Infusion of angiotensin II reduces loss of glomerular capillary area in the early phase of anti-Thy-1.1 nephritis possibly via regulating angiogenesis-associated factors

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Infusion of angiotensin II reduces loss of glomerular capillary area in the early phase of anti-Thy-1.1 nephritis possibly via regulating angiogenesis-associated factors.

Background. Although angiotensin II (Ang II) is involved in the progression of renal diseases, infusion of Ang II was reported to surprisingly ameliorate the early phase of anti-Thy-1.1 nephritis (Wenzel et al, *Kidney Int* 61:1020, 2002). Considering the known proangiogenic effect of Ang II and that angiogenic glomerular capillary repair is required for the recovery of damaged glomeruli in rat anti-Thy-1.1 nephritis, we hypothesized that Ang II infusion starting prior to the initiation of nephritis may induce the expression of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1), resulting in the increased glomerular capillary area in the early phase.

Methods. Ang II was infused (170 ng/min) in rats, and 5 days later, nephritis was induced by the administration of monoclonal 1-22-3 antibodies. Ang II type 1 or type 2 receptor antagonist (AT_1R or AT_2R, respectively) (losartan or PD123319, respectively) was coadministered.

Results. Ang II infusion affected on neither the deposition of Ig nor mesangiolysis in the initial phase, and resulted in the aggravation of creatinine clearance at day 14 and 35 after initiating anti-Thy-1.1 nephritis. Histologic alterations were ameliorated accompanied by reduced loss in rat endothelial cell antigen (RECA)-1(+) endothelial area in Ang II–infused nephritic rats on day 6 and 14 as compared to control nephritic group, and nephritic alterations were mostly resolved on day 35 in both groups. At the early stage (day 6), glomerular expression of VEGF and receptors flk-1 and flt-1 as well as Ang-1, and receptor Tie2 were increased, and glomerular monocyte infiltration and the expression of angiopoietin-2 (Ang-2), a natural antagonist of Ang-1, were reduced. Both Ang II receptors were involved in the regulation of angiogenic factors and receptors.

Conclusion. These results demonstrate that infusion of exogenous Ang II starting prior to the induction of nephritis activates VEGF and Ang-1 signaling regulated via both Ang II receptors, potentially leading to the accelerated recovery of injured glomerular endothelial cells in the early phase of anti-Thy-1.1 nephritis. Increased expression of VEGF and Ang-1 on podocytes further suggests the crucial association of endothelial cells and podocytes in maintaining proper glomerular capillary structures.

There are two major receptor isoforms for angiotensin II (Ang II), type 1 receptor (AT_1R) and type 2 receptor (AT_2R) [1]. AT_1Rs are expressed in afferent and efferent arterioles, glomeruli, and proximal tubules in normal animals and humans [2]. The known biologic functions of Ang II are exclusively mediated via AT_1R according to studies employing receptor-selective antagonists [1]. The mechanisms of Ang II–induced renal injuries are generally attributed to vasoconstriction, growth promoting as well as profibrotic effect leading to excess accumulation of extracellular matrix (ECM). Experimental and clinical studies using AT_1R antagonist (AT_1RA) or $AT_{1a}R$–deficient mice indicated the critical involvement of AT_1R in progressive renal disorders [3, 4].

Previous studies demonstrated the histologic abnormalities in renal arteries and arterioles accompanied by the thickening of vessel walls similar to hypertensive nephrosclerosis in angiotensinogen-deficient mice [5] or in AT_{1a} and $AT_{1b}R$ double knockout mice [6–8]. Blockade of AT_1R signal in peri- and neonatal period of spontaneously hypertensive rats (SHRs) results in the formation of vascular lesions similar to malignant hypertension [9]. In addition, activation of the renin-angiotensin system (RAS) might possess beneficial effects on kidney in certain pathophysiologic conditions.

For instance, blockade of the RAS resulted in exacerbated renal interstitial fibrosis of the stenosed kidney in a renal artery stenosis model [10]. A salutary role for Ang II was also demonstrated in a model of partial urinary tract obstruction [11]. These studies thus suggest the distinct physiologic role of the RAS in the development and maintenance of kidney apart from its known pathologic functions.

The proliferation of mesangial cells and the expansion of mesangial matrix are central features in various types of glomerular disorders [12, 13]. Anti-Thy-1.1 nephritis is one of the widely used models of mesangioproliferative glomerulonephritis [14]. Murine monoclonal 1-22-3 antibody binds to the mesangial cell surface and causes proteinuria and histologic alterations characterized by mesangiolysis followed by monocyte infiltration, mesangial cell proliferation, and accumulation of mesangial matrix [15]. Pharmacologic blockade of the RAS ameliorated the pathologic changes of the anti-Thy-1.1 nephritis [16, 17]. However, recent study by Wenzel et al [18] demonstrated that Ang II infusion starting 5 days prior to the induction of anti-Thy-1.1 nephritis surprisingly resulted in the amelioration of nephritic changes. Ang II infusion induced a significant reduction in glomerular monocyte infiltration, cell proliferation, and matrix expansion in nephritic rats compared to nephritic rats without Ang II infusion at 5 days after the induction of nephritis. In Ang II–infused nephritic rats, expression of monocyte chemotactic protein-1 (MCP-1) was inhibited and the glomerular expression of cell cycle inhibitor $p27^{kip1}$ and of transforming growth factor-β (TGF-β) was increased, potentially resulting in the amelioration of early nephritic changes in this model [18].

Angiogenesis, the development of new blood vessels from preexisting ones, is involved in physiologic events as well as pathologic disorders, including tumor growth and diabetic retinopathy [19]. Vascular endothelial growth factor (VEGF) promotes endothelial cell proliferation, migration, and tube formation and thus serves as a potent inducer of angiogenesis [20]. VEGF also induces vascular permeability and endothelium-dependent vasodilatation in association with endothelium-derived nitric oxide [21]. In the early phase of anti-Thy-1.1 nephritis following mesangiolysis, reduction in the number of glomerular endothelial cells is observed, followed by the angiogenic glomerular capillary repair process [22, 23]. The essential role of VEGF in promoting glomerular capillary repair in anti-Thy-1.1 nephritis has been reported utilizing $VEGF_{165}$ aptamer [24]. Angiopoietin-1 (Ang-1), a major physiologic ligand for Tie2 receptor, is responsible for the recruitment and stable attachment of pericytes leading to the maturation of newly formed blood vessels [25]. Platelet-derived growth factor (PDGF) and TGF-β are also known to play crucial roles in promoting pericyte attachment [26, 27]. In contrast, angiopoietin-2 (Ang-2),

a natural antagonist of Ang-1, loosens the attachment of pericytes rendering blood vessel to be "unstable" and resulting in promoting sprouting angiogenesis in the presence of VEGF [28]. During kidney development, Ang-1, Ang-2, and Tie2 are highly expressed and play pivotal roles in the maturation of glomeruli and renal blood vessels [29].

Recent studies have suggested that AT_1R-mediated signaling induced angiogenesis in hind limb ischemia or tumor models using $AT_{1a}R$-deficient mice [30, 31]. Based on the capacity of Ang II to induce angiogenic changes, we examined if glomerular capillary area is increased in the early phase of anti-Thy-1.1 nephritis by infusion of Ang II initiating 5 days prior to the induction of nephritis, similar to the experiments reported by Wenzel et al [18]. Since we observed increased glomerular endothelial area in Ang II–infused nephritic rats as compared to nephritic rats without Ang II infusion, we further investigated on the level of angiogenesis-associated factors, VEGF and angiopoietins as well as their receptors. Additionally, the involvement of AT_1R- and AT_2R-mediated signal in this regulatory mechanism was investigated using receptor selective antagonists. Coordinated regulation of angiogenesis-associated factors by Ang II may potentially lead to the accelerated recovery of glomerular endothelial cells in the early phase of anti-Thy-1.1 nephritis model, which is accompanied by glomerular capillary injury and loss following mesangiolysis [22]. In addition, glomerular injuries were not exacerbated at the "resolution" phase of anti-Thy-1.1 nephritis in Ang II–infused rats in spite of persistent hypertension, suggesting the importance of accelerated glomerular capillary repair in the early stage in this model.

METHODS

Experimental protocol

The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences. Studies were performed in male Wistar rats (CLEA Japan, Tokyo, Japan). The animals had free access to tap water and standard rat chow. Rats weighing 170 g received continuous infusion of Ang II (170 ng/min) (Sigma Chemical Co., St. Louis, MO, USA) via subcutaneous osmotic minipumps (Alzet model 2002) (Alza Corp., Palo Alto, CA, USA).

Immune-mediated mesangial cell injury was induced by the intravenous injection of monoclonal antibody 1-22-3 (1 mg/rat in 0.5 mL of saline) 5 days after the implantation of minipumps. On day 6, 14, or 35 after the injection of monoclonal antibody 1-22-3, animals were sacrificed.

In another series of experiments, the following groups of animals were studied on day 6 after initiating nephritis: (*1*) normotensive control, (*2*) normotensive nephritic

control, (3) Ang II infusion, (4) Ang II infusion with nephritis, (5) Ang II infusion with nephritis and losartan (30 mg/kg/day by the drinking water) (Merck, Rahway, NJ, USA), (6) Ang II infusion with nephritis and PD123319 (0.935 µg/min osmotic minipump) (Sigma Chemical Co.), and (7) norepinephrine (600 ng/min) (Sigma Chemical Co.) infusion with nephritis ($N = 5$ for each subgroup). No rats have died during the experimental period. Additionally, some rats were sacrificed at 24 hours following the initiation of anti-Thy-1.1 nephritis to examine the possible effect of Ang II infusion in the initial phase of nephritis.

Blood and urine examination

Blood urea nitrogen (BUN), serum and urinary creatinine levels, and urinary total protein levels were measured by SRL, Inc. (Okayama, Japan). Serum and urinary creatinine levels were measured by the enzymatic colorimetric method as described [32]. Urinary total protein concentration was measured by standard Pyrogallol red method using MicroTP-AR (Wako Pure Chemical, Osaka, Japan). The creatinine clearance was calculated and expressed as milliliters per minute.

Systolic blood pressure

Arterial blood pressure was measured before the implantation of the osmotic minipumps, induction of the nephritis and sacrifice using a programmable sphygmomanometer (BP-98A) (Softron, Tokyo, Japan) by the tail-cuff method as described previously [4, 33].

