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ET_B receptor protects the tubulointerstitium in experimental thrombotic microangiopathy

Masaomi Nangaku, Koei Yamada, Cheryl E. Gariepy, Toshio Miyata, Reiko Inagi, Kiyoshi Kurokawa, Masashi Yanagisawa, Toshiro Fujita, and Richard J. Johnson

Division of Nephrology and Endocrinology, University of Tokyo School of Medicine, Tokyo, Japan; Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas, USA; Molecular and Cellular Nephrology, Institute of Medical Sciences and Department of Medicine, Tokai University School of Medicine, Kanagawa, Japan; and Section of Nephrology, Baylor College of Medicine, Houston, Texas, USA

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Background. The characteristic features of thrombotic microangiopathy (TMA) include glomerular and peritubular capillary endothelial cell injury with thrombus formation and subsequent ischemic tubulointerstitial damage. The endothelin ET_B receptor has been shown to mediate both endothelial cell proliferation and vasodilation, and we therefore hypothesized that blockade of this receptor might promote more severe injury in this model.

Methods. TMA was induced in recently established transgenic rats that lack expression of ET_B receptor in the kidney; these animals were compared to control rats with TMA both in the short-term (days 1 and 3) when acute glomerular injury was most manifest, and the long-term (day 17) when glomeruli have recovered but tubulointerstitial injury is still present. Renal damage was assessed by histological analysis and blood urea nitrogen (BUN) measurements.

Results. No difference in the TMA model was observed between rats with and without ET_B receptor on days 1 or 3. At day 17, however, rats without the ET_B receptor showed more severe tubulointerstitial injury compared with those with ET_B receptor, which was associated with higher BUN levels. The tubulointerstitial damage was associated with a more severe loss of peritubular capillaries.

Conclusions. These findings suggest that the ET_B receptor may protect peritubular capillaries under the ischemic insult, and serve a defensive role in the tubulointerstitium induced by renal microvascular injury.

The hemolytic uremic syndrome and other related thrombotic microangiopathy (TMA) are characterized by injury to the renal microvascular endothelium. To elucidate the pathophysiology of renal TMA, we recently developed a model induced by the renal artery perfusion of anti-glomerular endothelial cell antibody [1, 2]. This model demonstrates features similar to hemolytic uremic syndrome in humans, including glomerular and peritubular capillary endothelial cell injury with platelet accumulation and fibrin deposition, microangiopathic hemolysis, mild thrombocytopenia, and renal failure [3, 4]. While some glomerular and peritubular capillary repair occurs after the initial endothelial damage, this repair process is not complete, and progressive tubulointerstitial damage occurs. Our current study was designed to investigate a role of ET_B receptor in the TMA model, because this endothelin receptor is thought to modulate both endothelial cell proliferation and vascular dilation [reviewed in 5–12].

Endothelins are a family of 21 amino acid peptides that include three distinct isoforms (ET-1, ET-2, and ET-3). ET-1 acts as a paracrine/autocrine factor and is secreted by vascular endothelial cells abluminally where it can act on the smooth muscle cell [13]. In the kidney, ET-1 is produced by glomerular epithelial cells, mesangial cells, endothelial cells, and tubular cells [14–16].

These isopeptides show different biological activities and bind to two subtypes of G protein-coupled receptors. One is ET_A receptor that shows a selective affinity for ET-1, and the other is ET_B receptor that shows a nonselective affinity for all three isoforms. The ET_B receptor is expressed in a variety of tissues, and ET_B receptor stimulation exerts a variety of biological effects including vasodilation and endothelial cell proliferation [17, 18]. Based on the actions of the ET_B receptor, we hypothesized that it may play a protective role in the kidney of TMA. To investigate the role of ET_B receptor in TMA model of rats, we employed recently established transgenic rats that lack expression of ET_B receptor in an organ-specific manner including the kidney [19].

Key words: ischemia, endothelium, hemolytic-uremic syndrome, hypoxia, endothelin, renoprotection, progressive renal disease.

