 1, fig. 1). The patients with other diseases (including other types of primary glomerulonephritis, diabetic nephropathy, and lupus nephritis) did not show a significantly higher incidence of the presence of serum HMGB1 when compared to the controls (table 1).

When serum HMGB1 positivity was used as an indicator of a diagnosis for patients who underwent renal biopsies, the sensitivity was 63.3% (95% confidence interval CI 43.9–80.1%), and the specificity was 71.1% (95% CI 64.7–76.9%) for diagnosing patients with either ANCA-GN or HSPN.

Presence or Absence of Anti-HMGB1 Antibodies in Sera

Anti-HMGB1 antibody was detected in 4 out of 85 patients (4.71%) who showed negative serum HMGB1 and in 10 out of 173 patients (5.7%) who showed positive serum HMGB1. Anti-HMGB1 antibody was not detected in any of the patients with ANCA-GN, HSPN, or IgAN. A representative result of the Western blot is shown in figure 2.

Association of Serum HMGB1 with Pathological and Clinical Parameters

The factors which were associated with positive serum HMGB1 were determined by logistic regression analysis. As shown in table 2, among the patients who underwent renal biopsies, ANCA-GN (odds ratio OR 3.29, 95% CI 1.34–8.04, $p = 0.009$) and HSPN (OR 6.49, 95% CI 1.28–23.89, $p = 0.023$) were associated with positive serum HMGB1. Minimal change disease (MCD) was negatively

associated with positive serum HMGB1 (OR 0.32, 95% CI 0.11–0.96, p = 0.042). None of the other clinical parameters, including proinflammatory cytokines such as CRP, IL-1β, IL-6, and TNF-α, or the presence of anti-HMGB1 antibody were associated with positive serum HMGB1. The presence of glomerular crescents showed a tendency to be associated with positive serum HMGB1, which however was not statistically significant (OR 1.71, 95% CI 0.96–3.04, p = 0.066).

Subanalysis of Patients with IgAN

As there were 62 IgAN patients participating in this study, this group was selected for subanalysis. As shown in table 1, patients with IgAN tended to be positive for HMGB1 (35.5%) compared to controls (12.2%), although the difference was not statistically significant. It is well known that IgAN is a heterogeneous disease, and we speculated that there would be some patients more likely to be positive for serum HMGB1 among this group. Therefore, we studied the association of the various clinical parameters with positive serum HMGB1. Table 3 shows that glomerular crescent formation (which suggests that the disease is in an active phase) was significantly associated with positive serum HMGB1 (OR 3.8, 95% CI 1.27–11.41, p = 0.016). In contrast, other pathological parameters, including glomerular hypercellularity, interstitial infiltration, and tubular atrophy, did not show a significant association with positive serum HMGB1. Further analysis revealed that out of 22 IgAN patients who were positive for serum HMGB1, 13 (59.1%) had glomerular crescents, while 11 (27.5%) out of 40 IgAN patients who were negative for HMGB1 showed crescent formation (p = 0.029). The levels of serum HMGB1 were significantly higher in IgAN patients with glomerular crescents as compared with IgAN patients without (minimum/median/maximum in ng/ml 0/1.4/66.1 and 0/0/5.7, respectively, p = 0.0067).

Localization of HMGB1 in the Kidney

Renal biopsy specimens from 11 patients with ANCA-GN (5 serum HMGB1 positive and 6 negative), from 2 patients with HSPN (both HMGB1 positive), and from 12 patients with IgAN (5 serum HMGB1 positive and 7 negative) were available for inclusion in our immunohistochemical studies. Three patients with MCD served as controls. Among the 11 patients with ANCA-GN, strong staining for HMGB1 was observed in the mononuclear cells of the interstitium in 1 out of the 5 patients who were serum HMGB1 positive and in 3 out of the 6 patients who were serum HMGB1 negative. Concerning the 12 patients with IgAN, HMGB1 was strongly stained in the interstitial mononuclear cells in 3 out of the 5 patients who were serum HMGB1 positive and in 2 out of the 7 patients who were serum HMGB1 negative. Among those who showed positive HMGB1 staining in the interstitium, 2 with ANCA-GN and 3 with IgAN also showed positive staining for HMGB1 in some of the mononucle-