Immunoblot

Immunoblot was performed as previously described with modifications [18, 34, 35]. Kidney sample was obtained from an individual rat, and five kidneys per group were employed for analysis by immunoblots. Briefly, glomeruli were isolated by differential sieving technique [14] from each kidney, and were resuspended in Laemmli buffer 1 containing 50 mmol/L Tris-Cl, pH 6.8, and 10% sodium dodecyl sulfate (SDS). Then, samples were boiled for 3 minutes and centrifuged at 14,000 rpm for 10 minutes at 4°C. Supernatant was collected and total protein concentration was determined by using the DC-protein determination system (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. To equal amounts of protein (100 µg), Laemmli buffer 2 containing dithiothreitol (DTT) (Sigma Chemical Co.), bromphenol blue (BPB) and glycerol was added to make the final concentration of DTT to be 100 µmol/L, that of BPB and glycerol to be 0.1% and 10%, respectively. Then, samples were denatured at 100°C for 7 minutes, and separated on SDS-polyacrylamide gels (Bio-Rad). Recombinant rat VEGF$_{164}$, human Ang-1 and human Ang-2 (R&D Systems, Minneapolis, MN, USA) were also applied. Samples

were then electrotransferred onto nitrocellulose membranes (Hybond-ECL) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were blocked with 5% nonfat dry milk in $1\times$ Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), incubated overnight with rabbit polyclonal anti-Ang-1 (1:1000) (Alpha Diagnostics, San Antonio, TX, USA), goat polyclonal anti-Ang-2 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-VEGF (1:1000) (Santa Cruz Biotechnology), rabbit polyclonal anti-flk-1 (1:100) (Santa Cruz Biotechnology), rabbit polyclonal anti-flt-1 (1:1000) (Santa Cruz Biotechnology) or rabbit polyclonal antirat tie2 (1:1000) (kindly provided by Regeneron Pharmaceuticals, Tarrytown, NY, USA) antibodies at 4°C. After incubation with horseradish peroxidase (HRP)-labeled-secondary antibodies for 1 hour, signals were detected with enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Membranes were re-probed with rabbit polyclonal antiactin antibodies (Bio-Rad) to serve as controls for equal loading. The density of each band was determined by using NIH image software, and expressed as a value relative to the density of the corresponding band obtained from the actin immunoblot.

Histologic analysis

At 1, 6, 14, and 35 days after the induction of nephritis, kidneys were removed, fixed in 10% buffered formalin and embedded in paraffin. Sections (3 µm thick) were stained with periodic acid-Schiff (PAS) for light microscopic observation. Total glomerular cell number was determined by counting the nuclei within the glomerular tuft as described [14]. Expansion of the mesangial matrix was quantitated by scoring (grade 0 to 4) using PAS-stained sections: 0, none; +1, mesangial matrix expansion observed in less than 25% of the glomerulus; +2, 25% to 50%; +3, 50% to 75%; and +4, greater than 75%. Mesangiolysis (day 1 after initiating nephritis) was quantitated by scoring (grade 0 to 3) using PAS-stained sections as previously described [24]: 0, no mesangiolysis; +1, segmental mesangiolysis; +2, global mesangiolysis; and +3, microaneurysm. In each kidney, more than 30 glomerular cross-sections were examined by two investigators and averaged. Histologic assessment was performed by the investigators in a blinded fashion.

Immunohistochemistry

Immunohistochemistry was performed as previously described [14, 33, 36]. For immunohistochemistry of infiltrating monocytes/macrophages, VEGF and Ang-1, paraffin-embedded sections (3 µm) were used. Sections were incubated with mouse monoclonal antibody to rat monocytes/macrophages (clone ED1) (Cymbus Biotechnology, Hants, UK), rabbit polyclonal antihuman VEGF
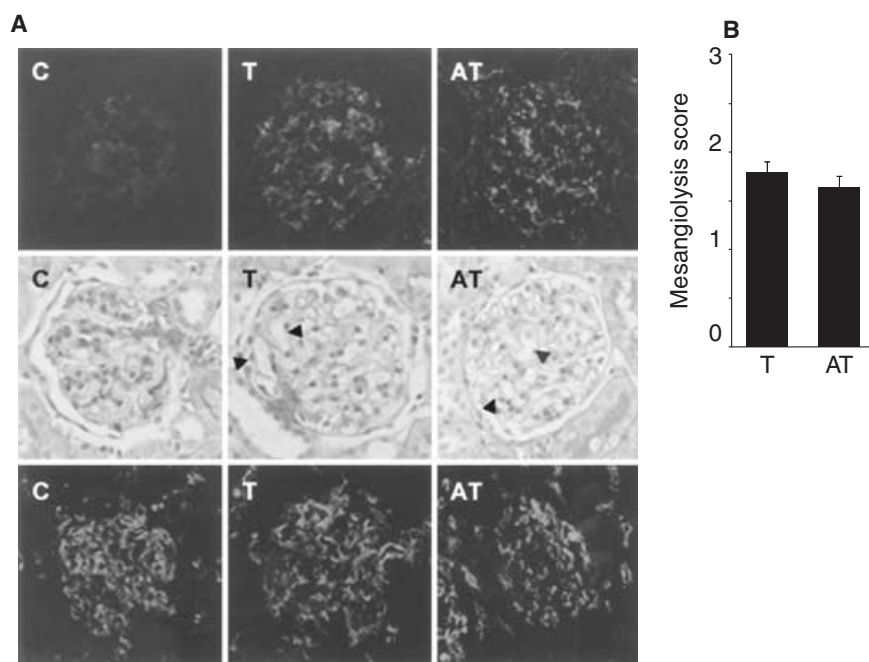
or anti-Ang-1 antibody followed by incubation with biotinylated secondary antibody, and immunoperoxidase staining was carried out utilizing the Vectastain ABC Elite Reagent Kit (Vector Laboratory, Burlingame, CA, USA) [14]. Diaminobenzidine (DAB) was used as a chromogen. All slides were counterstained with hematoxylin. Normal mouse or rabbit IgG was used as a negative control.

Immunofluorescence staining for mouse Ig, rat endothelial cell antigen (RECA)-1, and von Willebrand factor (vWF) was performed using frozen sections. Four micrometer sections were fixed in ethanol, and incubated with mouse monoclonal anti-RECA-1 antibody (Serotec, Oxford, UK) or rabbit polyclonal anti-vWF antibody (Dako, Carpenteria, CA, USA) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Frozen sections were incubated with FITC-conjugated antimouse Ig antibody (Chemicon, Temecula, CA, USA) to detect the binding of monoclonal antibody 1-22-3. Sections were observed by a confocal laser fluorescence microscope (LSM-510) (Carl Zeiss, Jena, Germany) and obtained image files were then analyzed using Lumina Vision software (Mitani, Fukui, Japan) to determine the RECA-1(+) area and vWF(+) area within glomeruli. In each kidney, more than 20 glomerular cross-sections were examined and averaged.

Statistical analysis

All values are expressed as mean ± SEM. A Kruskal-Wallis test with post hoc multiple comparisons using the Scheffe's test were employed for intergroup comparisons of clinical parameters and multiple variables. Statistical analysis was performed by StatView software (Abacus Concepts, Berkeley, CA, USA). Statistical significance is presented with P values by a Kruskal-Wallis test representing overall effect, and P values for post hoc multiple comparisons between groups by Scheffe's test. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Ang II infusion did not affect on the initial phase of anti-Thy-1.1 nephritis

Initial histologic changes following the administration of monoclonal antibody 1-22-3 were examined. At 24 hours after the induction of nephritis, glomerular deposition of mouse Ig was indistinguishable between rats pretreated with Ang II or untreated nephritic control rats (Fig. 1A). Control nonnephritic kidney section exhibited no deposition of mouse Ig. Histologic examination of the kidneys revealed marked mesangiolysis at 24 hours after the induction of nephritis, and the extent of mesangiolysis was indistinguishable between rats pretreated with Ang II or untreated nephritic control rats, suggesting that Ang II infusion prior to the induction of nephritis did not alter the initial phase of anti-Thy-1.1 nephritis (Fig. 1). Similar extent of glomerular endothelial RECA-1(+) area was observed in Ang II or nephrotic controls and both groups exhibited slightly diminished appearance as compared to normal control (Fig. 1A).

Table 1. Laboratory data of blood and urine samples (time course)

	Undiseased control	Anti-Thy-1.1 nephritis			Anti-Thy-1.1 nephritis with A-II-infusion		
		Day 6	Day 14	Day 35	Day 6	Day 14	Day 35
Blood urea nitrogen *mg/dL*[d]	17.96 ± 0.50	37.88 ± 8.01	25.64 ± 2.09	22.53 ± 1.59	56.38 ± 3.40[a]	48.98 ± 8.71	36.90 ± 5.50
Serum creatinine *mg/dL*[e]	0.22 ± 0.01	0.44 ± 0.05	0.33 ± 0.01	0.30 ± 0.01	0.60 ± 0.05[a]	0.61 ± 0.12[a]	0.53 ± 0.05[a]
Creatinine clearance *mL/min*[f]	2.44 ± 0.22	1.07 ± 0.10[a]	2.39 ± 0.30[b]	2.83 ± 0.04[b]	0.56 ± 0.06[a]	0.98 ± 0.31[a,c]	0.91 ± 0.26[a,c]
Daily urinary protein *mg/24hours*[g]	15.49 ± 0.68	85.42 ± 7.30[a]	53.58 ± 9.67	20.39 ± 0.64[b]	76.63 ± 9.58[a]	85.87 ± 17.73[a]	52.01 ± 5.72

Values are mean ± SEM. Urine samples were collected by placing rats in metabolic cages for 24 hours, $N = 5$ for each group. Laboratory data on day 6, 14, and 35 after the initiation of nephritis are shown. [a]$P < 0.05$ vs. undiseased control; [b]$P < 0.05$ vs. anti-Thy-1.1 nephritis on day 6; [c]$P < 0.05$ vs. anti-Thy-1.1 nephritis at the same time points; [d]$P = 0.0001$; [e]$P = 0.0001$; [f]$P = 0.0001$; [g]$P = 0.0001$; Kruskal-Wallis test.

Renal function and histologic alterations in time-course experiment

On day 6 after initiating nephritis, renal dysfunction and proteinuria were observed in anti-Thy-1.1 nephritis group, and Ang II infusion exacerbated renal dysfunction and diminished proteinuria without reaching to statistical significance (Table 1). Improvement of renal function and proteinuria was observed in anti-Thy-1.1 nephritis group on day 14. Although renal function of Ang II–infused nephritic group tended to be improved on day 14, creatinine clearance was significantly decreased as compared to nephritis group at this time point. On day 35 at the resolution phase of nephritis, renal function and proteinuria were markedly improved in anti-Thy-1.1 nephritis group close to the level of undiseased control. Although renal dysfunction and proteinuria of Ang II–infused nephritic group tended to be improved on day 35, creatinine clearance was still significantly decreased as compared to nephritis group at this time point. Ang II–infused nephritic animals showed comparable elevation of blood pressure on day 6, 14, or 35 after initiating nephritis (data not shown).

Control nephritic animals exhibited marked mesangial cell proliferation and matrix expansion at day 6 and 14 after initiating nephritis. These nephritic alterations were significantly diminished on day 35 (Fig. 2A, C, and D). Infusion of Ang II markedly ameliorated these alterations at day 6 after initiating nephritis as compared to control nephritic animals in consistent with the previous report [18]. On day 14, Ang II–infused nephritic rats exhibited further amelioration of histologic alterations as compared to control nephritic rats. At this point, histologic alterations in Ang II–infused nephritic rats were similar to those observed on day 35. There were no significant statistical difference of histologic alterations between Ang II–infused and control nephritic animals on day 35, both groups showing the appearance of "healing" of anti-Thy-1.1 nephritis.

