Angiotensin receptor blockade attenuates glomerulosclerosis progression by promoting VEGF expression and bone marrow-derived cells recruitment

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Abstract

Background. Previous studies have demonstrated that angiotensin Type I receptor blockade (ARB) reduces proteinuria, reverses glomerular injury and glomerulosclerosis in rat models of diabetic nephropathy and glomerulonephritis. However, the cellular and molecular mechanisms are unclear. To investigate the role of cells of the bone marrow (BM) in glomerular repair seen during ARB administration, we induced progressive glomerulosclerosis in enhanced green fluorescent protein BM chimeric rats by a single injection of anti-Thy 1.1 monoclonal antibody, followed by unilateral nephrectomy.

Methods. Cohorts of rats received valsartan or no treatment from Week 2 to Week 8 after induction of disease. Renal function, urinary protein excretion and histological changes were examined 8 weeks after anti-Thy-1.1 monoclonal antibody injection.

Results. Valsartan administration improved renal function, reduced severity of glomerulosclerosis and markedly reduced mortality. Valsartan administration promoted regeneration of the glomerular tuft, lowered proteinuria and resulted in enhanced vascular endothelial growth factor (VEGF) expression in the cortex and glomerular tuft. In addition, valsartan promoted increased recruitment of BM-derived cells (BMDCs) many of which expressed VEGF and likely contributed directly to glomerular repair. Nearly all BMDCs recruited to the glomerulus expressed the monocyte/macrophage marker CD68.

Conclusions. In conclusion, the data shows that ARB by valsartan prevents glomerulosclerosis progression by enhancing glomerular capillary repair which is associated with the recruitment of VEGF producing ‘reparative’ monocytes and macrophages from the BM.

Keywords: ARB; bone marrow-derived cells; glomerular capillary repair; glomerulosclerosis; VEGF

Introduction

Progressive glomerulosclerosis occurs in many renal diseases including diabetic nephropathy and chronic glomerulonephritis. Our previous studies have confirmed that angiotensin receptor Type I blockade (ARB) reduces proteinuria and reverses glomerular injury including glomerulosclerosis in rat models of glomerular nephropathy [1] and glomerulonephritis [2]. Other studies have demonstrated that inhibition of angiotensin is crucial in treatment of chronic kidney disease, presumably via effects on blood pressure and extracellular matrix [3–5].

Remodeling of glomerulosclerosis must involve regeneration and repair of glomerular cells and mesangial cells. There is increasing evidence that leukocytes from bone marrow (BM) can perform a wide range of reparative and regenerative functions in tissue injury [6] and a number of studies suggest that cells derived from BM can differentiate into certain parenchymal cells or endothelial cells [7, 8]. In the latter case, these are known as endothelial progenitor cells [9–11]. Our recent studies and other publications have demonstrated that BM stem cells could promote kidney repair and regeneration, but paracrine mechanism, not direct differentiation, plays a major role in this process [12, 13]. Increasing evidence has shown that macrophage/monocyte could enhance kidney and other organ repair and regeneration by releasing important cytokines and clear debris [12, 14]. In contrast, ablation
of macrophage resulted in slower kidney repair using CD11b-DTR transgenic mice [15].

In the present study, we explored the interactions of ARB and BM-derived cells (BMDCs) which may play potential important roles in procedure remodeling glomerulosclerosis. We selected the experimental model of progressive glomerulosclerosis which can be induced in the rat by a single injection of anti-Thy-1.1 monoclonal antibody, followed by unilateral nephrectomy using BM-chimeric rats [16]. This model is useful in observing whether BMDCs are involved in the regeneration and repair of injured glomeruli. Here, we also investigated the therapeutical effects of ARB on progressive glomerulosclerosis.

Materials and methods

Animals

Transgenic Sprague-Dawley (SD) rats carrying the enhanced green fluorescent protein (EGFP) transgene (EGFP rat) under control of the CAGGS promoter were purchased from Japan SLC, Inc. (Hamamatsu, Japan; by permission of Dr M. Okabe, Osaka University) and were maintained in a sterile environment and bred to obtain littermates of GFP-positive and -negative (wild-type) rats. All experimental protocols were approved by the animal committee of Harbin Medical University and Nigata University.