Table 2. OR for positive serum HMGB1 by logistic regression analysis in patients who underwent renal biopsies

	OR	95% CI	p
Diagnosis			
ANCA-GN	3.29	1.34–8.04	0.009
HSPN	6.49	1.28–23.89	0.023
IgAN	1.16	0.63–2.11	0.62
MN	1.21	0.60–2.43	0.58
FSGS	1.23	0.43–3.52	0.68
DN	1.01	0.24–4.17	0.97
SLE	0.51	0.18–1.41	0.19
MCD	0.32	0.11–0.96	0.042
Others	0.52	0.25–1.09	0.085
Clinical parameters			
Age	1.01	0.99–1.02	0.11
Gender (male)	1.56	0.91–2.71	0.11
Serum creatinine	1.03	0.79–1.34	0.79
CRP	1.08	0.97–1.21	0.14
Urinary protein	1.01	0.88–1.15	0.88
IL-1β	1.35	0.63–2.88	0.43
IL-6	0.99	0.99–1.01	0.48
TNF-α	0.98	0.83–1.17	0.88
Serum Anti-HMGB1 Ab	1.77	0.41–7.54	0.43
Glomerular crescents	1.71	0.96–3.04	0.066

Diagnosis: For explanation of abbreviations see footnote to table 1.

Table 3. OR for positive serum HMGB1 by logistic regression analysis in patients with IgAN

	OR	95% CI	p
Age	1.01	0.98–1.04	0.39
Gender (male)	1.75	0.58–5.22	0.31
Serum creatinine	0.95	0.31–2.95	0.93
CRP	0.89	0.07–10.30	0.92
Urinary protein	0.85	0.52–1.39	0.53
IL-1β	2.57	0.14–47.03	0.52
IL-6	5.1	0.65–39.55	0.11
TNF-α	2.57	0.14–47.03	0.52
Glomerular crescents	3.8	1.27–11.41	0.016