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METHODS

Experimental protocol and disease model

The renal TMA model was induced by the selective perfusion of the right kidney through the superior mesenteric artery with the purified goat anti-glomerular endothelial cell (GEN) antibody. The characteristics of the anti-GEN antibody and the technique of selective right renal artery perfusion were described previously [1]. Briefly, the right kidney was perfused through the superior mesenteric artery with 0.2 mL saline, followed by 30 mg/kg body weight of anti-GEN IgG. Ischemic time was less than six minutes in each rat.

In the first set of experiments, the role of the ET_B receptor was investigated in the TMA model at days 1 and 3. Three groups of rats were studied. Group I was composed of spotting lethal rats rescued by the transgene of the ET_B receptor with a neuron-specific promoter (EDNRB*sl* rats; $N = 7$). These rats show expression of ET_B receptor in a tissue-specific manner, with undetectable levels of ET_B receptor in the kidney [19]. Group II was composed of wild type Wistar-Imamichi rats ($N = 7$). Group III was composed of control EDNRB*sl* rats rats perfused with normal goat IgG ($N = 3$). In these rats, the unperfused left kidney was removed for evaluation of renal function prior to closing the abdominal incision when the disease was induced by selective renal artery perfusion. Twenty-four hours after perfusion, a blood sample was obtained, and a survival renal biopsy of the perfused kidney was performed in all groups. Three days after induction of disease, a blood sample was obtained via a cardiac puncture, and a sacrificial biopsy was performed for histologic analysis.

In the second set of experiments, the role of the ET_B receptor was investigated in the long term at day 17. Three groups of rats were studied. Group IV was composed of EDNRB*sl* rats ($N = 12$). Group V was composed of wild-type Wistar-Imamichi rats ($N = 12$). Group VI was composed of control EDNRB*sl* rats perfused with normal goat IgG ($N = 3$). In these experimental animals, the unperfused left kidney was removed at day 14 and rats were sacrificed at day 17.

All animal experimentation was conducted in accord with the Guide for Animal Experimentation, Faculty of Medicine, University of Tokyo.

Blood pressure measurement

Systolic blood pressure was measured in conscious, restrained rats using a tail-cuff sphygmomanometer (UR-5000; UEDA Company, Tokyo, Japan). The systolic blood pressure for each rat was calculated as the average of three separate measurements at each session.

Renal morphology

Methyl Carnoy's fixed tissue was processed and paraffin-embedded. Four-micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. An indirect immunoperoxidase method was used as previously described [4] to identify the following antigens: renal microvascular endothelial cells with monoclonal antibody JG-12 (a generous gift from Dr. Dontcho Kerjaschki, Vienna, Austria) [14]; monocyte-macrophages with antibody ED-1 (Bioproducts for Science, Indianapolis, IN, USA); osteopontin with goat anti-osteopontin (OPN; 199; Ceci Giachelli, University of WA, Seattle, WA, USA), proliferating cell nuclear antigen (PCNA) with PC10 (Dako A/S, Glostrop, Denmark), and platelets with monoclonal antibody PL-1 (generously provided by W.W. Baker, University of Groningen, Groningen, the Netherlands) [20]. Controls included omission of the primary antibody or substitution with an irrelevant antibody of the same species and isotype.

Tissue for immunofluorescence was embedded in O.C.T. compound and snap-frozen in isopentane. Fibrin was detected by staining with FITC-conjugated goat anti-fibrinogen IgG (Cappel Laboratory, Durham, NC, USA). Deposition of the pathogenic goat anti-GEN antibody was detected by biotinylated anti-goat IgG (Secondary antibody; Dako Corp., Carpinteria, CA, USA), followed by incubation with Oregon green/Neutralite Avidin (Molecular Probes Inc., Eugene, OR, USA).

Quantification was performed in a blinded manner on 30 randomly selected glomeruli per biopsy for each of the following variables: glomerular thrombi was assessed by immunostaining with anti-fibrinogen, glomerular endothelial cell loss by immunostaining for renal microvascular endothelium with JG-12 antibody; and platelet aggregation by PL-1 staining. For glomeruli thrombi and platelet infiltration, a semiquantitative scoring system was used as follows: 0 = normal; 1 = 0 to 25% of glomerular area involved; 2 = 25 to 50% of glomerular tuft involved; 3 = 50 to 75% of tuft area involved; and 4 = >75% of tuft area involved. A glomerular endothelial score was graded as follows: 0 = 0 to 25% glomerular tuft stains positive for endothelium; 1 = 25 to 50% positive; 3 = 50 to 75% positive; 4 = 75 to 100% positive. The number of glomerular macrophages (ED-1 positive) also was enumerated per glomerular cross section. Glomerular cross sections containing only a minor portion of the glomerular tuft (<20 discrete capillary segments per cross section) were not utilized. Glomerular sclerosis was assessed by periodic acid Schiff (PAS) staining, and was defined as segmental or global capillary collapse with increased matrix deposition in over 25% of glomerular surface area.

