Role of Thromboxane A₂, Endothelin-1 and Endothelin-3 in Rat Nephrotoxic Serum Nephritis

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ZHOU, L., IDA, T. and MARUMO, F. *Role of Thromboxane A₂, Endothelin-1 and Endothelin-3 in Rat Nephrotoxic Serum Nephritis.* Tohoku J. Exp. Med., 1996, 178 (4), 357-369 — To clarify the role of thromboxane A₂ (TxA₂), endothelin-1 (ET-1) and endothelin-3 (ET-3) in the progression of glomerular injury in accelerated nephrotoxic serum nephritis (NTN) in the rat, we studied the expression of ET-1 and ET-3 at the kidney by immunohistochemical method and examined the effect of a novel TxA₂ receptor antagonist, S-1452. The S-1452-treated group showed significantly lowered 24-hr proteinuria and milder glomerular cell proliferation and lobulation than the non-treated group (NT group) on experimental day 10. There was no significant difference in the glomerular polymorphonuclear cell (PMN) exudation between the 2 groups. Immunofluorescent findings revealed that ET-1 and ET-3 were seen along the glomerular capillary wall and partly in the mesangial area in all rats of the NTN group. The degree and positive rate of ET-1 and ET-3 staining were significantly higher in the NTN group than in the S-1452 group. These findings suggest that TxA₂ may be an important mediator in the development of NTN, and that TxA₂ receptor antagonist may be useful for the reduction of glomerular injury in this type of nephritis. In addition, local production of ET may contribute to the development of this nephritis. — thromboxane A₂; endothelin-1; endothelin-3; nephrotoxic serum nephritis

The immunological mechanism is thought to play a role in the pathogenesis of glomerular injury. Other factors that have been suggested to act as mediators of glomerular injury include platelets, complements, and coagulation-fibrinolysis system. Thromboxane A₂ (TxA₂), which is synthesized by platelets from arachidonic acid, has received particular attention. TxA₂ reduces renal blood flow by vasoconstriction (Baylis 1987) and controls glomerular ultrafiltration by contraction of mesangial cells (Scharschmidt et al. 1983; Mene and Dunn 1986). In addition, TxA₂ is a potent platelet aggregation factor and promotes the release of several inflammatory substances from platelets such as platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β).

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We previously reported that a TxA₂ receptor antagonist (S-1452) improved renal damage of female (NZB×NZW) F1 mice with spontaneous lupus nephritis and prevented the occurrence of proteinuria in rats with streptozotocin-induced diabetic nephropathy (Matsuo et al. 1995). Thrombocytopenia induced by anti-rabbit goat platelet antibody prevented renal injury and decreased proteinuria in acute serum sickness nephritis of rabbits (Ideura et al. 1992).

Concerning the relationship between endothelin (ET) and kidneys, the sites of ET production and ET receptor are reported to be vascular endothelium, tubular epithelium and mesangial cell (Ohta et al. 1990; Sakamoto et al. 1990; Terada et al. 1992). ET-1 has vasoconstrictive action like TxA₂, while ET-3 has vasorelaxing action (Hirata et al. 1989). Recent reports suggested that ET acts as a growth factor in mesangial cell proliferation and that its production is increased by TxA₂ (Rao et al. 1989; Sakamoto et al. 1990). In addition, Sirois et al. (1992) reported that the effect of ET-1 on vascular permeability is partly mediated and/or modulated by the secondary release of TxA₂. The renal concentrations of ET-1 and ET receptor were reported to increase significantly with age in NZB/W F1 mice (Nakamura et al. 1993). In addition, we reported that increased glomerular expression of ET-1 was observed in human lupus nephritis by immunohistochemical examination (Ida et al. 1990). These findings compelled us to study the role of TxA₂ and ET in glomerular injury.

S-145 ((±)-5(z)-7(3-endo-[(phenylsulfonyl)amino]bicyclo[2, 2.1]hept-2-exo-yl]heptenoic acid), a TxA₂ receptor antagonist, has a high affinity for platelets and vascular smooth muscle cells on culture (Hanasaki et al. 1988), and S-1452 is a stable calcium salt of the (+)-isomer of S-145.

In the present study, we examined the role of TxA₂, ET-1 and ET-3 in the pathogenesis of nephrotoxic serum nephritis of rats, and examined the effect of S-1452, a novel TxA₂ receptor antagonist, on this nephritis.