We next examined the changes in glomerular endothelial capillary area by immunohistochemistry with anti-RECA-1 antibody. On day 6 after initiating nephritis, glomerular RECA-1(+) endothelial area was markedly reduced in control nephritic animals suggesting insuffi-

cient endothelial cell repair (Fig. 2B and E) in contrast to Ang II–infused nephritic rats with significant suppression of the loss in glomerular RECA-1(+) area. On day 14, glomerular RECA-1(+) area was still greater in Ang II–infused nephritic animals, and it returned to be equivalent in both groups on day 35, close to the level of undiseased control animals.

Changes in blood pressure

Next, we examined the effect of Ang II infusion at day 6 after initiating anti-Thy-1.1 nephritis to further characterize the influence at relatively early stage of nephritis. In order to assess the specific effect of Ang II independent of elevated blood pressure, infusion of norepinephrine was performed on a distinct group of rats followed by the induction of nephritis. Infusion of Ang II or norepinephrine resulted in increased arterial systolic blood pressure at the point of initiation of nephritis. Systolic blood pressure was not significantly different between control and nephritic rats on day 6 after the initiation of nephritis (Fig. 3B). Ang II–infused nephritic rats exhibited significantly elevated systolic blood pressure compared to nephritic control rats. Treatment with losartan inhibited the increase of systolic blood pressure induced by Ang II infusion in nephritic rats in contrast to PD123319 not showing such an antihypertensive effect. There was no significant difference in body weight among each group (data not shown).

Renal function and histologic alterations (day 6)

In Ang II–infused nonnephritic animals, deterioration of renal function was observed (Table 2) in consistent with previous report showing decrease of creatinine clearance following Ang II infusion [37]. Proteinuria was not evident in Ang II–infused nonnephritic animals in contrast to the previous report [37] possibly due to the difference in animal strain (Wistar rat instead of Sprague-Dawley rat), dosage, or the extended time interval in the present study. Renal dysfunction of norepinephrine-infused nephritic rats was similar to that of anti-Thy-1.1 nephritis group, but exhibited increased proteinuria compared with anti-Thy-1.1 nephritis or Ang II–infused
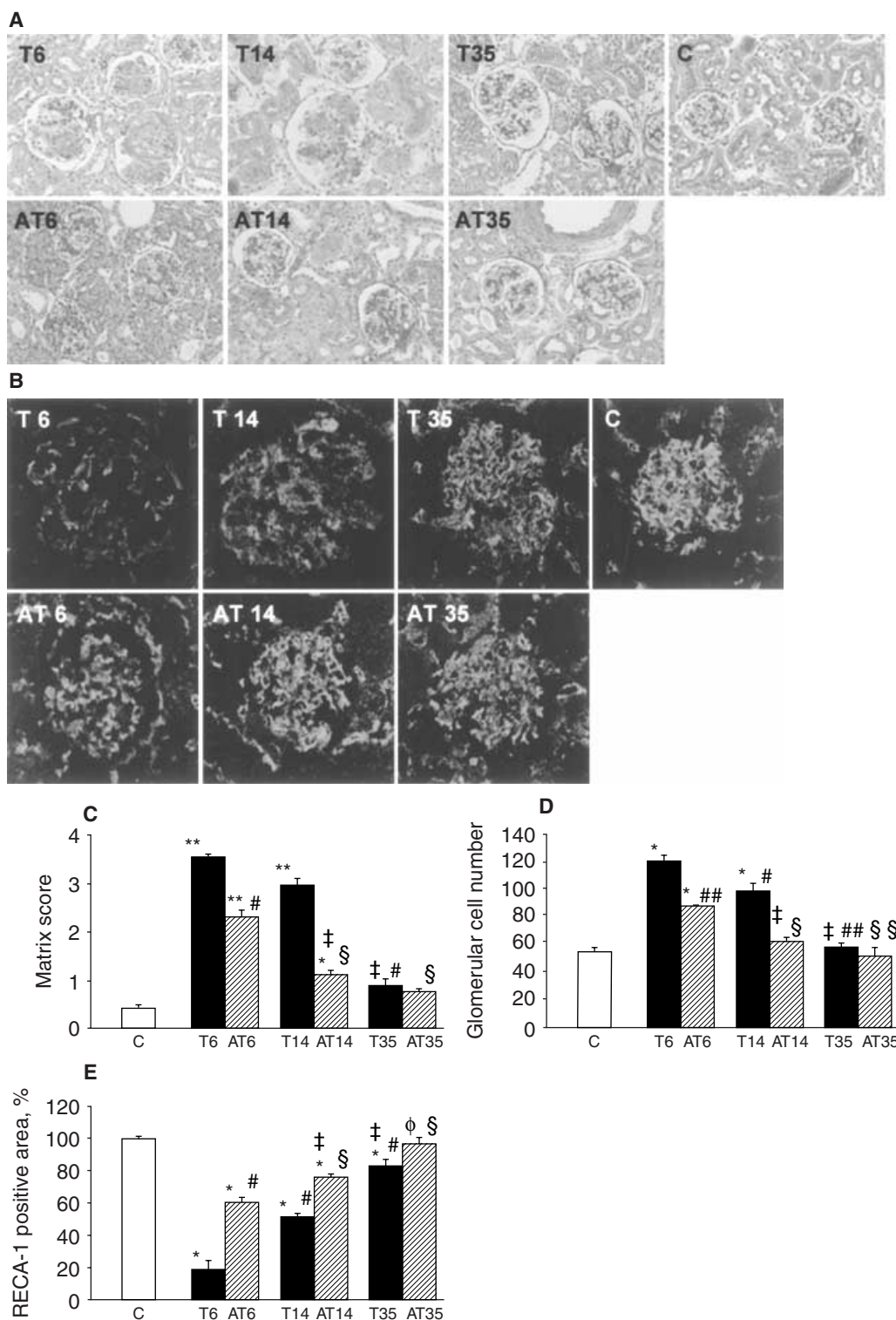
Fig. 2. Angiotensin II (Ang II) infusion ameliorated histologic alterations in anti-Thy-1.1 nephritis without affecting the healing process of nephritis. (*A*) Representative light microscopic appearance of glomeruli [periodic acid-Schiff (PAS) staining (original magnification 200×)]. (*B*) Immunofluorescent staining of glomerular endothelial cells (RECA-1) (original magnification 200×). (*C*) Matrix score. Control (C) $P = 0.012$ by Kruskal-Wallis test; $^*P < 0.05$ vs. C; $^{**}P < 0.01$ vs. C; $^#P < 0.05$ vs. T6; $^§P < 0.01$ vs. AT6; $^‡P < 0.01$ vs. T14 ($N = 5$ for each group). (*D*) Total glomerular cell number per glomerular cross section. Control (C) $P = 0.0054$ by Kruskal-Wallis test; $^*P < 0.01$ vs. C; $^#P < 0.05$ vs. T6; $^{##}P < 0.01$ vs. T6; $^§P < 0.05$ vs. AT6; $^§P < 0.01$ vs. AT6; $^‡P < 0.01$ vs. T14 ($N = 5$ for each group). (*E*) Relative ratio of glomerular RECA-1(+) area. Control (C) $P < 0.0001$ by Kruskal-Wallis test; $^*P < 0.01$ vs. C; $^#P < 0.01$ vs. T6; $^{§§}P < 0.01$ vs. AT6; $^‡P < 0.01$ vs. T14; $^ΦP < 0.01$ vs. AT14 or T35 ($N = 5$ for each group). All were determined as described in the **Methods** section. Abbreviations are: C, undiseased control; T6, T14, and T35, anti-Thy-1.1 nephritis on day 6, 14, and 35 after induction of nephritis, respectively; AT6, AT14, and AT35, anti-Thy-1.1 nephritis with Ang II infusion on day 6, 14, and 35 after induction of nephritis, respectively. Each column consists of mean ± SEM.
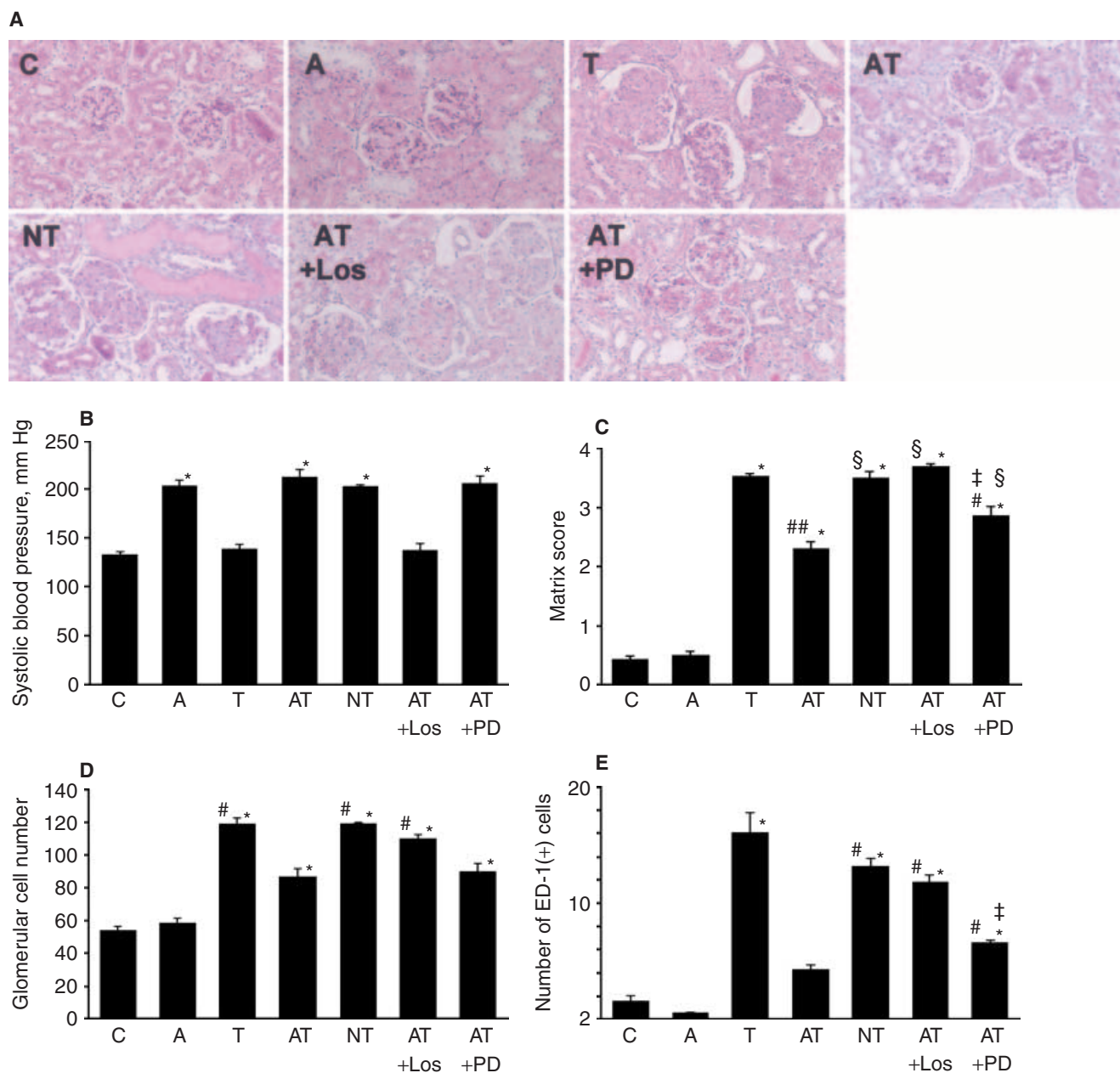
A