Peritubular capillary density was reported using a rarefaction score based on staining with JG-12 antibody as described previously [21, 22]. To quantify capillary rarefaction, JG-12 immunostained sections were examined through a 10 × 10-eyepiece grid under a ×10 objec-

tive. At this magnification, the grid covered an area of 1 mm^2. Each square (0.1 mm^2) within the grid that contained no JG-12-positive vessels was scored. At least 50 fields in the cortex were examined on the longitudinal section of each kidney. The minimum possible capillary rarefaction score was 0 and the maximum score 100, where 100 would indicate a complete absence of JG-12 positive cells.

Tubulointerstitial injury was scored semiquantitatively on thirty cortical fields of periodic acid-Schiff-stained biopsies using a ×20 objective, as described previously [23]. Tubulointerstitial injury was defined as tubular dilatation, tubular atrophy, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane and was scored on a scale of 0 to 4, as follows; 0 = no tubulointerstitial injury; 1 = <25% of the tubulointerstitium injured; 2 = 25 to 50% of the tubulointerstitium injured; 3 = 51 to 75% of the tubulointerstitium injured; 4 = >75% tubulointerstitium injured.

Osteopontin positive tubules were counted in ten randomly selected cortical fields using a ×10 objective. PCNA positive tubular cells and ED-1 positive cells in the tubulointerstitium were counted in twenty randomly selected cortical fields using a ×20 objective.

All assessments were performed in a blinded manner.

Statistical analysis

Values were expressed as mean ± SD and analyzed by the Student *t* test or analysis of variance (ANOVA) with the Bonferroni protected least significant difference test for multiple comparison. Nonparametric data were analyzed by Mann-Whitney test, when appropriate. Values were considered significant if P was <0.05.

RESULTS

Lack of ET_B receptor in the kidney does not alter binding of the anti-glomerular endothelial cell antibody in the TMA model

To test if EDNRB*sl* rats and wild-type Wistar-Imamichi rats show different binding of pathogenic anti-GEN IgG in the kidney, the biopsies obtained 24 hours after induction of disease were stained with anti-goat IgG. All the TMA rats (groups I and II) demonstrated bright linear staining in a capillary pattern with anti-goat IgG of the same intensity. The peritubular capillaries and vessels also showed positive staining in the two groups without any difference. Staining for goat IgG was negative in kidney tissues from Group III that had been perfused with normal goat IgG.

Lack of ET_B receptor in the kidney did not affect TMA in the short term

The primary manifestation of the TMA model in the short term is massive thrombus formation in glomeruli with acute renal failure. To examine whether the lack of ET_B receptor affects glomerular thrombus formation, fibrin deposition was assessed in glomeruli by immunofluorescence studies with samples obtained at day 1 and day 3. Semiquantitative analysis showed no difference of fibrin deposition between group I and group II at day 1 and at day 3 (Table 1). Rats perfused with normal goat IgG (group III) had no fibrin deposition in glomeruli.

Table 1. Histological assessments of glomerular damage at early time points

	ET_B receptor (−)	ET_B receptor (+)	P value
Fibrin deposition			
Day 1	2.4 ± 0.6	2.2 ± 0.6	0.44
Day 3	1.8 ± 0.5	1.7 ± 0.6	0.74
Platelet deposition			
Day 1	2.3 ± 0.6	2.2 ± 0.6	0.66
Day 3	1.2 ± 1.1	1.4 ± 0.5	0.63
Macrophage infiltration in glomeruli			
Day 1	3.7 ± 0.9	3.5 ± 0.8	0.75
Day 3	4.2 ± 1.3	4.1 ± 1.3	0.88
Glomerular endothelial damage			
Day 1	1.7 ± 1.1	1.8 ± 1.1	0.85
Day 3	2.5 ± 1.4	2.8 ± 1.1	0.70

Platelet accumulation in glomeruli was also assessed by immunostaining with platelet-specific monoclonal antibody, PL-1, using samples obtained one day and three days after induction of disease. Platelet aggregation was not different between group I and group II at day 1 and at day 3 (Table 1). No platelet accumulation was observed in rats perfused with normal goat IgG (group III).