Chimeric rats and BM transplantation

BM transplantation (BMT) was performed on 5-week-old SD rats (wild-type, EGFP⁺) as recipients and their siblings (EGFP⁻) as donors. As described in our previous study [17], EGFP⁺ rat BM was collected by flushing bone shafts of femurs, tibiae and humeri of EGFP rats with phosphate-buffered saline (PBS). After being sieved through 75- and 30-μm meshes, the cells were re-suspended at a concentration of 1–2 × 10⁸ cells/mL and kept on ice until use. Littermates of wild-type, EGFP⁻, were lethally irradiated [10 Gy per animal with an X-ray generator (PS-3000 SB Cs-137; Pony Industry co., LTD)] and within 4 h after irradiation, BM cells were administered via the tail vein. Six weeks later, the chimeric rats (wild-type rats that had received EGFP⁺ BMT) were divided into two groups treated or untreated with BMT after anti-Thy 1.1 antibody injection with unilateral nephrectomy.

Experimental design

Five weeks after BMT, experimental progressive glomerulosclerosis model was induced in 30 chimeric rats (body weight 120–135 g) by a single intravenous injection of monoclonal antibody anti-rat Thy-1.1 (1-22-3 mouse IgG3, 1.0 mg per rat) and then 30 min after injection, left nephrectomy was performed, according to the protocol described previously [16, 17]. These chimeric rats were divided into two groups. For the experimental group, on Day 7 after induction of disease, valsartan (gift from Novartis, Basel, Switzerland), a selective angiotensin receptor blocker, was given daily in the water (10 mg/kg) (ARB group, n = 14). For the control group, chimeric rats with unilateral nephrectomy after anti-Thy 1.1 antibody injection (untreated group, n = 16) were not given any treatment. The rats would induce progressive glomerulosclerosis and almost die by 11 weeks as reported previously [16, 17]. Rats were sacrificed on Day 56 after the administration of 1-22-3 in all groups.

Biochemical analysis of serum

Serum total protein as well as concentrations of albumin, total cholesterol, creatinine and blood urea nitrogen (BUN) were measured, respectively.

Urinary analysis

Chimeric rats with anti-Thy-1 nephritis were individually housed in metabolic cages, with free access to water, for collection of 24-h urine specimens on Days 3, 7, 14, 28, 42 and 56 after the injection of 1-22-3 antibody. The amount of urinary protein excreted was determined by the biuret method with bovine serum albumin as a standard.

Light microscopy

Tissue for light microscopy was fixed in 10% neutral-buffered formalin. The fixed material was embedded in paraffin, sectioned at a thickness of 4 μm and sections were stained with periodic acid-Schiff. Mesangial proliferation and glomerular sclerosis were graded semiquantitatively on a scale of 0 to 4+, as reported previously [18].

Examination of cell markers on frozen sections by immunofluorescence

Immunofluorescent staining was performed on 3 μm frozen sections followed by fixation with cold acetone. Sections were stained with the primary antibody at 4°C overnight and washed with PBS. Incubation with the second antibody was for 1 h at room temperature. The primary antibodies were mouse anti-rat OX-7 antibody (A hybridoma producing OX-7 IgG1 was purchased from European Collection of Animal Cells Porton Down, Salisbury, UK) for identifying glomerular endothelial cells (GECs) and rabbit anti-rat ZO-1 antibody (Zymed Laboratories, San Francisco, CA) for identifying podocyte, mouse anti-rat CD45 (Abcam, Cambridge, UK) antibody for identifying leukocyte, mouse anti-rat CD68 (Serotec Ltd) antibody for identifying monocyte/macroage and mouse anti-vascular endothelial growth factor (VEGF) monoclonal antibody (Abcam). The secondary antibody was tetramethyl rhodamine B isothiocyanate (TRITC)-conjugated anti-mouse or anti-rabbit antibody (Dako, Glostrup, Denmark). For conventional immunofluorescence of the sections, we used confocal laser scanning immunofluorescent microscopy, with the MRC-1024 confocal imaging system (Bio-Rad laboratories, Hemel Hempstead, UK). The glomerular capillary density was calculated by Photoshop software, RECA-1-positive area (RA) and whole glomerular area (GA) can be calculated. The glomerular capillary density = RA/GA * 100%. The GFP-positive cells, BM-derived endothelial cells and mesangial cells were counted in a blinded manner by two unrelated researchers in at least 30 glomeruli per kidney.

Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting were carried out as previously described [19]. For detection of VEGF, 20 μg protein extracted from kidney cortex was applied to each lane. After blotting, and blocking blots were incubated with anti-VEGF (Abcam) overnight, blots washed then incubated with anti-mouse-HRP antibodies (1:5000) (Jackson Immunoresearch). After detection and stripping, blots were re-detected with anti-β-actin (Santa Cruz Biotechnology).

Statistical analysis

All values are given as mean ± SD. One-way analysis of variance with Steel-Dwass test was employed to analyze multiple comparison. Kaplan–Meier analysis was used to analyze survival rate. Comparisons between two groups were carried by unpaired t-test. P-values < 0.05 were considered significant in all statistical tests. All tests were carried out using GraphPad Prism (GraphPad Software).

Results

Administration of valsartan decreases mortality and decreases urinary protein excretion in a rat model of progressive glomerulosclerosis

As reported in our previous study [17], this experimental rat model was easier to induce progressive glomerulosclerosis and die from chronic renal failure than other kinds of rats. In the present study, only 5 of the 16 untreated rats survived. The other 11 rats died at Weeks 2, 4, 5, 6, 7 and 8 after 1-22-3 antibody injection. However, 10 of the 14 ARB treated rats survived until Week 8 and only 4 rats died at Weeks 5, 6 and 7 (Figure 1A). The surviving numbers in the two groups can be seen in Figure 1A. This meaningful evidence indicates that administration of ARB enhances survival and could be a useful therapeutic strategy for progressive glomerulosclerosis.
Urinary protein excretion was determined on Days 3, 7, 14, 28, 42 and 56 after anti-Thy 1.1 monoclonal antibody injection. Until 28 days after Thy-1 antibody injection, urinary protein excretion in both treated with ARB and untreated groups was obviously higher than that in control group, but no significant difference existed between the two groups. In the untreated group, urinary protein excretion sharply increased with time and was up to 190.6 ± 47.5 mg/day. In the ARB-treated group, urinary protein excretion did not increase with time. It was significantly lower than that in the untreated group from Days 42–56 after injection of Thy 1 antibody (Figure 1B), suggesting that ARB treatment could partly repair renal injury and lower urinary protein excretion.

Valsartan treatment partly ameliorates renal dysfunction

After 5 weeks of irradiation, we determined the renal function and other general characteristics in chimera rats. As shown in Table 1, serum creatinine (0.28 ± 0.17 mg/dL), BUN (18.3 ± 3.1 mg/dL) and 24-h protein excretion (2.39 ± 2.87 mg/day) in chimera rats after 5 weeks of irradiation were at levels similar to those observed in control rats (0.27 ± 0.28 mg/dL, 18.1 ± 5.8 mg/dL and 2.43 ± 2.17 mg/day, respectively). In addition, we also found no significant difference in serum total protein, albumin and total cholesterol between control and chimera rats (Table 1). These data show that chimera rats display no evidence of radiation-induced renal dysfunction.

We examined whether ARB treatment has an impact on renal function. All rats after antibody injection with nephrectomy had renal dysfunction with significant increase in serum creatinine from levels of 0.28 ± 0.17 to 2.21 ± 1.19 mg/dL in untreated group, 1.61 ± 0.56 mg/dL in ARB-treated group. Also, all rats have a significant increase in serum BUN from levels of 18.3 ± 3.1 to 201.3 ± 87.2 mg/dL in untreated group, 129.5 ± 48.7 mg/dL in ARB-treated group. However, renal function in animals with ARB treatment was significantly improved than in untreated animals throughout the study (Table 1).