MATERIALS AND METHODS

Experimental animals

The experiment was performed using 6-week old, 200–220 g male Wistar rats obtained from Japan Laboratory Animal Inc. (Tokyo). The rats were maintained on standard pelleted chow and water ad libitum in air-conditioned rooms at 25°C with a 12-hr light/12-hr dark cycle (lights on from 8:00 to 20:00). The principles of laboratory animal care noted in NIH publication number 85-23, revised in 1985, were followed in all experiments.

Chemicals

S-1452, a potent TxA₂ receptor antagonist, was kindly supplied by Shionogi Developmental Research Laboratories (Osaka).

Preparation of nephrotoxic serum. Nephrotoxic serum (NTS) was obtained by the modified method from Krakower and Greenspon (1951). Immediately

after sacrificing rabbits, the kidneys were removed and perfused with saline, and the cortex was isolated. Glomeruli was obtained by passing the specimens through three meshes of different sizes. One mg of glomeruli mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) was injected subcutaneously on the back and foot pads of albino rabbits 4 times every 2 weeks. Ten days after the last injection, blood was drawn from the ear arteries of the rabbits. The specificity of the serum was certified by the fact that linear staining along the glomerular capillary wall of a normal rat was observed by indirect fluorescein isothiocyanate (FITC) staining using FITC-conjugated anti-rabbit IgG antibody from goat (Wako Pure Chemical, Osaka).

Experimental protocol

For inducing accelerated nephrotoxic serum nephritis (NTN), 1 mg of normal rabbit IgG (Organon Teknika NV-Cappel Products, Durham, NC, USA) with 0.5 ml of complete Freund's adjuvant was preimmunized subcutaneously into 38 rats. These rats were divided into 3 groups. In the S-1452 group, 15 rats were injected with 1 ml of NTS through the tail vein 7 days after the preimmunization, and orally administered 10 mg/4ml/kg body weight S-1452 in 0.5% carboxymethyl cellulose sodium salt (CMC) every day throughout the experiment. In the NTN without treatment group (non-treated group: NT group), 15 rats were injected with 1 ml of NTS through the tail vein 7 days after the preimmunization, and administered 4 ml of 0.5% CMC/kg body weight orally every day throughout the experiment. In the control group, 8 rats were injected with 1 ml of normal saline through the tail vein 7 days after the preimmunization. All rats were sacrificed under anesthesia by somnopeny1 (Pitman Moore Inc., Mundelein, IL, USA) on the 17th experimental day.

Blood samples

On the 17th experimental day, blood was obtained by cardiopuncture. Blood urea nitrogen (BUN), serum creatinine (Cr), serum sodium (Na) and total protein (TP) were autoanalyzed by an autoanalyzer (736; Hitachi, Tokyo).

Urine samples

On the 15th or 16th experimental day, the rats were placed in a stainless steel metabolic cage and 24 hr urine was obtained. After centrifugation at 3,000 rpm for 15 min at room temperature, urine protein (UP), creatinine (UCr) and sodium (UNa) concentration were determined by the biuret method, alkaline picric acid method, and fluorometry, respectively.

Histological evaluation

A part of the renal cortex was divided into two pieces immediately after nephrectomy; one piece was for light microscopic examination, and the other for

immunofluorescent microscopic examination.

For light microscopy, tissues were fixed in 10% formaldehyde and embedded in paraffin, and 2 μ m-thick sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and periodic acid methenamine silver (PAM).

For histological evaluation by light microscopy, the sections from all the rats were reviewed and classified into the following 3 grades based on qualitative difference: grade 1, mild change; grade 2, moderate change; and grade 3, severe change.

To confirm the reliability of the grading, the evaluation was performed twice by two of the investigators without prior knowledge of experimental information. Histological findings were evaluated as glomerular cell proliferation, polymorphonuclear cell (PMN) exudation and lobulation.

For immunofluorescent microscopy, parts of the renal cortex were frozen in *n*-hexane at -70°C . Three- μ m-thick sections were made by a cryostat and stained by indirect immunofluorescence with anti-ET-1 antibody (1:100 in PBS) (rabbit antiserum to endothelin-1, Organon Teknika NV-Cappel Products, Durham, NC, USA) and ET-3 (1:100 in PBS) (rabbit anti serum to endothelin-3, Cambridge Research Biochemicals, Wilmington, DE, USA). The sections of all rats with nephritis were stained by FITC-conjugated goat anti-rabbit IgG and rabbit anti-rat IgG (1:50 in PBS, respectively) (Wako Pure Chemical). The degree of staining by anti-ET-1, anti-ET-3 antibody, goat anti-rabbit IgG and rabbit anti-rat IgG was graded into the following grades: 0, $\pm$, partial; negative; 1+, mildly positive; 2+, moderately positive; 3+, highly positive. The sites of staining were also evaluated.