Macrophage infiltration was evaluated by ED-1 staining. TMA rats (group I and group II) showed more macrophage infiltration in glomeruli at day 1 (3.7 ± 0.9 and 3.5 ± 0.8, respectively) and at day 3 (4.2 ± 1.3 and 4.1 ± 1.3, respectively) compared with control rats (group III, 1.4 ± 0.3 at day 1 and 1.3 ± 1.0 at day 3), but there was no difference in glomerular macrophage infiltration between group I and group II (Table 1).

To assess endothelial cell damage, we performed staining with an endothelial cell-specific monoclonal antibody, JG-12, using samples obtained at day 1. Rats perfused with control goat IgG (group III) showed an intact staining pattern of JG-12, while some of the glomeruli in TMA rats (groups I and II) showed a decrease in staining. Semiquantification of JG-12 staining of glomeruli suggested that TMA rats of group I and group II showed equivalent glomerular endothelial damage (Table 1). Furthermore, the number of peritubular capillaries did not differ either at day 1 (capillary rarefaction score 33.3 ± 13.5 vs. 36.6 ± 16.0, $P = 0.68$) or at day 3 (30.9 ± 12.4 vs. 27.6 ± 11.7, $P = 0.62$).

In these rats, the left unperfused kidneys were removed for assessment of renal function when TMA was induced. TMA rats (group I and group II) developed

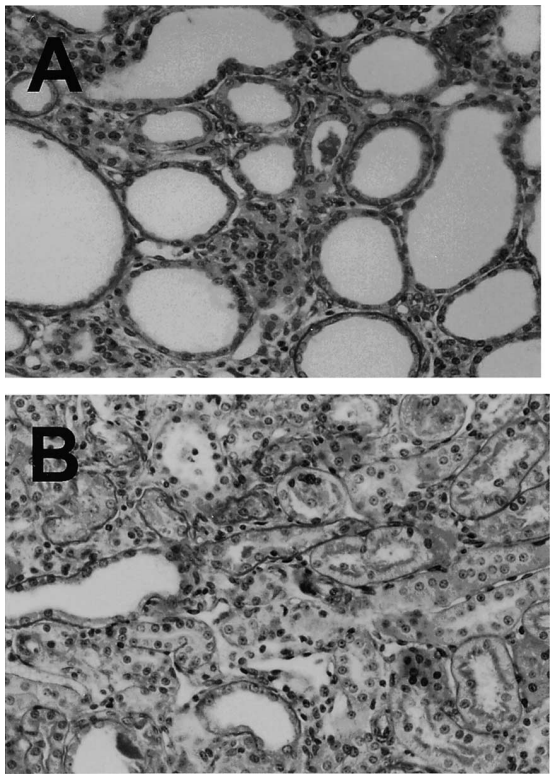

Fig. 1. Persistent tubulointerstitial damage in thrombotic microangiopathy (TMA) rats at day 17. ET_B receptor-deficient TMA rats showed severe tubulointerstitial damage (*A*) compared to wild-type TMA rats (*B*) at day 17 (PAS, ×200).

acute kidney failure [blood urea nitrogen (BUN) levels 58.3 ± 15.2 mg/dL and 49.4 ± 11.7 mg/dL at day 1; 94.6 ± 42.7 mg/dL and 83.5 ± 54.1 mg/dL at day 3]. There was no difference in the degree of kidney failure between the two groups ($P = 0.25$ and 0.68 at day 1 and day 3, respectively). Rats perfused with normal goat IgG (group III) had markedly lower BUN levels at day 1 (30.9 ± 5.5 mg/dL) or at day 3 (22.0 ± 7.2 mg/dL).

In summary, ET_B receptor deficiency in the kidney did not change any disease manifestations examined in this study in the short term until day 3.