Valsartan administration ameliorates histological alterations

Glomerular alterations were examined on Day 56 after anti-Thy 1 antibody injection by light microscopy. Sequential renal histology during the disease course, as described recently in the same experimental schedule [16], was abbreviated here. On Day 56, in the untreated group, severe mesangial cell proliferation with matrix expansion was observed in the majority of the glomeruli. Many glomeruli showed crescentic lesions and diffuse interstitial fibrosis.
tubular atrophic changes with interstitial cell infiltration (Figure 2A). Only moderate mesangial cell proliferation with mesangial matrix and mild crescentic lesions were observed in the ARB treatment group (Figure 2B). The mesangial proliferation index and glomerular sclerosis index in the untreated group were significantly higher than the ARB administration group (Figure 2C and 2D). Light microscopic findings in the present study also showed that progressive glomerulosclerotic lesions, associated with renal insufficiency, occurred in the untreated model, as described previously [18]. These findings indicate that ARB treatment can ameliorate histological changes.

**Valsartan treatment promoted glomerular capillary repair and regeneration by enhanced VEGF expression**

The maintenance of the microvasculature would appear to be critical for the prevention of progressive renal disease [20]. To analyze glomerular capillary regeneration, we used RECA-1 as markers for endothelial cells to evaluate the glomerular capillary's density. As shown in Figure 3A, evident loss of glomerular endothelium occurred in the untreated group (Figure 3A). However, treatment with ARB (Figure 3B) significantly increased the glomerular capillary density. The same result could be expressed graphically, as shown in Figure 3C.

VEGF is a potent angiogenic factor that maintains the glomerular and peritubular capillary network in the kidney [21]. VEGF is required for endothelial cell differentiation, vasculogenesis, angiogenesis and mesangial cell survival and differentiation. Here, we examined the expression of VEGF by immunofluorescence. We found that treatment with ARB (Figure 3E) caused significant increased intensity of VEGF expression in glomerulus compared with the untreated group (Figure 3D). In addition, we also found some BMDCs co-stain with VEGF indicated with small arrows in Figure 3E and 3I, more GFP/VEGF double-positive cells could be observed in ARB group than in untreated group (Figure 3J). These data indicate that BMDCs might promote glomerular repair by paracrine mechanism to some extent. The enhancement of VEGF expression by ARB administration has also been demonstrated by western blot (Figure 3F and 3G). Because podocytes are the major source of VEGF production in the glomerulus, we investigated it by double staining using podocyte marker (ZO-1) and VEGF. As shown in Figure 3H and 3I (indicated by the larger arrows), most of VEGF co-stain with podocyte in both groups. Therefore, we demonstrated that ARB treatment promotes glomerular repair by increased VEGF expression produced by podocyte and recruited BMDCs.