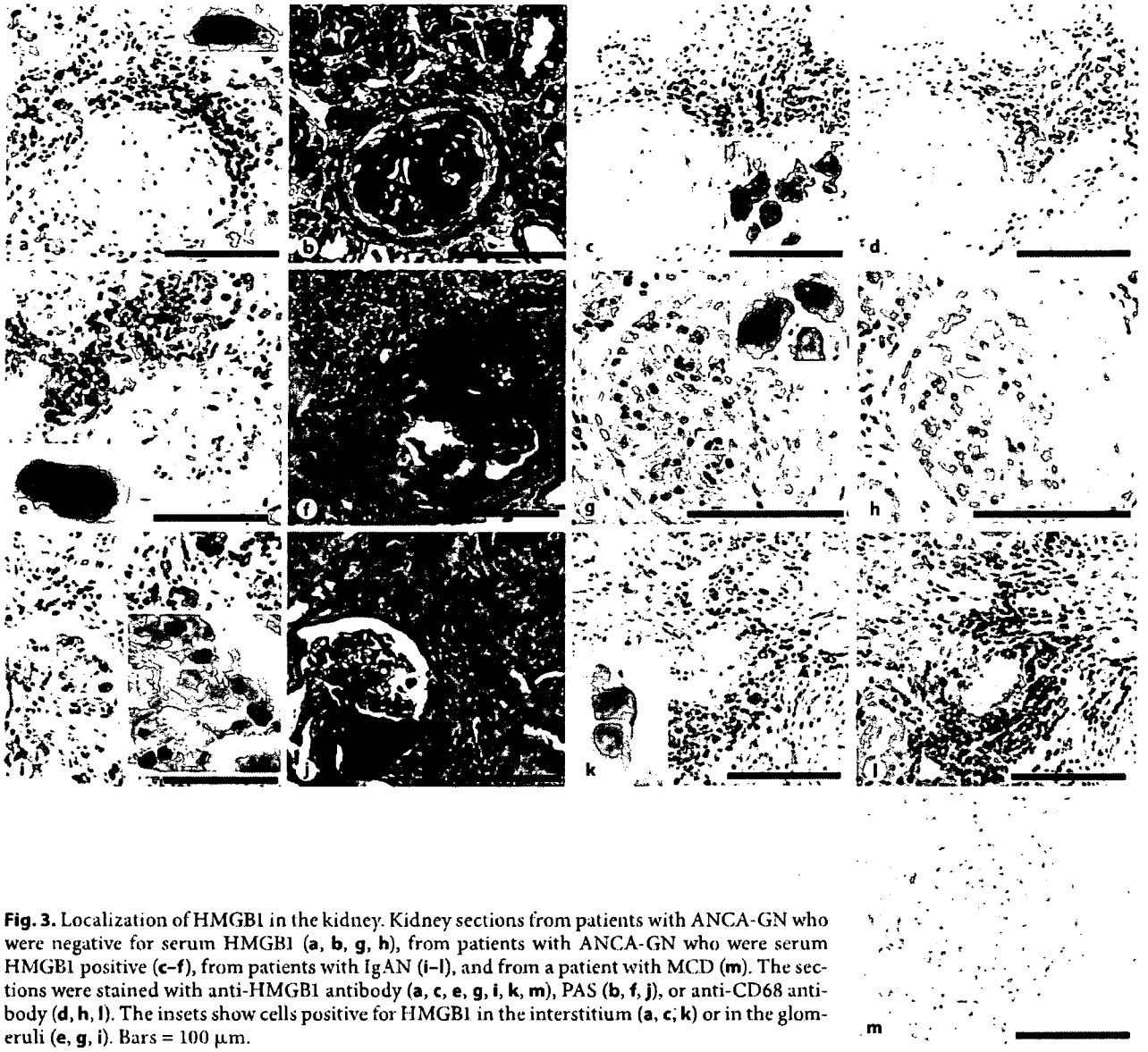

Fig. 3. Localization of HMGB1 in the kidney. Kidney sections from patients with ANCA-GN who were negative for serum HMGB1 (**a**, **b**, **g**, **h**), from patients with ANCA-GN who were serum HMGB1 positive (**c–f**), from patients with IgAN (**i–l**), and from a patient with MCD (**m**). The sections were stained with anti-HMGB1 antibody (**a**, **c**, **e**, **g**, **i**, **k**, **m**), PAS (**b**, **f**, **j**), or anti-CD68 antibody (**d**, **h**, **l**). The insets show cells positive for HMGB1 in the interstitium (**a**, **c**, **k**) or in the glomeruli (**e**, **g**, **i**). Bars = 100 μm.

ar cells of the glomeruli. In both the interstitium and the glomeruli, HMGB1 was localized in the nuclei as well as in the cytoplasm of the mononuclear cells, which were most likely to be macrophages (fig. 3). Only weak staining was observed in the mononuclear cells of the interstitium in the other 7 patients with ANCA-GN, in the other 7 patients with IgAN, and in the 2 patients with HSPN. The localization was similar regardless of the presence or absence of serum HMGB1. No staining was observed in the patients with MCD (fig. 3).

Discussion

This is the first study showing the presence of HMGB1 in the sera of patients with renal diseases. The results showed that serum HMGB1 was more likely to be positive in patients who underwent renal biopsies than in controls. The criteria used in the present study may suggest that the patients in the biopsy group would have some renal damage, leading to a higher incidence of positive HMGB1. The results of the present study showed that

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