Statistical analysis

Each measured value was expressed as mean $\pm$ s.d. Statistical analysis was performed using Student's *t*-test for blood and urine data, and the Mann-Whitney test for histological evaluation. Statistical significance was defined as $p < 0.05$.

RESULTS

Two of the 15 rats in the NT-group and 1 of the 15 rats in the S-1452 group died during the experimental period. Although there was no death in the control group, there was no significant difference in the death rate among the 3 groups. These drop-out rats were excluded from the analysis.

Blood chemistry

Table 1 shows the data of BUN, Cr, Na and TP of the NT and S-1452 groups. BUN and Cr were significantly higher in the NT group than in the control group ($p < 0.05$). There was no significant difference in serum levels of Na and TP among the 3 groups.

TABLE 1. *BUN, Cr, Na and TP after the administration of NTS or physiological saline in the NT, the S-1452, and the control groups*

Group	Number of rats	BUN (mg/100 ml)	Cr (mg/100 ml)	Na (mEq/liter)	TP (g/100 ml)
NT	13	19.6 ± 4.7 ^{a,b}	0.1 ± 0.1 ^b	137.9 ± 8.8	4.7 ± 0.3
S-1452	14	16.0 ± 4.1	0.5 ± 0.2 ^b	136.6 ± 9.0	4.6 ± 0.6
Control	8	16.1 ± 2.9	0.3 ± 0.1	143.8 ± 3.4	5.0 ± 0.2

mean ± s.d.

^a $p < 0.05$ compared with the S-1452 group.^b $p < 0.05$ compared with the control group.*Urinary protein, Cr, Na and urine volume*

Table 2 shows the UP, UCr, UNa and urine volume (UV). The UP in the NT group was significantly higher than in S-1452 group and in the control ($p < 0.05$). The UCr level of the NT group was significantly higher than that of the S-1452 group ($p < 0.05$). UNa was significantly higher in the NT group than in the S-1452 group ($p < 0.05$). There was no significant difference in the UNa between the NT and control groups. No significant difference was found in UV among the 3 groups.

Light microscopic findings

Table 3 shows the grade of histological findings, including glomerular cell proliferation, PMN exudation and lobulation. Light microscopic examination revealed glomerular cell proliferation, exudation of PMNs and monocyte and lobulation in all rats in both the NT and S-1452 groups. Neither widening of the mesangial matrix nor interstitial changes were observed.

The degree of lobulation and glomerular cell proliferation was higher in the NT group than in the S-1452 group ($p < 0.05$, $p < 0.01$, respectively). There was no significant difference in the PMN exudation between the 2 groups. The histological findings of rat No. 8 from the NT group reveal severe glomerular

TABLE 2. *24-hr UP, UV, UCr and UNa after the administration of NTS or physiological saline in the NT, the S-1452, and the control groups*

Group	Number of rats	UP (mg/100 ml)	UV (ml/24 hr)	UCr (mg/100 ml)	UNa (mEq/liter)
NT	13	252.4 ± 82.4 ^a	7.8 ± 3.2	131.1 ± 29.6 ^b	192.9 ± 62.5 ^a
S-1452	14	158.3 ± 89.4	9.5 ± 6.2	63.6 ± 18.1	127.6 ± 41.4
Control	8	118.9 ± 88.4 ^c	8.9 ± 2.4	59.9 ± 20.3	157.1 ± 46.2

mean ± s.d.

^a $p < 0.05$ compared with the S-1452 group.^b $p < 0.05$ compared with the S-1452 and the control group.^c $p < 0.05$ compared with the NT group.

TABLE 3. *Histological grading of glomerular injuries by light microscopy after the administration of NTS in the NT and the S-1452 groups*

Group	Rat No.															Statistical results ^c
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
NT group																
Cellular proliferation	3 ^a	3	3	2	3	3	2	3	3	2	3	— ^b	— ^b	2	2	<0.01
Leukocyte infiltration	1	2	1	1		1	1	3	3	2	3	—	—	2	2	n.s. ^d
Lobulation	2	3	2	1	2	2	1	3	3	1	3	—	—	3	2	<0.05
S-1452 group																
Cellular proliferation	2	2	1	1	2	2	2	1	1	2	2	1	3	1	— ^b	
Leukocyte infiltration	3	1	1	1	1	1	2	1	1	2	2	1	3	2	—	
Lobulation	2	1	1	1	1	2	2	1	1	2	2	1	3	1	—	

^aNumbers and histological gradings: 1, mild change; 2, moderate change; 3, severe change.

