Regression of Renal Disease by Angiotensin II Antagonism Is Caused by Regeneration of Kidney Vasculature

Andrea Remuzzi,*† Fabio Sangalli,* Daniela Macconi,* Susanna Tomasoni,* Irene Cattaneo,* Paola Rizzo,* Barbara Bonandrini,* Elena Bresciani,† Lorena Longaretti,* Elena Gagliardini,* Sara Conti,* Ariela Benigni,* and Giuseppe Remuzzi*‡

*IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Centro Anna Maria Astori, Science and Technology Park Kilometro Rosso Bergamo, Italy; †Department of Industrial Engineering, Bergamo University, Dalmine (Bergamo), Italy; and ‡Unit of Nephrology and Dialysis, Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy

ABSTRACT

Chronic renal insufficiency inexorably progresses in patients, such as it does after partial renal ablation in rats. However, the progression of renal diseases can be delayed by angiotensin II blockers that stabilize renal function or increase GFR, even in advanced phases of the disease. Regression of glomerulosclerosis can be induced by angiotensin II antagonism, but the effect of these treatments on the entire vascular tree is unclear. Here, using microcomputed tomography and scanning electron microscopy, we compared the size and extension of kidney blood vessels in untreated Wistar rats with those in untreated and angiotensin II antagonist–treated Munich Wistar Frömter (MWF) rats that spontaneously develop kidney disease with age. The kidney vasculature underwent progressive rarefaction in untreated MWF rats, substantially affecting intermediate and small vessels. Microarray analysis showed increased Tgf-β and endothelin-1 gene expression with age. Notably, 10-week inhibition of the renin-angiotensin system regenerated kidney vasculature and normalized Tgf-β and endothelin-1 gene expression in aged MWF rats. These changes were associated with reduced apoptosis, increased endothelial cell proliferation, and restoration of Nrf2 expression, suggesting mechanisms by which angiotensin II antagonism mediates regeneration of capillary segments. These results have important implications in the clinical setting of chronic renal insufficiency.


The progression of renal diseases leads invariably to end stage organ failure in patients as well as experimental models.1,2 This translates into one half million patients reaching ESRD each year globally and >700,000 deaths.2 The scale of the problem has been underestimated, whereas the costs for RRT are becoming problematic, even for wealthy nations. In the last 10 years, we and others have provided evidence that this progression can be significantly delayed and even reversed in rats and humans by the use of drugs that interfere with the renin-angiotensin system (RAS).3 However, despite important clinical implications, the mechanisms by which RAS inhibition induces reversal of renal lesions have not been established so far.

In this study, using imaging of rat kidney by microcomputed tomography (microCT) and scanning electron microscopy (SEM), we analyzed extension and organization of micro- and macrovasculature in Munich Wistar Frömter (MWF) rats, a model of progressive glomerular injury, and normal control Wistar rats. We also investigated the effect of RAS inhibition on kidney vasculature in MWF rats, treatments known to induce regression of renal structural and functional changes that develop spontaneously with age in these animals.4 Other than morphologic and morphometric evaluations of kidney vasculature, we finally elucidated the potential molecular mechanisms by gene and protein expression that could account for loss of vascular segments and changes in glomerular capillary (GC) organization in MWF rats and successful regeneration of kidney vasculature obtained by RAS inhibition.

We investigated kidney vasculature in normal Wistar and MWF rats at different ages and with different treatments as reported in the diagram in Figure 1A. In MWF rats, the spatial density of arteries

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Correspondence: Dr. Andrea Remuzzi, Department of Biomedical Engineering, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Centro Anna Maria Astori, Science and Technology Park Kilometro Rosso Via Stezzano, 87, 24126 Bergamo, Italy. Email: andrea.remuzzi@marionegri.it

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and veins was importantly reduced as kidney disease progressed at 50 weeks of age, when histologic damage at glomerular and tubular levels was already marked (Supplemental Figure 1, Supplemental Table 1) and important proteinuria developed, compared with Wistar rats (444±89 versus 6.6±1.5 mg/24 h in MWF50 versus Wistar50, respectively). The same pattern was observed for small arterial and venous vessels as well as the microcirculation (Figure 1B). Thus, the volume density of intermediate-sized vessels (80–180 μm diameter) was significantly reduced in aged MWF rats compared with controls (Figure 1, B and C). Both angiotensin–converting enzyme inhibitor (ACEi) and angiotensin II receptor antagonist (AIIRA) treatments markedly improved renal histology (with regression of glomerular and tubular damage) (Supplemental Figure 1, Supplemental Table 1), significantly reduced proteinuria (499±216 MWF60, 69±36 MWF+LIS60, and 101±42 mg/24 h MWF+LOS60), and robustly regenerated kidney vasculature (Figure 1D). Of note, both treatments importantly increased vasculature extension (Figure 1, C and D), which was higher than in control animals at same age. The regeneration of new vascular segments is also shown by the statistically significant increase in estimated length of small caliber blood vessels (Figure 1E). We also verified (as reported in Supplemental Material) that angiotensin–converting enzyme inhibition did not affect the volume density and estimated capillary length in two additional groups of Wistar rats (data not shown). Vascular rarefaction in MWF rats and regeneration after RAS inhibition were also confirmed by evaluating capillary volume density with rat endothelial cell antigen (Reca1) staining in both interstitial and glomerular areas (Figure 1F).