ET_B receptor has a protective role in the tubulointerstitium in TMA in the long term

While glomeruli recover from the initial TMA in this model, the characteristic manifestation later in the course of the disease is chronic tubulointerstitial injury [1, 22]. Many studies emphasized that the extent of tubulointerstitial damage correlates better with the impairment of renal function than the degree of glomerular damage [24–32]. Therefore, we examined the severity of tubulointerstitial damage at day 17. To assess renal function, the left non-perfused kidneys were removed at day 14.

The main pathologic features of TMA rats (groups IV and V) were marked atrophy or dilatation of tubules and interstitial fibrosis. The tubulointerstitial injury was particularly severe in rats of group IV (Fig. 1). Semiquantitative analysis confirmed more severe tubulointerstitial damage in rats of group IV compared with that in rats of group V (Table 2). Furthermore, immunohistological analysis employing osteopontin as a marker of tubular injury demonstrated more osteopontin-positive tubules in rats of group IV compared with that of group V (Fig. 2 and Table 2). Quantitative analysis of tubular cell proliferation showed more PCNA-positive tubular cells in rats of group IV compared with group V (Table 2). Macrophage infiltration in tubulointerstitium was more severe in group IV rats compared with those of group V (Table 2). This was associated with more severe renal dysfunction in EDNRB*sl* rats than in wild-type Wistar-Imamichi rats at day 17 (BUN levels 72.7 ± 23.9 vs. 50.7 ± 20.2 mg/dL, $P < 0.05$). No renal dysfunction or histological damage was observed in control EDNRB*sl* rats (group VI, BUN levels 25.1 ± 5.1 mg/dL).

Table 2. Histological assessments of tubulointerstitial damage at day 17 points

	ET_B receptor (−)	ET_B receptor (+)	*P* value
TI damage	2.5 ± 0.4	2.0 ± 0.5	<0.01
OPN(+) tubules	7.5 ± 4.1	2.9 ± 2.0	<0.01
PCNA(+) tubules	20.9 ± 12.8	3.8 ± 3.1	<0.005
Macrophage infiltration	8.9 ± 5.1	3.5 ± 2.1	<0.005
Capillary rarefaction	6.3 ± 1.9	4.0 ± 1.6	<0.05

Abbreviations are: TI, tubulointerstitial; OPN, osteopontin; PCNA, proliferating cell nuclear antigen.

In contrast, no significant difference in glomeruli was observed between the two groups of TMA rats. Glomerular sclerosis was not different between rats of group IV and group V (3.5 ± 2.8 vs. 3.3 ± 3.2%, $P = 0.87$). There was also no difference in the number of macrophages in glomeruli (1.0 ± 0.5 in group IV rats and 1.0 ± 0.4 in group V rats, respectively; $P = 0.84$).

Peritubular capillary damage

The tubulointerstitial damage in group IV rats was associated with more severe disruption of the peritubular capillary network (Fig. 3). While capillary rarefaction was improved at day 17 compared with day 1 and day 3, peritubular capillary loss was more severe in ET_B receptor deficient rats (group IV) than in control rats (group V; Table 2). Double staining with JG-12 and PCNA showed rare proliferating peritubular capillary endothelial cells (Fig. 3 inset), but there was no statistical difference between the two groups (0.01 ± 0.01 cells/mm^2 vs. 0.01 ± 0.01, P = NS).