![Image](https://example.com/image.jpg)
Fig. 3. Valsartan administration promotes glomerular capillary repair and regeneration by enhanced VEGF expression. Representative images of immunofluorescent staining for glomerular capillary (RECA-1) from either untreated rat (A) or ARB treatment rat (B) on Day 56 after injection of 1-22-3 antibody. (C) Graphs showing mean glomerular capillary density in glomeruli of two groups (n = 20 each group). Representative confocal images of immunofluorescent staining VEGF in glomeruli from either untreated rat (D) or ARB treatment rat (E) on Day 56 after injection of 1-22-3 antibody. Double staining identifies VEGF produced by BMDCs marked with smaller arrows. (F) Western blot (upper) for VEGF (40-kDa) and loading control (β-actin, 45-kDa) in kidney cortex from chimera, untreated or ARB treatment rat on Day 56 after injection of 1-22-3 antibody. (G) VEGF protein expression was determined by western blot from chimera rat (normal), untreated rat (untreated) and ARB treatment rat (ARB) and normalized for β-actin expression. *P < 0.01 versus chimera group, #P < 0.05 versus untreated group. The representative confocal images of co-stain for podocyte (ZO-1, light green) and VEGF (red) in glomeruli on Day 56 after the injection of 1-22-3 antibody from either untreated rat (H) or ARB treatment rat (I). Double staining identifies VEGF produced by podocytes marked with bigger arrows. (J) Graph showing mean number of GFP/VEGF double-positive cells per glomerulus of two groups. *P < 0.05 versus untreated group. Data are mean values ± SD (bars, 50 μm).
Valsartan administration promoted BMDCs recruitment and nearly all expressed positive for the monocyte/macrophage marker positive. The representative confocal images of BMDCs recruitment into glomeruli from either untreated rat (A) or ARB treatment (B) on Day 56 after injection of 1-22-3 antibody. (C) Graph showing mean number of BMDCs in glomeruli of two groups. *P < 0.05 versus untreated group. The representative confocal images of immunofluorescent staining for monocytes/macrophages (CD68) with glomerular sections from either untreated rat (D) or ARB treatment (E) on Day 56 after injection of 1-22-3 antibody. (F) Graph showing the percentage of BM-derived monocytes/macrophages (CD68+), CD45-positive cells and CD45-negative cells both untreated and ARB group. The representative confocal images of immunofluorescent staining for leukocytes (CD45) with glomerular sections from untreated group (G) or ARB treatment group (H) on Day 56 after injection of 1-22-3 antibody. BMDCs with CD45—marked with arrows.

**Valsartan administration promoted BMDC recruitment and nearly all expressed positive for the monocyte/macrophage marker**

In the chimeric control rats without administration of 1-22-3 antibody, very few cells were observed within glomeruli (1.0 ± 0.2), shown in our previous study [17]. Eight weeks after administration of 1-22-3 antibody in chimeric rats, we found that treatment with ARB (Figure 4B) caused a significant increase in the number of glomerular BMDC compared with the untreated group (Figure 4A). The same result could be expressed graphically, as shown in Figure 4C. These findings indicate that treatment with ARB increases BMDC recruitment to injured glomeruli, which partly increases VEGF production involved in glomerular capillary repair and improved kidney function.

In the present work, we also investigated whether BMDCs recruited to injured glomeruli could differentiate into glomerular cells. We examined the recruitment of BMDCs into the glomeruli in the two groups by immunofluorescence with cell-specific markers. We used CD68 as a monocyte/macrophage marker, CD45 as a leukocyte marker, RECA-1 as an endothelial cell marker and OX-7 as a mesangial cell marker. In both untreated and ARB treatment group, nearly all recruited BMDCs expressed CD68 (Figure 4D and 4E) and CD45 (Figure 4G and 4H). We did not find convincing evidence that BMDCs expressed OX-7 and RECA-1 (data not shown), but we noticed that a minority do not express CD45 indicated with arrow (Figure 4H) and therefore require further characterization in future research. The percentage of different kind of cells from BM was shown in
Discussion

The present study shows that valsartan ameliorates progressive glomerulosclerosis in an experimental rat model, as exemplified by reduced mortality, improved renal function and pathological findings. Administration of valsartan causes increased intensity of glomerular VEGF expression and promotes the glomerular capillary repair and regeneration. In addition, valsartan promotes monocyte/macrophage recruitment into glomeruli from BM that express VEGF and are therefore likely to contribute to glomerular repair.

Progressive glomerulosclerosis is induced by many kinds of renal diseases including diabetic nephropathy, glomerulonephritis and others. However, no effective therapeutic strategy has been found to recovery progressive glomerulosclerosis. Angiotensin II (Ang II) triggers cell proliferation and accumulation of extracellular matrix through its Type 1 receptor (AT1), leading to glomerulosclerosis. Ang II is also an important mediator of oxidative stress that is involved in renal pathophysiology and vascular injury [22]. Inhibition of Ang II is therefore an important target in the treatment of chronic kidney disease. In the present study, we demonstrated that ARB therapy interrupts these pathogenic mechanisms in the glomerulus, indicating that angiotensin plays an important role in the progressive glomerulosclerosis.