Vascular rarefaction is expected to translate in GC injury, which resulted in proteinuria, glomerulosclerosis, and

**Figure 1.** RAS blockade regenerates kidney vasculature. (A) Diagram of the experimental design. (B) Representative three–dimensional views of kidney vasculature by ex vivo microCT in 50- and 60-week-old MWF rats compared with 50-week-old Wistar rats and representation of intermediate–and small–sized capillary beds. (C) Quantification of vascular and kidney volume (top panel), vessels with diameter ranging from 80 to 180 μm (middle panel), and vessels with diameter <80 μm (bottom panel). (D) Representation of kidney vasculature in MWF rats on treatment with lisinopril and losartan. (E) Equivalent vessel length of blood vessels with diameters ranging from 80 to 180 μm (upper panel) and <80 μm (lower panel). (F) Quantification of capillary volume density (Vv) as a percentage of Reca1 positivity in interstitial (upper panel) and glomerular (lower panel) areas. Values are means±SDs; n=5 for Wistar and MWF50 rats, n=10 for untreated MWF60 rats and MWF rats treated from 50 to 60 weeks of age with lisinopril, and n=7 for MWF rats treated from 50 to 60 weeks of age with losartan. *P<0.05 versus MWF60; **P<0.01 versus MWF60; °P<0.05 versus MWF50; °°P<0.01 versus MWF50. LIS, lisinopril; LOS, losartan; Reca1, rat endothelial cell antigen.
loss of kidney function. We also reported that RAS inhibition induced regression of glomerular lesions and enhanced the tuft area devoid of sclerotic lesions. Here, we have now characterized the effects of ACEi and AIIRA on aged MWF rats at the level of GC ultrastructure by virtue of quantitative SEM. As shown in Figure 2A (and more extensively in Supplemental Figure 2), major changes characterized the glomerular population in MWF rats. Thus, mean GC volume ($V_G$) increased with age compared with that in Wistar rats, with remarkable broadening of $V_G$ distribution (Figure 2B), likely for a compensatory mechanism. Similarly, the shape of the GC tuft in MWF rats was more irregular compared with that in Wistar rats, which was shown by the distribution of the circularity parameter (Figure 2C). Morphometric estimation of GC length (Figure 2D) showed a more heterogeneous distribution in the MWF50 group, with some glomeruli characterized by very long (up to 32 mm) and short (<3 mm) capillaries. Of interest, the mean GC diameter was higher in MWF rats compared with Wistar rats (10.9±0.3 versus 7.4±0.4 μm). RAS inhibition in MWF rats by lisinopril did not reduce but actually, increased the glomerular enlargement and heterogeneity observed with age in these rats. Actually, $V_G$ was higher in lisinopril-treated MWF rats than in untreated animals, with a higher number of glomeruli with large volume (up to 8 $\mu\text{m}^3 \times 10^{-6}$). The same was observed for GC length (median [range]; 22.7 [4–51] in MWF +LIS60 versus 17.6 [3–61] mm in MWF60), with a larger fraction of glomeruli characterized by longer capillary length. On the contrary, capillary diameter remained constant (9.8±0.7 versus 9.4±0.6 μm). This detailed analysis of GC structure suggests that the kidney disease in this model modifies (to a major extent) the structure of functioning glomerular capillaries and ACEi treatment, likely affecting the remaining functional nephrons and further increasing the heterogeneity of the glomerular tufts with formation of new capillary segments in already enlarged capillary tufts. These changes likely provide higher volumes of functional GC segments and consequently, increased filtering surface area. Our results of increased mean diameter as well as segment length indicate that hydraulic resistance of GC networks may not be changed by treatments. Actually, enlargement of capillary diameter is expected to reduce hydraulic resistance, whereas elongation of capillary segments is expected to increase it. These two effects may cancel each other.