B

C

D

E

Fig. 3. Angiotensin II (Ang II) infusion ameliorated histologic alterations and the infiltration of monocyte/macrophage in anti-Thy-1.1 nephritis mainly mediated via angiotensin type 1 receptor (AT$_1$R). (*A*) Representative light microscopic appearance of glomeruli [periodic acid-Schiff (PAS) staining (original magnification 200×)]. (*B*) Systolic blood pressure in experimental groups. Systolic blood pressure in conscious rats was measured on day 6 after induction of nephritis. Control (C) $P = 0.036$ by Kruskal-Wallis test; *$P < 0.05$ vs. C, T, or AT + Los. (*C*) Matrix score. Control (C) $P = 0.0004$ by Kruskal-Wallis test; *$P < 0.01$ vs. C or A; #$P < 0.05$ vs. T; ##$P < 0.01$ vs. T; §$P < 0.01$ vs. AT; ‡$P < 0.05$ vs. AT or AT + Los. (*D*) Total glomerular cell number per glomerular cross section. Control (C) $P = 0.0044$ by Kruskal-Wallis test; *$P < 0.05$ vs. C or A; #$P < 0.05$ vs. AT or AT + PD ($N = 5$ for each group). All were determined as described in the **Methods** section. (*E*) Number of mean glomerular ED-1(+) cells per glomerular cross-section. Control (C) $P = 0.002$ by Kruskal-Wallis test; *$P < 0.05$ vs. C, A, or AT; #$P < 0.05$ vs. T; ‡$P < 0.05$ vs. NT or AT + Los ($N = 5$ for each group). Abbreviations are: C, undiseased control, A, Ang II–infused; T, anti-Thy-1.1 nephritis; AT, anti-Thy-1.1 nephritis with Ang II infusion; NT, anti-Thy-1.1 nephritis with norepinephrine infusion; Los, losartan; PD, PD123319. Each column consists of mean ± SEM.

anti-Thy-1.1 nephritis group. Treatment with losartan resulted in significant improvement of renal dysfunction and proteinuria as compared to Ang II–infused anti-Thy-1.1 nephritis group, but treatment with PD123319 failed to show such remarkable therapeutic effects.

Histologic alterations characterized by mesangial cell proliferation and matrix expansion were not improved in norepinephrine-infused nephritic animals in contrast to Ang II–infused nephritic rats (glomerular cell number, undiseased control, 53.6 ± 2.9; anti-Thy-1.1 nephritis,

Table 2. Laboratory data of blood and urine samples from rat

	Day 6						
	Control	Ang II	Thy-1	Ang II+Thy-1	Norepinephrine + Thy-1	Anti-Thy-1.1 nephritis with Ang II-infusion + losartan	Anti-Thy-1.1 nephritis with Ang II-infusion + PD123319
Blood urea nitrogen mg/dL[e]	17.96 ± 0.50	30.18 ± 6.49	37.88 ± 8.01	56.38 ± 3.40[a]	33.56 ± 5.25	20.26 ± 0.72[b]	51.00 ± 12.15[a,c]
Serum creatinine mg/dL[f]	0.22 ± 0.01	0.34 ± 0.05	0.44 ± 0.05	0.60 ± 0.05[a]	0.35 ± 0.03	0.25 ± 0.01[b]	0.49 ± 0.09
Creatinine clearance mL/mn[g]	2.44 ± 0.22	1.16 ± 0.19	1.07 ± 0.10[a]	0.56 ± 0.06[a]	1.29 ± 0.07	1.95 ± 0.14[b]	0.80 ± 0.34[a]
Daily urinary protein mg/24hours[h]	15.49 ± 0.68	11.81 ± 2.88[a]	85.42 ± 7.30[a,d]	76.63 ± 9.58[a,d]	145.61 ± 17.69[a,b]	43.00 ± 8.51[b]	70.50 ± 15.37[a]

Values are mean $\pm$ SEM. Urinary samples were collected by placing rats in metabolic cages for 24 hours ($N = 5$ for each group). Data on day 6 after the initiation of nephritis are shown.

[a]$P < 0.05$ vs. control; [b]$P < 0.05$ vs. Ang II + Thy-1; [c]$P < 0.05$ vs. Anti-Thy-1.1 nephritis with Ang II-infusion + losartan; [d]$P < 0.05$ vs. Ang II; [e]$P = 0.0001$; [f]$P = 0.000$; [g]$P = 0.0001$; [h]$P = 0.0001$; by Kruskal Wallis test.

118.5 ± 3.8; nephritis with norepinephrine infusion, 119.0 ± 1.3; nephritis with Ang II infusion, 86.4 ± 5.5; matrix index undiseased control, 0.42 ± 0.07; anti-Thy-1.1 nephritis, 3.52 ± 0.06; nephritis with norepinephrine infusion, 3.49 ± 0.12; nephritis with Ang II infusion, 2.30 ± 0.12) (Fig. 3A, C, and D). Although treatment with losartan in addition to Ang II infusion exacerbated nephritic alterations (glomerular cell number 109.7 ± 2.9, matrix index; 3.68 ± 0.05, losartan), PD123319-treatment showed suppressive effect (glomerular cell number 89.2 ± 5.2, matrix index; 2.87 ± 0.15, PD123319), suggesting that antinephritic effect was mediated mainly via AT_1R, and partially via AT_2R.

### Glomerular accumulation of monocytes/macrophages

We next examined glomerular infiltration by monocytes/macrophages since it is a hallmark of mesangioproliferative glomerulonephritis [38]. Marked glomerular accumulation of ED-1–positive monocyte/macrophage was observed in nephritic rats (Fig. 3E). Infusion of Ang II significantly decreased the influx of monocyte/macrophage, although norepinephrine infusion did not show such inhibitory effect (control 1.6 ± 0.4, nephritis 16.0 ± 1.8, nephritis with norepinephrine infusion 13.2 ± 0.7, and nephritis with Ang II infusion 4.3 ± 0.4). Suppression of monocyte/macrophage recruitment following

Ang II infusion was inhibited by losartan, but not by PD123319 (losartan 11.8 ± 0.7 and PD123319 6.6 ± 0.2), suggesting that the inhibitory effect on monocyte accumulation was mainly mediated via AT_1R, and partially via AT_2R.

Ang II suppresses the loss of glomerular capillary area dependent on both AT_1R and AT_2R in the early phase of anti-Thy-1.1 nephritis

The specific effect of Ang II to suppress the loss in glomerular endothelial capillary area was determined by immunohistochemical detection of RECA-1. Although Ang II infusion resulted in the suppression of the loss in glomerular endothelial area in nephritic animals on day 6, norepinephrine infusion failed to show such an effect. The effect of Ang II infusion on maintaining glomerular endothelial area was equally suppressed by losartan and by PD123319 (Fig. 4A and B). To further support the effect of Ang II infusion in suppressing the loss of glomerular capillary area in the early phase of anti-Thy-1.1 nephritis, we also examined immunostaining for vWF [39]. Immunoreactivity for vWF was dominantly observed in glomerular capillary area in normal control and Ang II–infused control rats. Immunoreactivity for vWF was maintained in Ang II–infused nephritic rats in parallel with results observed in RECA-1 immunostaining

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Fig. 4. Angiotensin II (Ang II) induces glomerular capillary repair through angiotensin type 1 and type 2 receptor (AT_1R and AT_2R). (*A*) Immunofluorescence staining of glomerular endothelial cells (RECA-1). Glomerular endothelial cell damage and recovery were assessed by indirect immunofluorescence method using anti-RECA-1 antibody as described in the **Methods** section. (*B*) Glomerular RECA-1(+) area was determined by using image analyzer (Lumina Vision) as described in the **Methods** section. Relative ratio of glomerular RECA-1(+) area. Control (C) $P = 0.0087$ by Kruskal-Wallis test; *$P < 0.05$ vs. C or A; #$P < 0.05$ vs. AT. (*C*) Immunofluorescence staining of glomerular endothelial cells [von Willebrand factor (vWF)]. Immunofluorescence staining was performed using anti-vWF antibody as described in the **Methods** section. (*D*) Glomerular vWF-positive area was determined by using image analyzer (Lumina Vision) as described in the **Methods** section. Relative ratio of glomerular vWF-positive area. Control (C) $P = 0.0002$ by Kruskal-Wallis test; *$P < 0.01$ vs. C, A, or AT. Each column consists of mean $\pm$ SEM ($N = 5$ for each group). Abbreviations are: C, undiseased control; A, Ang II–infused; T, anti-Thy-1.1 nephritis; AT, anti-Thy-1.1 nephritis with Ang II infusion; NT, anti-Thy-1.1 nephritis with norepinephrine infusion; Los, losartan; PD: PD123319 (original magnification 400×).

A

B

C

D
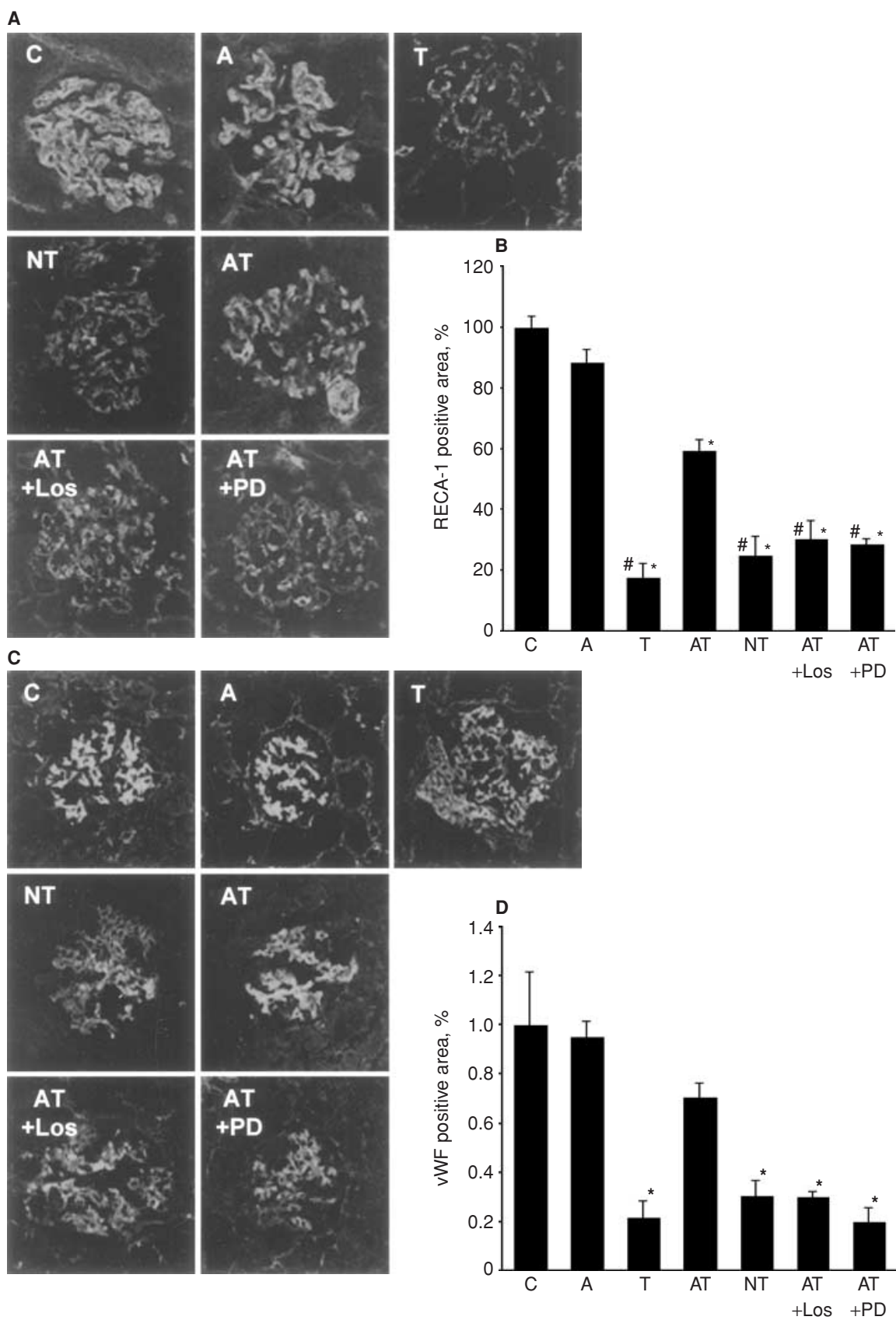
(Fig. 4C and D). Suppressive effect of Ang II infusion on the loss in vWF immunoreactivity was inhibited by treatment with losartan or PD123319. Combining these results obtained from RECA-1 and vWF immunostaining, the effect of Ang II in suppressing the loss in glomerular capillary area was considered to be mediated via both AT_1R and AT_2R.