^bRats died after the second booster injection.

^cComparison between the NT and the S-1452 group.

^dn.s., Not significant.

Fig. 1. Light microscopic findings of a rat in the NT group. Severe glomerular cell proliferation, PMNs exudation and lobulation were observed (PAS $\times 400$).

endocapillary proliferation with PMN exudation and lobulation (Fig. 1). The histological findings of rat No. 13 from the S-1452 group reveal mild glomerular cell proliferation (Fig. 2). In the control group, which did not receive NTS administration, there were no histological abnormalities 10 days after saline injection.

Fig. 2. Light microscopic findings of a rat in the S-1452 group. Mild glomerular cell proliferation was observed (PAS $\times 400$).

Immunofluorescent microscopic findings

Table 4 shows the degree of staining by anti-ET-1, anti-ET-3, anti-rabbit IgG, and anti-rat IgG antibody examined by immunofluorescent microscopy. ET-1, ET-3, rabbit IgG and rat IgG were stained along the capillary walls and in the mesangial area in both the NT group and S-1452 groups. No obvious staining of ET-1 and ET-3 could be observed in the tubular epithelial cells or vascular endothelial cells in the present study.

Contrary to the result that all rats in the NT group showed positive staining by the anti-ET-1 and anti-ET-3 antibodies, 6 and 5 rats in the S-1452 group

TABLE 4. *Immunofluorescent findings of glomeruli after the administration of NTS in the NT and the S-1452 groups*

Group	Number of rats	ET-1				ET-3				Rabbit IgG				Rat IgG			
		$\pm^a$	+	2+	3+	$\pm$	+	2+	3+	$\pm$	+	2+	3+	$\pm$	+	2+	3+
NT	13	0	5	4	4	0	7	4	2	0	2	10	1	0	10	3	0
S-1452	14	6	6	2	0	5	8	1	0	0	3	11	0	0	12	2	0

^aImmunofluorescent findings: $\pm$, partial staining; +, mildly staining; 2+, moderately staining; 3+, highly staining.

^b $p < 0.01$ comparison between the NT and the S-1452 group in anti ET-1 antibody and anti ET-3 antibody.

^cNot significant.

showed negative staining by anti-ET-1 and anti-ET-3 antibodies, respectively. There were significant differences between the two groups in the degrees of staining by anti-ET-1 and anti-ET-3 antibodies (Figs. 3, 4, 5, 6), respectively. No significant difference in the degree of staining by either anti-rabbit IgG or anti-rat

Fig. 3. Immunofluorescent findings of a rat in the NT group by anti-ET-1 antibody. Highly positive staining along glomerular capillary walls and partial mesangial staining were observed (original magnification $\times 200$).

Fig. 4. Immunofluorescent findings of a rat in the S-1452 group by anti-ET-1 antibody. Mild and segmental staining was observed (original magnification $\times 200$).

Fig. 5. Immunofluorescent findings of a rat in the NT group by anti-ET-3 antibody. Highly positive staining along glomerular capillary walls and partial mesangial staining were observed (original magnification $\times 200$).

Fig. 6. Immunofluorescent findings of a rat in the S-1452 group by anti-ET-3 antibody. Weak staining at glomeruli was observed (original magnification $\times 200$).

IgG was observed between the two groups. There was no obvious staining by anti-ET-1, anti-ET-3, anti-rabbit IgG or anti-rat IgG antibody in the control group. There was no correlation between the degree of staining of ET-1, ET-3, rabbit IgG, and rat IgG.

Staining with anti-rabbit IgG prior to staining with anti-ET-1 and ET-3 did not change the staining pattern or the degree of staining of ET-1 and ET-3.

DISCUSSION

This study suggests that S-1452, a novel TxA₂ receptor antagonist, improved the renal histological changes and lessened the glomerular staining of ET-1 and ET-3. While TxA₂ production was reported to increase in the NTN model, TxA₂ reduced the ultrafiltration coefficient (K_f) of the glomerular capillary and glomerular filtration rate (GFR) in addition to its platelet aggregability (Takahashi et al. 1990). Platelet aggregation induces the release of several inflammatory substances, including PDGF, β -thromboglobulin, platelet factor 4 (Tomura et al. 1982), heparitinase (Wasteson et al. 1976; Knawar and Farquhar 1979) and interleukin-1 (Johnson et al. 1992; Ketteler et al. 1994). The elevated production of TxA₂ probably has a great influence on the glomerular hemodynamics (Scharschmidt et al. 1983; Mene and Dunn 1986; Baylis 1987) and is involved in platelet aggregation for the increase in glomerular inflammation (Hawkins 1972).