To clarify the molecular mediators underlying the regeneration of the kidney vasculature elicited by RAS blockade, we first analyzed by PCR array the renal expression profile of 84 genes involved in the modulation of the biologic processes of angiogenesis (Figure 3A). Unexpectedly, gene expression of vascular growth-promoting factors, such as Vegf and related receptors or angiopeptin-1 and -2, did not change between MWF60 and Wistar rats, whereas genes related to fibrosis, inflammation, and extracellular matrix remodeling were differentially expressed between the two strains. This framework is consistent with hallmarks of renal scarring that characterize advanced nephropathy in MWF rats when vasculature rarefaction was strongly evident. Upregulated profibrotic genes included the three Tgf–isoforms, with Tgf–2 being the most expressed, and endothelin-1 (Et–1). Validation of the array data by quantitative RT-PCR (Figure 3B) confirmed renal overexpression of Tgf–2 and Et–1 genes in MWF rats. Of interest is the observation that abnormal expression of such genes was almost normalized by both ACEi and AIIRA. These findings are in line with previous evidence showing that renoprotection induced by ACEi in MWF rats was associated with normalization of TGF– protein in glomeruli and the cortical interstitium and paralleled the reduction in urinary excretion of ET–1, which likely reflects the renal synthesis of the peptide. ET–1, synthesized

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Figure 2. ACE inhibition enhances GC volume and length. (A) Representative SEM images of GC lumen in Wistar and MWF rats. (B) $V_G$ distribution histograms in Wistar and MWF rats. (C) Distribution histogram of glomerular circularity. (D) Distribution histogram of estimated individual capillary length. Values are median (range; n=225 glomeruli per group). **p<0.01 versus MWF60; ***p<0.01 versus MWF50. LIS, lisinopril.
predominantly (although not exclusively) in endothelial cells (ECs), exerts proinflammatory, mitogenic, and profibrotic effects through the ET₁ receptor (ET₁R). At the level of glomerular microcirculation, it has been reported that podocyte-specific activation of TGF-β signaling results in the release of ET-1 by visceral epithelial cells that act as paracrine stimulus for EC dysfunction through ET₁R activation, setting in motion a vicious cycle that leads to podocyte depletion that eventuates in segmental glomerular damage. This evidence prompted us to investigate the role of endothelial deregulation of ET₁/ET₁R signaling in MWF rats, a well known model of progressive endothelial and podocyte loss. To identify the cellular sources of ET-1, we performed multiple immunostaining and found that ET-1 protein was highly expressed by both ECs and podocytes, which were documented by costaining of Reca1 and α-smooth muscle actin (α-SMA) in kidneys of MWF compared with Wistar rats (Supplemental Figure 3A). Renal ET₁R expression in the vascular endothelium of MWF rats was also increased in a time-dependent manner (Supplemental Figure 3B). Angiotensin II inhibition markedly reduced ET-1 renal expression to control levels, particularly in cortical interstitium (Supplemental Figure 3A), and normalized ET₁R endothelial expression, which was indicated by reduced ET₁R staining in Reca1-positive cells (Supplemental Figure 3B).

Both TGF-β2 and ET-1 promote the endothelial-to-mesenchymal transition,
which generates matrix-producing fibroblasts and/or myofibroblasts, while directly leading to EC loss.\textsuperscript{12-15} Although we cannot exclude the contribution of pericyte detachment from EC to scar-forming myofibroblasts, to assess the contribution of the endothelial-to-mesenchymal transition to vascular rarefaction, we evaluated the expression of the mesenchymal marker $\alpha$-smooth muscle actin ($\alpha$SMA) at the vascular level by means of colocalization with the Reca1 antigen. Glomerular and interstitial peritubular capillaries of MWF rats strongly expressed $\alpha$SMA, whereas only occasional staining was observed in Wistar rats. RAS blockade by ACEi and AIIRA significantly reduced $\alpha$SMA expression in kidney microvasculature of MWF rats to an equal extent (Figure 3, C and D). A similar mechanism seems to operate at the level of larger vessels to the extent that $\alpha$SMA staining progressively increased, reaching a peak at 60 weeks in MWF rats. RAS blockade did not simply lower $\alpha$SMA accumulation in large vessels but even normalized it in most animals (Figure 3E).

Collectively, these data indicate that inhibition of TGF-$\beta$ and ET-1/ET$_{\alpha}$R signaling likely explains the beneficial effect of RAS blockade on the regeneration of kidney vasculature in advanced nephropathy. Because on RAS blockade, new vessel segment and capillary formation occurs, we further explored how the balance between apoptosis and proliferation at the EC level could account for vessel regeneration. Activated caspase3 was strongly upregulated in Reca1-positive ECs in MWF rats at 50 weeks of age and even more at 60 weeks of age (Figure 4A). However, ECs were induced to proliferate, which was confirmed by the increased number of Ki67-positive cells in the renal vascular compartment, possibly to counterbalance the onset of the apoptotic events (Figure 4B). Angiotensin II inhibition significantly