Immunohistochemical analysis of glomerular VEGF expression

We next examined glomerular expression of angiogenesis-associated factors, VEGF and Ang-1, considering the effect of Ang II to increase glomerular endothelial cell area potentially through regulating the expression of these factors. In glomeruli of undiseased normal rats, VEGF was detected on glomerular capillaries, most likely on podocytes based on the distribution pattern (Fig. 5A). In nephritic control rats, VEGF was observed in mesangial area and on podocytes, and Ang II infusion further increased the expression of VEGF mainly on podocytes and less markedly in mesangial area (Fig. 5A). The glomerular immunoreactivity for VEGF was partially diminished in losartan-treated rats, but not in PD123319-treated rats.

Expression of VEGF and receptors, flk-1 and flt-1

The expression of VEGF, and receptors, flk-1, flt-1 in glomeruli, was further examined by immunoblot. Although Ang II infusion alone only showed modest increase of VEGF as compared to normal control, the expression of VEGF was significantly increased in nephritic rats (Fig. 5B to E). The level of VEGF was elevated by norepinephrine infusion (2.4-fold), and further increased by 3.4-fold in Ang II–infused nephritic rats. Increase in the protein level of VEGF induced by Ang II infusion was partially blocked by losartan (62.6% inhibition), or PD123319 (43.1% inhibition), suggesting the involvement of both receptors. The glomerular expression of flk-1, a high affinity receptor of VEGF, was induced by Ang II infusion in control rats and in nephritic rats (4.2-fold increase). The level of glomerular flk-1 was also increased in nephritic control rats (Fig. 6A and C). Increase in the level of flk-1 induced by Ang II infusion was blocked by losartan and by PD123319, suggesting the involvement of AT_1R and AT_2R. The protein level of flt-1, a low-affinity receptor for VEGF, was also increased by Ang II infusion in nephritic rats (1.8-fold increase). Increase in the level of flt-1 induced by Ang II infusion was partially blocked by losartan or PD123319, suggesting the role of both AT_1R and AT_2R (Fig. 6A and B). These results indicate that Ang II infusion induced VEGF expression mainly from podocytes and mesangial cells, and induced the expression of flk-1 and flt-1 most likely on glomerular endothelial cells, thus facilitating the promotion of angiogenic response in glomeruli.

Immunohistochemical analysis of glomerular Ang-1 expression

The immunoreactivity for Ang-1 was mainly detected on podocytes in normal control rats, and Ang II infusion markedly increased immunoreactivity for Ang-1 protein on podocytes (Fig. 7A). Increased immunoreactivity for Ang-1 was observed in Ang II–infused nephritic animals, but not in norepinephrine-infused nephritic animals suggesting the specific effect of Ang II independent of elevated blood pressure (Fig. 7A). The glomerular immunoreactivity for Ang-1 was diminished in losartan-treated or PD123319-treated rats as compared to Ang II–infused nephritic rats (Fig. 7A).

Expression of Ang-1, Ang-2, and tie2

The glomerular expression of Ang-1 was markedly increased (3.3-fold) in Ang II–infused nonnephritic rats as compared to normal control. The level of Ang-1 was also significantly increased in nephritic control rats, and Ang II further induced (3.6-fold) the expression of Ang-1 in nephritic rats (Fig. 7B to E). The increase of Ang-1 protein induced by Ang II was almost completely blocked by losartan (94.0% inhibition), and markedly blocked by PD123319 (78.3% inhibition), suggesting the involvement of both AT_1R and AT_2R. In contrast, Ang-2, an endogenous antagonist of Ang-1, was suppressed by Ang II infusion in nonnephritic rats as compared to normal control rats ($P < 0.01$). The protein level of Ang-2 in Ang II–infused nephritic rats was significantly lower than normal control or nephritic control rats ($P < 0.05$). Infusion of norepinephrine resulted in a moderate reduction of Ang-2 protein level in nephritic animals. Treatment with losartan (2.3-fold) or PD123319 (2.1-fold) increased the level of Ang-2 as compared to Ang II–infused nephritic rats suggesting the role of both AT_1R- and AT_2R-mediated signal on the inhibition of Ang-2 expression (Fig. 8A to D). The expression of receptor tie2 was not significantly altered in control nephritic rats compared to normal control rats ($P < 0.05$). The expression of tie2 was markedly induced by Ang II infusion (1.9-fold increase), but not significantly by norepinephrine infusion in nephritic rats. Increase in the level of tie2 protein induced by Ang II infusion was significantly blocked by PD123319, but not by losartan, suggesting the dominant role of AT_2R over AT_1R (Fig. 8E to H). Accordingly, Ang II infusion resulted in the increase of Ang-1/Ang-2 ratio and tie2, thus conceivably leading to "stable" vascular microenvironment resistant to inflammatory stimuli as well as reduced monocyte/macrophage infiltration.

DISCUSSION

Angiogenesis is composed of several steps: (*1*) the degradation of vascular basement membrane matrix by protease, (*2*) migration and proliferation of endothelial cells, (*3*) endothelial tube formation, (*4*) recruitment
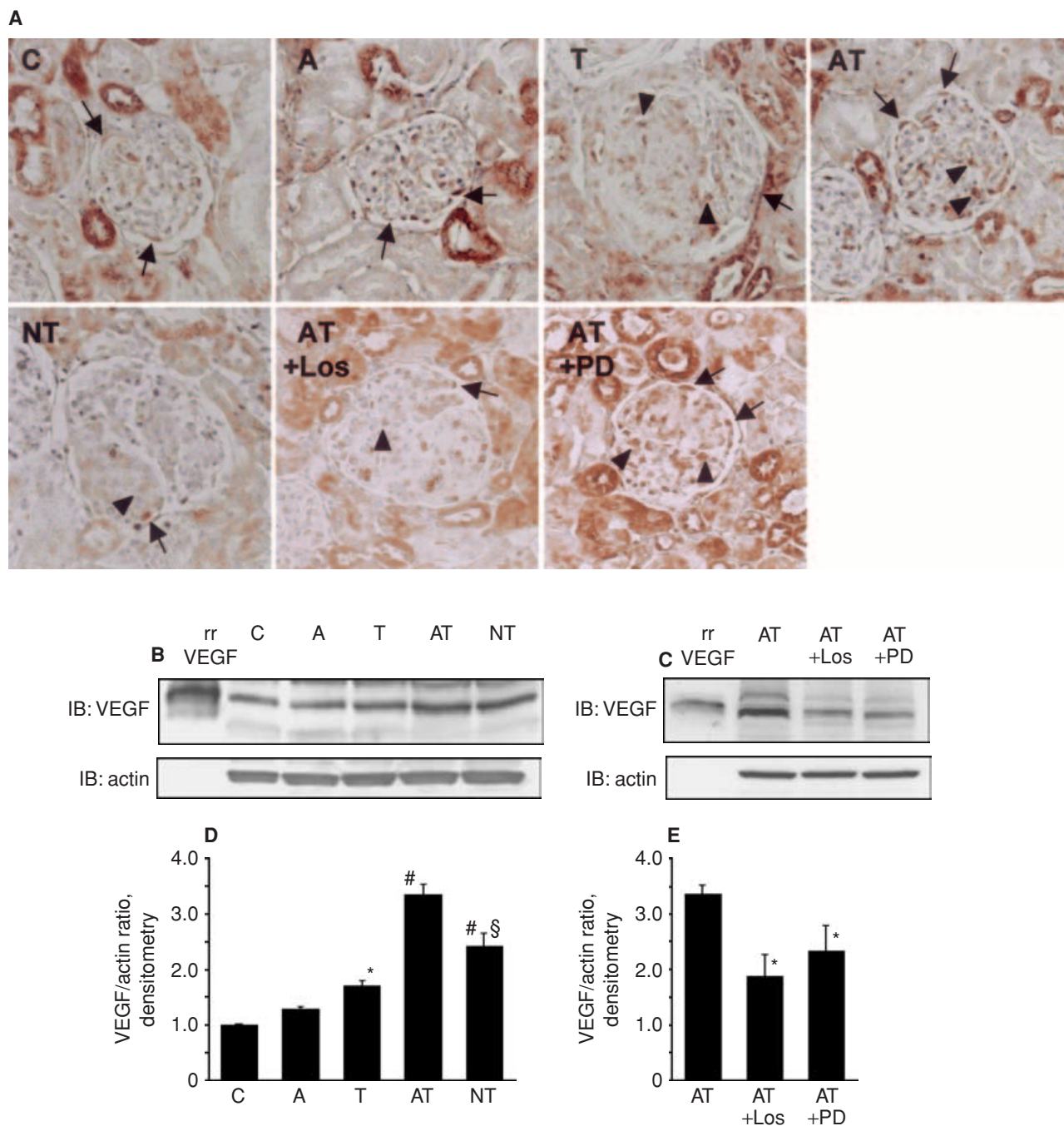
Fig. 5. Angiotensin II (Ang II) infusion induces glomerular vascular endothelial growth factor (VEGF) expression. (*A*) Immunohistochemistry of VEGF. Distribution of VEGF was determined as described in the **Methods** section by indirect immunohistochemistry. VEGF was observed in glomerular podocytes (arrows) and in mesangial area (arrowheads). Representative light microscopic appearance of glomerulus (original magnification 400×). (*B*) Immunoblot analysis of VEGF (30 kD). (*C*) Immunoblot analysis for actin (42 kD). In each lane, 100 μg of protein obtained from isolated glomeruli was loaded. The band of recombinant rat $VEGF_{164}$ (rrVEGF) was observed around 30 kD in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each band was scanned and subjected to densitometry. (*D*) Intensities of VEGF protein relative to actin. Control (C) $P < 0.0001$ by Kruskal-Wallis test; *$P < 0.05$ vs. C; #$P < 0.01$ vs. C, A, or T; §$P < 0.05$ vs. AT. (*E*) Intensities of VEGF protein relative to actin. Control (C) $P = 0.0222$ by Kruskal-Wallis test; *$P < 0.05$ vs. AT. Abbreviations are: C, undiseased control; A, Ang II–infused; T, anti-Thy-1.1 nephritis; AT, anti-Thy-1.1 nephritis with Ang II infusion; NT, anti-Thy-1.1 nephritis with norepinephrine infusion; Los, losartan; PD, PD123319 ($N = 5$ for each group). Each column consists of mean ± SEM.