Several studies have demonstrated that impairment of the capillary repair process in glomerular damage could be associated with the development of glomerular sclerosis and renal dysfunction [18, 20, 23]. Ang II infusion induces endothelial dysfunction [24], triggers reactive oxygen species by stimulating NAD(P)H oxidase [25] and causes vascular inflammation [26, 27]. In hypertensive humans, interruption of the renin–angiotensin system with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers restores endothelial function. Studies have shown that VEGF plays an important role in endothelial cell proliferation and capillary repair in damaged glomeruli [28]. The findings presented here show that ARB therapy significantly promotes regeneration of capillary loops and also enhances the expression of VEGF in glomeruli, consistent with previous study [29]. This evidence indicates that ARB enhances the glomerular capillary repair and regeneration not only by inhibition of Ang II but also by upregulation of VEGF expression.

Accumulating data demonstrate that adult neovascularization actually require BMDCs [30, 31]. To address this issue, we employed an EGFP transgenic rat for investigating the role of BMDCs with or without ARB treatment. We found that ARB administration resulted in increased BMDC recruitment into the injured glomerulus. To our knowledge, this is the first presentation of data showing that ARB can enhance the recruitment of BMDCs to injured glomeruli and promote the glomerular capillary repair and regeneration. Several studies have shown strong evidence that VEGF could promote the mobilization and recruitment of BM cells to target organ [32]. We examined whether ARB increases the expression of VEGF by immunofluorescent staining and western blot. It could be interpreted that ARB promotes BMDCs recruitment by VEGF pathway. Recently, our study demonstrated that hematopoietic stem cells could enhance the injured kidney repair following kidney ischemia–reperfusion injury by releasing multiple angiogenic factors, such as VEGF, hepatocyte growth factor [12]. But, the precise mechanism underlying BMDCs homing is still unclear. Further studies are required to understand homing. The majority of VEGF was produced by podocytes, as has been shown by other studies [33]. We found that macrophages can also secrete VEGF and promote glomerular repair and regeneration. VEGF, whether produced by podocytes or BMDCs may play a major role in promoting endothelial cell proliferation and glomerular capillary repair. Nearly all BMDCs recruited into glomeruli express monocyte/macrophage marker both control diseased rats and valsartan-treated rats (ARB group). Our recent publications have demonstrated that macrophage can promote kidney repair and regeneration following ischemia/reperfusion kidney injury [15]. We have also shown data that transfused CD34+ progenitor cells, fromBM, can promote kidney repair after ischemia injury [12]. In contrast, kidney repair and regeneration would be prevented if macrophage is deleted [14, 15, 34]. The present study suggests that ARB administration may enhance monocyte/macrophage recruitment that promotes glomerular repair by releasing local factors including VEGF. It is well-known that macrophages have heterogeneous phenotypes as they play diverse roles in the development and recovery of renal diseases. Some subpopulations of macrophages (M1) have a pathogenic function in renal inflammation. Alternatively, M2 macrophage subpopulations may resolve inflammation and repair injury. Different macrophage subpopulations release different kinds of cytokines that induce inflammation and fibrosis or promote repair [35]. Recently, some studies have demonstrated that transfused reprogrammed macrophages could protect against adriamycin nephropathy [36] and contribute to kidney repair and regeneration [37]. Further studies are required to characterize the macrophages in these kidneys to determine their phenotype and choose right macrophage as therapeutic tool. To determine whether BMDCs can differentiate into glomerular cells, we used RECA-1 as endothelial cell marker and OX-7 as mesangial cell marker. We did not find convincing evidence that BMDCs expressed OX-7 and RECA-1 (data not shown), but we noticed that a minority do not express CD45 and therefore require further characterization in future research. These data suggest that paracrine mechanism, not direct differentiation, plays a major role in kidney repair, which is consistent with our recent research [12]. Taken together, these data indicate that increased BMDCs recruited into glomeruli in response to ARB treatment express monocyte/macrophage that might contribute to kidney repair by releasing reparative cytokines including VEGF. Further studies are required to determine the reparative mechanism of monocyte/macrophage in this model.
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