We previously reported that thrombocytopenia induced by anti-rabbit goat platelet antibody suppressed renal injury and decreased proteinuria in acute serum sickness nephritis of rabbits (Ideura et al. 1992), and that S-1452 retarded the progression of renal injury in female (NZB $\times$ NZW) F1 mice with spontaneous lupus nephritis and prevented the occurrence of proteinuria in rats with streptozotocin-induced diabetic nephropathy (Matsuo et al. 1995). These findings suggested that TxA₂ has an important role in the pathogenesis of several types of nephritis. S-1452 ameliorated increase of BUN, glomerular cell proliferation, lobulation and proteinuria in the present study. These results further support the role of TxA₂ in nephritis. However, there were no significant differences in serum Cr or glomerular PMN exudation between the NT and S-1452 groups. The glomerular thromboxane synthetase activity of PMNs increases in accelerated nephrotoxic serum nephritis (Wu et al. 1993), and cause glomerular capillary wall damage in combination with the adhesion molecule (intercellular adhesion molecule: ICAM-1) (Kawasaki et al. 1992). In addition, PMNs release a proteolytic enzyme (Hawkins 1972). These findings suggested that factors other than platelets, including PMNs, participate in this nephritis. The inability of the TxA₂ receptor antagonist to completely inhibit renal injury in this study may be attributable to the participation of factors other than platelets, including PMNs.

We successfully showed ET-1 and ET-3 staining along the glomerular capillary walls and at mesangium in this nephritis. S-1452 administration significantly diminished the staining of ET-1 and ET-3. Although ET-like immunoreactivity at the glomeruli can only be graded semiquantitatively, the differences between the control and NT groups and between the NT and S-1452 groups were clear.

ET is a vasoactive amino-acid peptide that has 3 types of isopeptides; ET-1,

ET-2 and ET-3. It has been reported that ET production is localized in the endothelial and epithelial cells of the glomerulus (Koseki et al. 1989; Kasinath et al. 1992; Marsden and Brenner 1992), mesangial cells (Sakamoto et al. 1990; Zoja et al. 1991) and tubular epithelial cells (Ohta et al. 1990) confirmed by ETmRNA appearance or autoradiography in vitro. In addition, the production rate of ET-1 by mesangial cells was high at the time of their proliferation phase and depended on protein kinase C activity, which is important in the regulation of cell proliferation (Sakamoto et al. 1992). We previously reported that glomerular expression of ET-1 increased in human lupus nephritis, especially diffuse proliferative lupus nephritis (Ida et al. 1990). These findings suggested that ET-1 promotes mesangial cell proliferation in an autocrine or paracrine fashion (Sakamoto et al. 1990; Zoja et al. 1991). Recently, ET-3 has received attention in combination with endothelium-derived relaxing factors, which is thought to be the same as nitric oxide (NO) (Yamashita et al. 1991). While ET-1 shows vasoconstrictive and ET-3 vasodilating action, both ET-1 and ET-3 seem to play a role in the progression of this nephritis.

S-1452 lessened ET expression at glomeruli. The correlation between TxA2 and ET was not directly analyzed in this study. However, the TxA2 analogue, thrombin and TGF- β accelerated the appearance of pre-pro ET-1 mRNA and secretion of ET-1 by human mesangial cells (Zoja et al. 1991). ET-1 itself promoted the release of TxA2 from rabbit kidney and spleen (Rao et al. 1989), and the effect of ET-1 on vascular permeability is partly mediated and/or modulated by the secondary release of TxA2 (Sirois et al. 1992). In addition, renal expression of mRNAs for ET-1, ET-3 and ET receptors was reported to increase in NZB/W F1 mice (Nakamura et al. 1993). These findings could indicate that the mechanism by which S-1452 lessened ET expression at glomeruli is attributable to the downregulated synthesis of ETs accompanied by a reduction of TxA2, and that TxA2 correlated with ET has a role in the progression of this nephritis cooperatively or cumulatively. Although the precise mechanism by which ETs act pathophysiologically in this type of nephritis remains to be clarified, the present study provides important and new information about alteration in renal ET expression.

In conclusion, the findings of the present study strongly suggest that TxA2 in combination with ET plays a role in the progression and the pathogenesis of nephrotoxic serum nephritis, while S-1452, a novel TxA2 receptor antagonist, prevents the renal injury significantly, but not completely. Further study, including investigation of the availability of specific endothelin receptor antagonist or inhibitor may clarify the pathogenesis of glomerulonephritis.

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