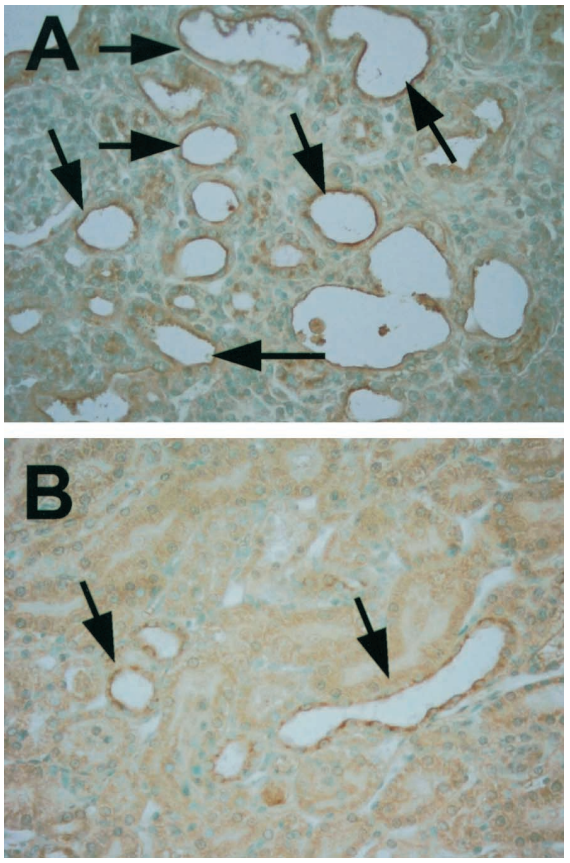

Fig. 2. Osteopontin (OPN) expression by damaged tubules in TMA rats at day 17. ET_B receptor-deficient TMA rats (*A*) show greater expression of the tubular injury marker, osteopontin, compared to wild type TMA rats (*B*). Representative OPN-positive tubules are indicated by arrows (×200).

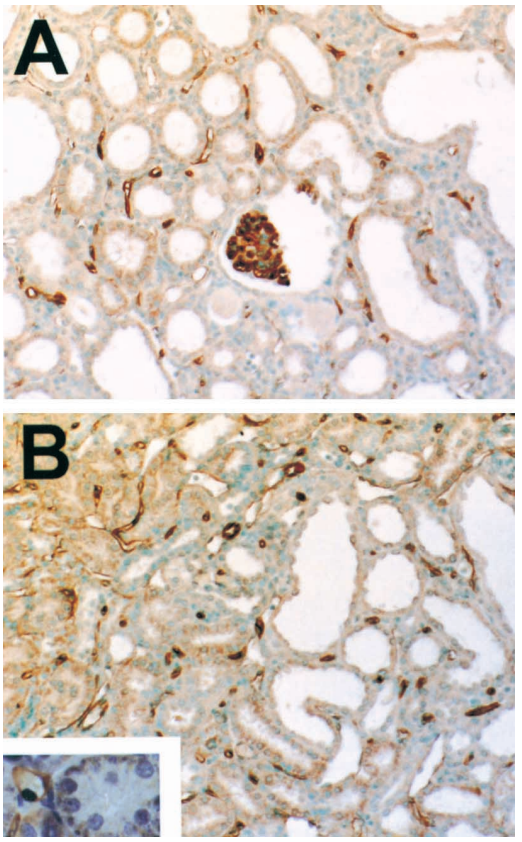

Fig. 3. Disruption of the peritubular capillary network in TMA rats at day 17. JG-12 staining demonstrated greater peritubular capillary density in wild type TMA rats (*B*) than in ET_B receptor deficient TMA rats at day 17 (*A*; ×200). Double-staining with PCNA (black) and JG-12 (brown) revealed occasional proliferating peritubular capillary endothelial cells (inset, ×400).

No blood pressure changes were observed throughout the experimental course

To examine the changes of systemic blood pressure due to lack of ET_B receptor in the kidney, blood pressure of the experimental animals was measured before induction of the disease, and 3 days and 17 days after the induction of the model. We did not find any changes of systolic blood pressure levels throughout the experimental course (Table 3).

Table 3. Systolic blood pressure levels

	ET_B receptor-deficient TMA rats	Wild-type TMA rats	ET_B receptor control rats
Pre-study	142 ± 10	142 ± 12	136 ± 8
Day 3	141 ± 13	146 ± 20	139 ± 20
Day 17	144 ± 15	145 ± 13	152 ± 10

DISCUSSION

A naturally occurring null mutation of the gene encoding the ET_B receptor in spotting lethal (*sl*) rats exhibit aganglionic megacolon associated with white color [33]. These deficits result from failure of the neural crest-derived epidermal melanoblasts and enteric nervous system precursors to completely colonize the skin and intestine, respectively. Homozygous animals die within 35 days after birth due to intestinal obstruction [33]. Transgenic expression of the ET_B receptor with the human dopamine-beta-hydroxylase (DBH) promoter prevents the intestinal defect in these rats [19]. In the rescued rats, expression of ET_B receptor was not detected in the kidney while ET_B receptor was strongly expressed in the kidney of wild-type rats [19]. This can be explained by the tissue-specific promoter activity of the transgene [34] and enabled us to utilize rats that lack expression of ET_B receptor in a tissue specific manner.