and attachment of mesenchymal cells to the endothelial cell tube, and (*5*) maturation of blood vessels [19]. The essential role of VEGF in the development of renal vasculatures and glomeruli had been reported [40], and VEGF also plays an important role in glomerular capillary repair in nephritis model [24, 41]. The role of Ang-1, Ang-2, and tie2 in the process of renal development is well-known, and the loss of VEGF and Ang-1
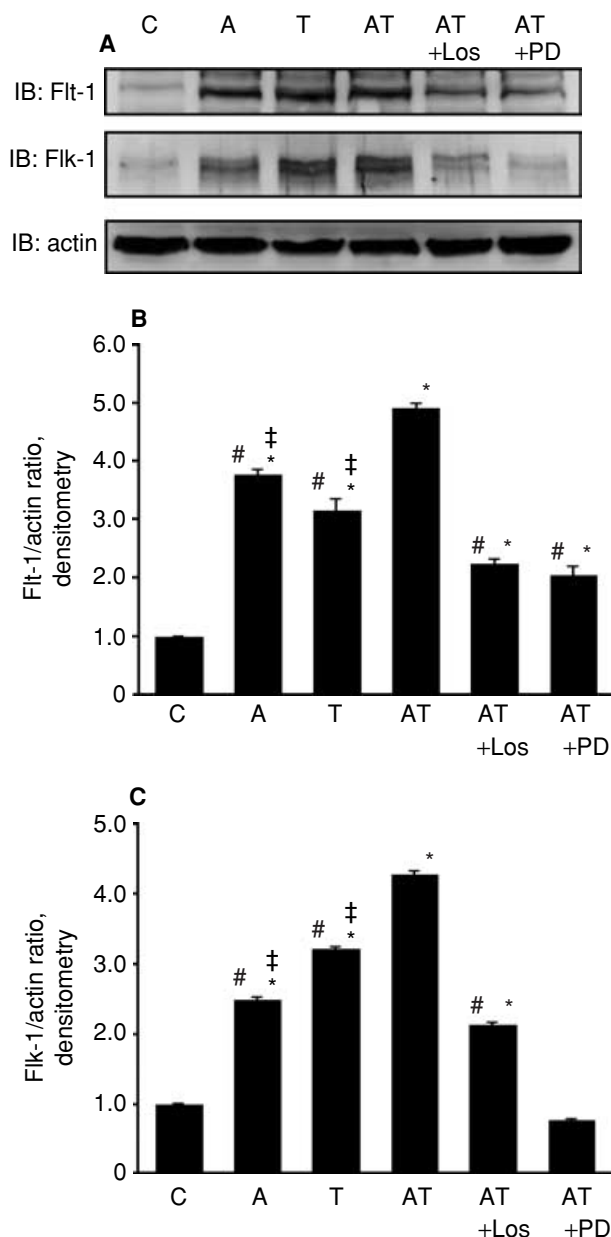
Fig. 6. Immunoblot analysis of Flt-1 and Flk-1. (*A*) Immunoblots for Flt-1 (170 kD), Flk-1 (160 kD) and actin. In each lane, 100 µg of protein obtained from isolated glomeruli was loaded. Each band was scanned and subjected to densitometry. (*B*) Intensities of Flt-1 protein relative to actin. Control (C) $P = 0.0062$ by Kruskal-Wallis test; *$P < 0.05$ vs. C; #$P < 0.05$ vs. AT; ‡$P < 0.05$ vs. AT + Los or AT + PD. (*C*) Intensities of Flk-1 protein relative to actin. Control (C) $P = 0.0054$ by Kruskal-Wallis test; *$P < 0.05$ vs. C or AT + PD; #$P < 0.05$ vs. AT; ‡$P < 0.05$ vs. AT + Los. Two bands were observed around 160 kD in immunoblots for Flk-1. Abbreviations are: C, undiseased control; A, angiotensin II (Ang II)–infused; T, anti-Thy-1.1 nephritis; AT, anti-Thy-1.1 nephritis with Ang II infusion; Los, losartan; PD, PD123319 ($N = 4$ for each group). Each column consists of mean ± SEM.

accompanied by the up-regulation of Ang-2 had been observed in anti-glomerular basement membrane (GBM) nephritis model in a temporal manner in association with glomerular capillary loss [42].

In the present study, we observed that Ang II infusion resulted in the amelioration of characteristic histologic features in rat anti-Thy-1.1 nephritis model similar to the previous report [18]. The surprising effect of Ang II infusion in ameliorating early changes in anti-Thy-1.1 nephritis is in contrast to previous studies that described the beneficial effect of blockade of the RAS in renal disorders [16, 17]. However, we speculate the milieu caused by the infusion of exogenous Ang II is considerably apart from the interference with a local endogenous RAS by angiotensin-converting enzyme (ACE) inhibitor or AT_1RA. In addition, the essential role of the RAS in renal vascular development and maintenance of blood vessels had also been reported [5–7]. Studies using mice deficient in both $AT_{1a}R$ and $AT_{1b}R$ revealed delayed maturity in glomerular growth and renal arterial hypertrophy [6, 7]. More recently, SHRs treated with ACE inhibitor or AT_1RA from the last week of gestation until 8 weeks of age exhibited smooth muscle cell hyperplasia and increased wall thickness in renal cortical arteries, ultimately resulting in malignant hypertension [9]. These previous studies may suggest that the blockade of Ang II formation and AT_1R-mediated actions suppress the synthesis of Ang II–induced another trophic factors essential for the development and maintenance of renal vascular structures. We speculate VEGF and Ang-1 may be such trophic factors induced by Ang II mediating accelerated glomerular endothelial cell repair in the early phase of anti-Thy-1.1 nephritis. In addition, activation of the RAS might possess beneficial effects on kidney in certain pathophysiologic conditions such as renal artery stenosis [10] and partial urinary tract obstruction [11].

In the present study with extended time points, Ang II infusion neither resulted in the exacerbation of histologic alterations nor interfered with healing process of anti-Thy-1.1 nephritis. Previous study of two-kidney, one-clip hypertension rat model followed by the induction of anti-Thy-1.1 nephritis revealed no significant influence of renovascular hypertension on healing of the glomerular lesions [43]. Since two-kidney, one-clip Goldblatt hypertension model is an Ang II–dependent model of hypertension, our results may be considered to share consistency with these previous findings. Although renal function tended to be impaired in Ang II–infused nephritic animals as compared to control nephritic animals, histologic alterations were less remarkable in Ang II–infused animals. Previous report by Aizawa et al [37] demonstrated that infusion of Ang II for a shorter term (7 days) in Sprague-Dawley rats decreased glomerular filtration rate (GFR) (as demonstrated by creatinine clearance) and increased proteinuria [37]. They described that AT_1R-mediated action of Ang II was responsible for the decrease of creatinine clearance, whereas the synergistic action of Ang II and pressor overload was critical for the increased proteinuria. Decreased creatinine
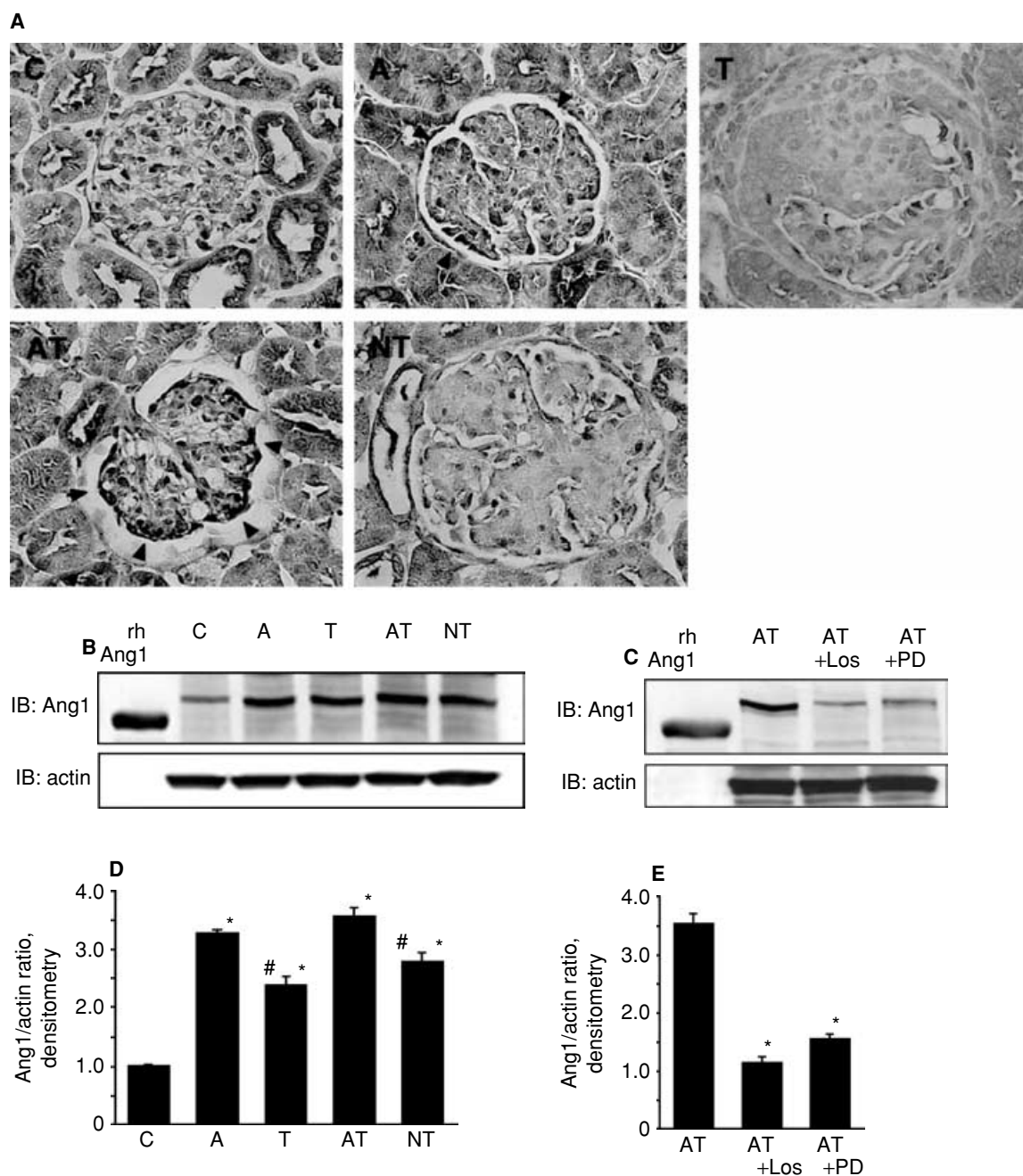
Fig. 7. Angiotensin II (Ang II) infusion induces glomerular angiopoeitin-1 (Ang-1) expression. (*A*) Immunohistochemistry of Ang-1. Distribution of Ang-1 was determined as described in the **Methods** section by indirect immunohistochemistry. Ang-1 was observed in glomerular podocytes (arrowheads) and less frequently in mesangial area. Representative light microscopic appearance of glomerulus (original magnification 400×). Immunoblot analysis of Ang-1. (*B* and *C*) Immunoblots for Ang-1 (100 kD, most likely presenting as dimer) and actin. In each lane, 100 μg of protein obtained from isolated glomeruli was loaded. Recombinant human Ang-1 (rhAng-1) migrated as an approximately 70 kD bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each band was scanned and subjected to densitometry. (*D*) Intensities of Ang-1 protein relative to actin. Control (C) $P = 0.0001$ by Kruskal-Wallis test; *$P < 0.01$ vs. C; #$P < 0.01$ vs. A or AT. (*E*) Intensities of Ang-1 protein relative to actin. Control (C) $P = 0.0009$ by Kruskal-Wallis test. *$P < 0.01$ vs. AT. Abbreviations are: C, undiseased control; A, Ang II–infused; T, anti-Thy-1.1 nephritis; AT, anti-Thy-1.1 nephritis with Ang II infusion; NT, anti-Thy-1.1 nephritis with norepinephrine infusion; Los, losartan; PD, PD123319 ($N = 5$ for each group). Each column consists of mean ± SEM.