Figure 4. RAS blockade reduces EC apoptosis, while increases proliferation, and restores Nrf2 expression. (A) Immunostaining for Reca1 (white) and caspase3 (red). Apoptotic ECs were evaluated by semiquantitative score (from zero to three in each field). (B) Immunostaining and quantification of Reca1 (white) and Ki67 (red) expression in untreated and lisinopril–or losartan-treated MWF rats. Nuclei were stained with DAPI (blue), and renal structures were stained with WGA (green). The enlarged details are shown in Insets in A and B. (C) Immunostaining for Nrf2 in formalin–fixed, paraffin–embedded kidney sections by immunoperoxidase technique. Nrf2 expression was evaluated by semiquantitative score (from zero to three in each field). Values are means±SDs (A and B, $n=3$; C, $n=4$). Scale bar, 50 $\mu$m. **$P<0.01$ versus MWF60; ***$P<0.01$ versus MWF50. DAPI, 4',6-diamidin-2-fenilindolo LIS, lisinopril; LOS, losartan; Reca1, rat endothelial cell antigen1; WGA, wheat germ agglutinin.
reduced the number of apoptotic cells and sustained the compensating protective mechanism fostering EC proliferation, which could account for the important increase in the density and length of the microvessels observed by microCT (Figure 1, C and D) in treated MWF rats. NF-E2–related factor2 (Nrf2) has been recently recognized as a critical intracellular regulator of EC dynamics, governing angiogenic sprouting and vascular branching.16 Such pathways can be theoretically targeted therapeutically to counteract the effects of oxidative stress and TGF-β/Smad–mediated renal fibrosis.17–20 Finding here that RAS blockade rescues Nrf2 expression (as shown in Figure 4C), which was significantly decreased in untreated MWF rats, is consistent with this possibility.

In conclusion, our findings of vascular rarefaction in progressive renal disease in the MWF rat and vascular regeneration on RAS inhibition disclose a new paradigm for renal disease progression and offer room for therapeutic interventions. Vascular regeneration by angiotensin II antagonism, documented here for the intrinsic reparative capability of the kidney by halting TGF-β and ET-1–mediated damage. This phenomenon enhances the intrinsic reparative capability of the kidney by balancing regeneration and fibrosis and promoting angiogenesis, Nrf2–dependent vascular remodeling, and EC proliferation. These effects translate into improvement of tissue blood perfusion and filtration capacity and regression of kidney fibrosis. These results have obvious important implications for human medicine, showing effective improvement of an important clinical problem2 that has costs are becoming problematic, even for wealthier nations.

CONCISE METHODS

Animal Studies
The study design is graphically reported in Figure 1A, and more detailed information is reported in Supplemental Material. Briefly, one group of Wistar rats was studied at 30 weeks of age, and four groups of MWF rats were studied at 50 and 60 weeks of age with and without treatment with lisinopril or losartan. Kidney vasculature was investigated by microCT imaging with the use of a radio-opaque resin (Microfil; Flow Tech) injected in the kidney under general anesthesia (Supplemental Material). Imaging by microCT was obtained by three–dimensional numeric reconstruction using the NRecon software (Bruker-MicroCT), and quantification of vasculature components was performed using CTAnalyser software (Bruker-MicroCT). Details on calculations and statistics of geometric parameters of kidney blood vessels are reported in Supplemental Material. Glomerular microcirculation was investigated by morphologic and quantitative SEM using a casting technique and imaging on a scanning electron microscope (Cross-Beam 1540ESB; Carl Zeiss). Morphometric analysis was performed on digital images to estimate GC dimensions as described in Supplemental Material.

Gene Expression Analysis and Immunohistochemistry
Total RNA was extracted with the Qiagen RNeasy Mini Kit and reverse transcribed with the RT2 First-Strand Kit (Qiagen). The Rat Angiogenesis Pathway RT2 Profiler PCR Array (Qiagen) was used to determine the differentially expressed genes. Validation of Tgf-β2 and E2a–1 gene expression was performed by quantitative RT-PCR. Reca1, αSMA, Ki67, caspase3, ETα, R, ET-1, and α-actinin4 protein expression was evaluated by immunofluorescence experiments, whereas Nrf2 expression was detected by the immunoperoxidase technique, which is specified in Supplemental Material.

Statistical Analyses
Data are expressed as means ± SDs or medians and ranges as specified. Statistical analysis was performed using ANOVA (Prism 6.0; GraphPad Software Inc., San Diego, CA). Bonferroni post hoc analysis was adopted to estimate statistical significance between groups’ comparisons. Statistical significance was defined as $P<0.05$.

Study Approval
Animal care and treatment were conducted in accordance with the institutional guidelines and compliance with national (DE 116, GU 18/2/1992, Circ. 8, and G.U 14/7/1994) and international laws and policies (Dir. 2010/63/EU and 9/22/2010). All animal studies were approved by the Institutional Animal Care and Use Committee of Istituto di Ricerche Farmacologiche Mario Negri.

REFERENCES