Rats without ET_B receptor in the kidney developed glomerular TMA essentially similar to that observed in rats with ET_B receptor at earlier time points. In contrast, the tubulointerstitial injury that is observed in a late stage of the TMA model was aggravated by ET_B receptor

deficiency. This was associated with a decline in the number of the peritubular capillaries (PTC). Although we cannot rule out the possibility that the differences in the phenotypic responses between the two groups of animals could be due to the disparate genetic background, as we could not use the *sl* homozygous rats because of the lethal nature, the lack of ET_B receptor in the kidney may provide an explanation for the more severe renal injury observed in the transgenic animals.

Among the various components making up the tubulointerstitial regions of the kidney, peritubular capillaries (PTC), which are a network of interstitial vessels, are thought to play a major role in maintaining renal function and hemodynamics. Several recent studies showed a strong correlation between the degree of loss of PTC and the progression renal disease [35–38]. Renal tubules, especially by the proximal (S3 segments) and thick ascending limbs, are particularly susceptible to ischemic injury because of the low oxygen tensions and the high energy demands [39, 40]. Because PTC are essential for maintaining proper renal hemodynamics and for supplying oxygen to the entire kidney, it is possible that PTC injury causes localized hypoxia, resulting in scarring of the tubulointerstitium. Recent observations utilizing the same model emphasized an important role of PTC in the kidney, as we found that treatment with vascular endothelial growth factor (VEGF) of the TMA model accelerated the recovery of PTC and ameliorated the subsequent tubulointerstitial fibrosis and renal failure [22]. Although we cannot exclude the possibility that a decline in PTC in ET_B receptor deficient animals was merely a result of more severe tubulointerstitial damage, it is tempting to speculate that the ET_B receptor in the kidney protects PTC and maintains oxygenation in the kidney, thereby reducing the fibrotic response.

The lack of the ET_B receptor in the kidney may lead to severe tubulointerstitial injury via several different mechanisms. ET-1 has been implicated in the pathogenesis of the sustained regional vasoconstriction following ischemic acute renal failure [reviewed in 11, 41]. The renal vasculature is 10 times more sensitive to ET-1 than other vascular beds [42–45], and ET-1 potently constricts both afferent and efferent arterioles in the kidney [46–48]. Vasoconstriction is mediated by ET_A receptor, while the compensatory vasodilating effect is mediated by ET_B receptor. While we could not detect any difference in the degree of acute renal failure or glomerular damage between ET_B receptor-deficient and wild-type rats of TMA model until day 3 in this study, a lack of the vasodilating effect via the ET_B receptor may have aggravated tubulointerstitial injury under the chronic ischemic conditions that were likely present.

While ET_B receptor mediates vasodilation in the kidney, previous observations demonstrated that ET-1 has a dose-dependent stimulatory, proliferative and migratory effect on endothelial cells isolated from bovine adrenal capillaries and human umbilical vein endothelial cells (HUVECs) via the ET_B receptor [49–51]. Recently Salani and colleagues showed that the angiogenic effects of endothelin-1 was additive to that of VEGF [52]. These findings provide another possibility that the severe tubulointerstitial injury observed in ET_B receptor deficient rats might be due to impaired PTC proliferation secondary to a lack of the ET_B receptor. While we could not detect a significant difference in the number of proliferating PTC between the two groups of rats, it is possible that a difference in endothelial cell proliferation may have been present if tissue had been biopsied at an earlier time point.

Previous reports had emphasized a role of endothelin in tubulointerstitial damage [53]. Our findings, to our knowledge for the first time, suggested a protective role of ET_B receptor on PTC and tubulointerstitial injury in chronic ischemic damage. Our studies are consistent with a critical role for the microvasculature in progressive renal disease.

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Reprint requests to Masaomi Nangaku, M.D., Ph.D., Division of Nephrology and Endocrinology, University of Tokyo School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: mnangaku-tky@umin.ac.jp

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