clearance levels in Ang II–infused nephritic animals as compared to control nephritic rats in the present study are in line with this previous observation. Proteinuria tended to be exacerbated in Ang II–infused animals on day 14

and 35, but was slightly diminished on day 6 as compared to control nephritic rats, possibly due to reduced GFR at the proteinuric stage. We failed to observe proteinuria in Ang II–infused nonnephritic animals possibly due to
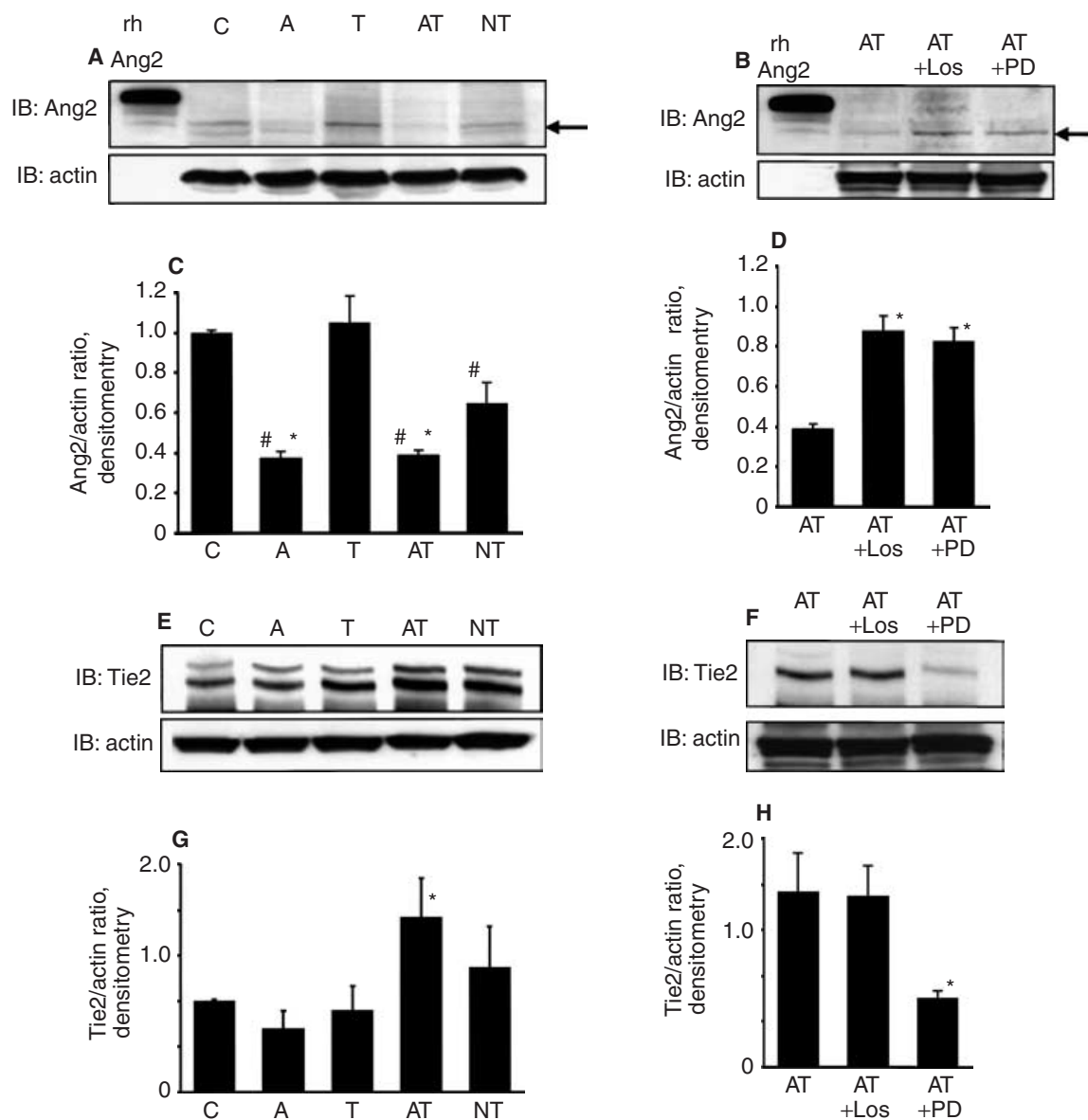
Fig. 8. Immunoblot analysis of angiopoeitin-2 (Ang-2) and tie2. (*A* and *B*) Immunoblots for Ang-2 (50 kD) and actin are shown. In each lane, 100 μg of protein obtained from isolated glomeruli was loaded. Recombinant human Ang-2 (rhAng-2) migrated as an approximately 60 kD bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each band was scanned and subjected to densitometry. (*C*) Intensities of Ang-2 protein relative to actin (*N* = 5 for each group). Control (C) *P* = 0.0004 by Kruskal-Wallis test; **P* < 0.01 vs. C; #*P* < 0.05 vs. T. (*D*) Intensities of Ang-2 protein relative to actin (*N* = 5 for each group). Control (C) *P* = 0.0033 by Kruskal-Wallis test; **P* < 0.01 vs. AT. Each column consists of mean ± SEM. (*E*) Immunoblots for tie2 (140 kD) and actin. In each lane, 100 μg of protein obtained from isolated glomeruli was loaded. Each band was scanned and subjected to densitometry. (*F*) Immunoblots for tie2 (140 kD) and actin. In each lane, 100 μg of protein obtained from isolated glomeruli was loaded. Each band was scanned and subjected to densitometry. (*G*) Intensities of tie2 protein relative to actin. Control (C) *P* < 0.0001 by Kruskal-Wallis test; **P* < 0.01 vs. C, A or T. (*H*) Intensities of tie2 protein relative to actin. Control (C) *P* = 0.0032 by Kruskal-Wallis test; **P* < 0.01 vs. AT or AT + Los. Abbreviations are: C, undiseased control; A, angiotensin II (Ang II)–infused; T, anti-Thy-1.1 nephritis; AT, anti-Thy-1.1 nephritis with Ang II infusion; NT, anti-Thy-1.1 nephritis with norepinephrine infusion; Los, losartan; PD, PD123319 (*N* = 5 for each group). Each column consists of mean ± SEM.

the different strains of animals used in the present study or distinct time course. Norepinephrine served as controls for Ang II showing comparable elevation of systolic blood pressure. Creatinine clearance of norepinephrine-infused nephritic rats was close to that of control nephritic rats, and Ang II–infused nephritic rats exhibited significant deterioration. Treatment with losartan markedly im-

proved creatinine clearance of Ang II–infused nephritic animals in contrast to PD123319 lacking such effects. Although the experimental settings are different between the present study and the previous study by Aizawa et al [37], these results again suggest that Ang II infusion decreases creatinine clearance via AT_1R-mediated action. Collectively, the discrepancy between renal function and

histologic findings observed in the present study may, at least in part, depend upon the direct role of Ang II infusion on GFR. Proteinuria in norepinephrine-infused nephritic rats was increased as compared to nephritic control rats. Treatment with losartan significantly improved proteinuria of Ang II–infused nephritic animals in contrast to PD123319 lacking such effects. These results again suggest the involvement of Ang II (mainly via AT_1R) and pressor load on proteinuria.

Although Ang II infusion ameliorated increase of glomerular cellularity and the expansion of mesangial matrix, norepinephrine failed to show such effects. Our results are in consistent with previous reports by Wenzel et al, using antithymocyte serum–induced nephritis and antithymocyte serum nephritis with renovascular hypertension [18, 44]. These findings indicate that elevated blood pressure did not ameliorate nephritic alterations, but this effect was attributable to the specific action of Ang II. The glomerular accumulation of monocyte/macrophage is one of the common pathologic findings in various forms of glomerular disorders. Previous report demonstrated the Ang II–induced decrease of monocyte/macrophage mediated by the suppression of MCP-1 at 24 hours after the induction of antithymocyte serum nephritis [18]. We observed that Ang II infusion suppressed the glomerular accumulation of monocyte/macrophage on day 6 after initiating nephritis. This effect was mainly dependent on AT_1R, similar to the previous study [18]. Although it is hard to assess the reason why we could observe the inhibitory effect of Ang II on day 6, we speculate the discrepant findings might be partly attributable to the difference in disease models (using monoclonal 1-22-3 antibody in the present study instead of antithymocyte serum) or to the difference in the genetic background of rats (using Wistar rats instead of Sprague-Dawley rats). Infusion of norepinephrine failed to show inhibitory effect on monocyte/macrophage recruitment, again suggesting the effect of Ang II independent of blood pressure. Similarly, Ang II–induced suppression of mesangial matrix expansion was dominantly mediated via AT_1R. We speculate that reduced infiltration of monocyte/macrophage in Ang II–infused nephritic rats may, at least in part, account for the reduction in the accumulation of mesangial matrix, since chemokines produced by monocyte/macrophage are involved in the accumulation of ECM [45].

In the present study, we used immunostaining for RECA-1 and vWF to determine the glomerular capillary endothelium, and rats with anti-Thy-1.1 nephritis exhibited marked reduction in the level of these endothelial cell markers in consistent with previous report showing similar reduction in the number of glomerular capillary-lumina at the early stage of anti-Thy-1.1 nephritis [23]. Deposition of Ig as well as mesangiolysis in the initial phase of anti-Thy-1.1 nephritis were similarly observed

in Ang II–infused and control nephritic animals, suggesting that Ang II did not influence on the initial alterations in the course of anti-Thy-1.1 nephritis. Treatment with Ang II resulted in the suppression of the loss in RECA-1- and vWF-positive area suggesting that Ang II infusion caused accelerated recovery of glomerular endothelial cells following loss of endothelial cells caused by the lack of capillary support due to mesangiolysis [22]. The effect of Ang II to maintain glomerular endothelial area in the early stage of anti-Thy-1.1 nephritis was mediated via both AT_1R and AT_2R. Suppression of the loss in glomerular capillary area in Ang II–infused nephritic animals in the early stage might have lead to the accelerated healing of nephritic alterations on day 14.

Recent reports clarified the involvement of Ang II in inducing angiogenesis in various setting of diseases. Ang II induced VEGF and Ang-2, and stimulated angiogenesis via AT_1R in cardiac microvascular endothelial cells [46]. In AT_{1a} knockout mice, suppressed angiogenic response to ischemic injury was observed [30]. AT_2R negatively modulated ischemia-induced angiogenesis using AT_2R-deficient mice [47]. These reports indicated the involvement of Ang II in inducing angiogenesis mainly via AT_1R. However, recent report demonstrated the possible action of AT_2R in inducing angiogenesis using alginate implant angiogenesis model in AT_2R-deficient mice [48]. In diabetic retinopathy model and Ang II infusion model, increased retinal expression of VEGF was blocked with AT_1RA or PD123319 [49].

Our present results demonstrated that the protein level of VEGF was significantly elevated in control nephritic rats compared to normal control animals. Several growth factors and cytokines such as TGF-β, PDGF, Ang II, interleukin (IL)-1 and IL-6, are known to up-regulate VEGF expression [50]. The observed elevation in the level of VEGF in control nephritic rats may be attributed to the effects caused by these factors, at least in part, since some of these factors are considered to be increased in anti-Thy-1.1 nephritis. Ang II infusion markedly induced VEGF protein level compared to control nephritic rats, and norepinephrine also induced VEGF to a lesser extent. Previous reports demonstrated the involvement of mechanical stretch in inducing VEGF [51]. Our results may suggest that systemic hypertension induced by norepinephrine infusion might have resulted in up-regulation of VEGF possibly mediated via mechanical stretch. Up-regulation of VEGF induced by Ang II infusion was inhibited by losartan, but not by PD123319, suggesting the involvement of AT_1R similar to the reported results on mesangial cells [51]. Recent study demonstrated that Ang II infusion up to 2 weeks resulted in increased expression of VEGF protein, and that AT_1RA (valsartan) moderately suppressed VEGF production, in contrast to PD123319 almost completely blocking Ang II–induced increase of VEGF [52]. First, we speculate

the involvement of distinct regulatory system in different two disease models resulted in the discrepancy between two studies. Second, since we obtained cell lysates from isolated glomeruli, our results precisely reflected the alterations in glomeruli distinct from results obtained by utilizing whole kidney sample [52], considering that renal tubular epithelial cells and macrophages also produce VEGF [53].

The protein level of flk-1 was increased in Ang II–infused control, nephritic control and Ang II–infused nephritic rats. Previous reports demonstrated up-regulated kinase domain region (KDR) and flt-1 expression in glomeruli of human mesangioproliferative glomerulonephritis [54] and increased expression of flk-1 in association with glomerular endothelial cell growth and thus capillary repair in anti-Thy-1.1 nephritis [55]. Up-regulation of flk-1 as well as flt-1 in nephritic control rats thus may suggest the mechanism in relation to glomerular capillary repair. Infusion of Ang II resulted in further increase of flk-1 and flt-1 in nephritic rats as compared to nephritic control or Ang II–infused control rats. Up-regulation of flt-1 and flk-1 in Ang II–infused nephritic rats was mediated via both AT_1R and AT_2R.

We also examined changes in the expression of Ang-1 and Ang-2, since angiopoietins play crucial roles in the process of angiogenesis and vascular maturation. The level of Ang-1 protein was significantly increased in anti-Thy-1.1 nephritis compared to normal control rats. Although not described previously in the setting of anti-Thy-1.1 nephritis, the increase in the level of Ang-1 together with the increased VEGF/flk-1/flt-1 expression suggest the self-protective mechanism to promote angiogenic repair against glomerular capillary injury in the early phase of nephritis. The level of Ang-1 protein was also increased in glomeruli of Ang II–infused control and nephritic rats (approximately threefold). In contrast to norepinephrine infusion, Ang II infusion showed significantly increased Ang-1 suggesting the role of Ang II in inducing Ang-1 independent of elevated blood pressure. In previous report by Rizkalla et al [52], Ang II infusion resulted in the increase of Ang-1 protein (approximately twofold) mediated via AT_1R [52]. In our experiments, both AT_1R and AT_2R were involved in inducing Ang-1. The observed discrepancy in the extent of increase in the level of Ang-1 following Ang II infusion and the repertoire of responsible Ang II receptors might be explained again by the distinct materials (i.e., glomeruli vs. whole kidney) used in obtaining protein extracts, and by distinct disease models employed. The expression of Ang-2 was suppressed by Ang II infusion in control rats. In contrast, results reported by Rizkalla et al [52] showed up-regulation of Ang-2 following Ang II infusion, again considered to be attributable to the absent glomerular isolation step in their experiments, potentially suggesting

increased production of Ang-2 in extraglomerular cells following Ang II infusion. Infusion of norepinephrine in nephritic rats resulted in the decrease of glomerular Ang-2 protein level, suggesting the inhibitory mechanism induced by elevated blood pressure. However, results obtained by using receptor antagonists revealed that PD123319 treatment exhibiting similar extent of systemic hypertension with Ang II–infused nephritic rats, failed to down-regulate Ang-2, suggesting that the inhibitory mechanism on Ang-2 expression following Ang II infusion was independent of elevated blood pressure. The inhibitory effect of Ang II on the expression of Ang-2 was considered to be mediated via both AT_1R and AT_2R in anti-Thy-1.1 nephritis rats. The expression of tie2 was not significantly altered in Ang II–infused control and control nephritic rats as compared to normal control rats. Although infusion of norepinephrine showed similar results, Ang II infusion markedly increased tie2 expression in nephritic rats compared to other controls. The effect of Ang II in increasing the level of tie2 protein was mainly mediated via AT_2R. These results implicate that Ang II infusion increased the relative ratio of Ang-1/Ang-2 and the expression of tie2 in anti-Thy-1.1 nephritis, and thus triggering the signal mediated by Ang-1/tie2 system. This regulatory mechanism on the expression of Ang-1/Ang-2/tie2 was mediated via both AT_1R and AT_2R.

Although both VEGF and Ang-1/tie2 are essential factors for vascular development and angiogenesis, the appropriate balance between these factors are considered to be very important for proper formation of blood vessels. Thurston et al [56] reported the formation of leaky blood vessels in mice overexpressing VEGF, and the development of nonleaky blood vessels in mice overexpressing Ang-1, and that coexpression of Ang-1 and VEGF resulted in enhanced angiogenic effect with the formation of nonleaky blood vessels. We recently reported that the increase of VEGF and Ang-2 in the model of diabetic nephropathy had been inhibited by tumstatin peptide, an angiogenesis inhibitor derived from type IV collagen, accompanied by therapeutic effects [35]. In the present study, Ang II induced signaling via VEGF/flk-1/flt-1 and Ang1/tie2 in the early phase of anti-Thy-1.1 nephritis following glomerular endothelial cell damage, suggesting the orchestrated environment toward promoting angiogenesis, potentially leading to the formation of nonleaky blood vessels. We consider this microenvironment resulted in the accelerated recovery of glomerular capillary structures in Ang II–infused nephritic rats. Additionally, decreased infiltration of monocyte/macrophage in Ang II–infused nephritic rats may be attributed to the formation of nonleaky glomerular capillaries in the presence of enhanced Ang-1/tie2 system accompanied by up-regulation of VEGF. Ang-1 stabilizes blood vessels rendering them nonleaky to inflammatory insult by

inducing the attachment of pericytes (mesenchymal cells) around endothelial cell tube. In the setting of glomerular capillary microenvironment, mesangial cells may be considered to act like pericytes since they provide support for maintaining glomerular capillary structures. Coordinated up-regulation of VEGF and Ang-1 by Ang II infusion initiated prior to the induction of nephritis might have mediated glomerular endothelial cell repair in a "stable" manner, and thus, at least in part, resulted in the stabilization of mesangial cells with the amelioration of proliferative and fibrogenic changes in the early phase of anti-Thy-1.1 nephritis. Although we could not observe significant exacerbation of tubulointerstitial injuries in Ang II–infused nephritic rats compared to nephritic control rats, a previous report by Wenzel et al [18] described the observed tubulointerstitial lesion in this model similar to previously described focal tubulointerstitial injuries induced by Ang II infusion for longer time [57].

According to the results obtained by immunohistochemistry of VEGF and Ang-1, both of these factors were mainly localized to glomerular podocytes. The crucial role of glomerular podocytes had been demonstrated in the recovery phase of anti-Thy-1.1 nephritis. Signals from podocytes are considered to attract the sprouting of capillaries to sites beneath the GBM, because at the sites forming an adhesion to Bowman's capsule and at which the GBM was devoid of podocytes, capillaries never assembled [22]. Our results suggest that Ang II stimulated the production of VEGF and Ang-1 in podocytes and increased the expression of flk-1, flt-1 and tie2 on glomerular endothelial cells, properly orchestrating the angiogenic capillary repair process. Also, the indirect influence of glomerular endothelial cells toward glomerular podocytes possibly mediated via secreted factors or alteration on matrix microenvironment might be involved in the ameliorated glomerular changes in Ang II–infused anti-Thy-1.1 nephritis rats in the early phase. Mice with podocyte-specific overexpression or deletion of VEGF demonstrated the development of proteinuria and pathologic changes in glomerular endothelial cells, suggesting the importance of appropriate level of VEGF in maintaining intact glomerular capillary structures [58]. The suggested role of Ang-1 in maintaining glomerular endothelium and regulating the action of VEGF on glomerular permselectivity [59] further support the importance of interaction between glomerular endothelial cells and podocytes in the repair and the maintenance of glomerular capillary structures.

CONCLUSION

We demonstrated that Ang II infusion initiated prior to the induction of anti-Thy-1.1 nephritis suppressed characteristic histologic alterations as previously described by Wenzel et al, induced the increase of glomerular endothelial cell area possibly in association with the induction of angiogenic factors such as VEGF and Ang-1 and their receptors. Our results further suggested the accelerated histologic healing process of nephritic alterations in Ang II–infused animals potentially due to the increase of endothelial area in the early stage of nephritis. The specific setting utilized in the present study revealed unknown biological function of Ang II in association with angiogenic response in glomeruli, similar to the reported proangiogenic effect of Ang II on extrarenal blood vessels [30, 46]. Our results however do not question the well-established beneficial effect of ACE inhibitors or AT_1R blocker in "chronic" renal disorders in physiological settings. Further clarification of downstream signaling pathways involved in the regulation of VEGF, Ang-1, Ang-2, and flk-1/flt-1/tie2 receptors mediated by AT_1R or AT_2R stimulation may lead to the development of novel specific therapeutic approaches to promote glomerular capillary repair.

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