CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Nature and Mediators of Parietal Epithelial Cell Activation in Glomerulonephritides of Human and Rat

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Bowman's capsule parietal epithelial cell activation occurs in several human proliferative glomerulonephritides. The cellular composition of the resulting hyperplastic lesions is controversial, although a population of CD133⁺CD24⁺ progenitor cells has been proposed to be a major constituent. Mediator(s) involved in proliferation and migration of progenitor cells into the Bowman's space have been poorly explored. In a series of 36 renal biopsies of patients with proliferative and nonproliferative glomerulopathies, dysregulated CD133⁺CD24⁺ progenitor cells of the Bowman's capsule invade the glomerular tuft exclusively in proliferative disorders. Up-regulation of the CXCR4 chemokine receptor on progenitor cells was accompanied by high expression of its ligand, SDF-1, in podocytes. Parietal epithelial cell proliferation might be sustained by increased expression of the angiotensin II (Ang II) type-1 (AT₁) receptor. Similar changes of CXCR4, SDF-1, and AT₁ receptor expression were found in Munich Wistar Frömter rats with proliferative glomerulonephritis. Moreover, an angiotensin-converting enzyme inhibitor normalized CXCR4 and AT₁ receptor expression on progenitors concomitant with regression of crescentic lesions in a patient with crescentic glomerulonephritis. These results suggest that glomerular hyperplastic lesions derive from the proliferation and migration of renal progenitors in response to injured podocytes. The Ang II/AT₁ receptor pathway may participate, together with SDF-1/CXCR4 axis, to the dysregulated response of renal precursors. Thus, targeting the Ang II/AT₁ receptor/CXCR4 pathways may be beneficial in severe forms of glomerular proliferative disorders. (*Am J Pathol* 2013, 183: 1769–1778; <http://dx.doi.org/10.1016/j.ajpath.2013.08.008>)

Glomerular injury caused by multiple etiologies can lead to activation and accumulation of parietal epithelial cells within the Bowman's space as a common response to damage.¹ The most diffuse lesions are found in rapidly progressive idiopathic glomerulonephritis, Wegener's granulomatosis, and anti-glomerular basement membrane antibody disease. Extracapillary proliferation can also be observed in lupus nephritis, IgA nephropathy, and membranoproliferative glomerulonephritis,^{2,3} and if left untreated, can result in rapidly progressive renal failure.⁴

Understanding the mechanism(s) for the formation of these multilayered cellular lesions would lead to the development of more targeted therapies than the currently used cytotoxic agents. Although extracapillary proliferation is a relatively straightforward pathological change to recognize, more

controversial has been determining its cellular components and their possible pathogenic role. The traditional concepts have come largely from immunohistochemical studies that have concluded that multilayered cellular lesions are a mixture of glomerular parietal epithelial cells, macrophages, and myofibroblasts,^{5–7} the proportion of such cells in the lesion being variable. In both animal models and human tissues, parietal epithelial cells predominate when Bowman's capsule is intact, whereas macrophages and myofibroblasts

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prevail when Bowman's capsule is ruptured,^{5,8,9} an event reported to be rare.¹⁰ More recent studies also suggested the podocytes as key constituents of the crescents,^{11–14} but their role remains unclear. Given their terminally differentiated phenotype,^{15–17} they could not theoretically migrate and proliferate, limiting their contribution, if any, in extracapillary lesion formation. Recently, a heterogeneous population of renal progenitor cells, previously identified in normal human Bowman's capsule,¹⁸ has been documented in hyperplastic lesions of human crescentic glomerulonephritis.¹⁹ It has been suggested that these extracapillary lesions could be the result of dysregulated proliferation of renal progenitor cells in response to the injured podocytes.¹⁹ This possibility is supported by our finding in Munich Wistar Frömter (MWF) rats, which are genetically programmed to develop renal damage characterized by excessive progenitor cell migration and proliferation leading to their accumulation into cellular lesions and glomerulosclerosis.²⁰ Nevertheless, the mechanisms and mediators responsible for the intra-glomerular accumulation of renal progenitor cells in proliferative glomerulonephritis remain ill-defined. Evidence in SCID mice with acute renal failure indicates an important role of stromal cell-derived factor-1 (SDF-1) and its receptor C-X-C chemokine receptor type 4 (CXCR4) in the therapeutic migration of renal progenitor cells.²¹ Because activation of the angiotensin II (Ang II)/angiotensin II type-1 (AT₁) receptor pathway may contribute to cell proliferation and migration processes,²² and based on previous findings that angiotensin-converting enzyme (ACE) inhibition reduced glomerular lesions by limiting renal progenitor cell migration,²⁰ we reasoned that the AT₁ receptor could also play a key role in the abnormal proliferation of renal progenitors underlying the hyperplastic lesions.

Therefore, building on the previous experimental work suggesting that both the SDF-1/CXCR4 and the Ang II/AT₁ receptor pathway play a role in sustaining the migratory/proliferative properties of renal progenitor cells, we sought to get further insights into the cellular mechanism(s) and mediators contributing to extracapillary proliferation in humans. In particular, our efforts focused on i) further highlighting the key role of renal progenitor cells in the multilayered accumulation of proliferating cells in the Bowman's space of patients with proliferative glomerulonephritis; ii) dissecting the contribution of the SDF-1/CXCR4 axis to the migratory property of dysregulated progenitor cells; and iii) testing whether the Ang II/AT₁ receptor pathway critically participates, together with the chemokine/receptor, to migration and proliferation of renal progenitors, resulting in the progression of extracapillary lesions.

Materials and Methods

Patients

Thirty six patients with proliferative and nonproliferative glomerulopathies from the archives of the Unit of

Nephrology, Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy, were enrolled in the study. Patients with glomerular extracapillary proliferative lesions had either extracapillary glomerulonephritis ($n = 9$) or IgA nephropathy ($n = 9$). Patients with nonproliferative diseases were diagnosed as either membranous nephropathy ($n = 7$) or diabetic nephropathy ($n = 11$). Written informed consent was obtained from all these patients. Demographic, clinical, and hematological parameters at the time of renal biopsy were retrieved from the hospital database. In addition, to further address the possible participation of the Ang II/AT₁ receptor pathway and renal progenitor cells in the development of glomerular hyperplastic lesions, we analyzed in-depth renal tissue specimens from one of these patients with antineutrophil cytoplasm antibody—positive crescentic glomerulonephritis from whom two biopsies were collected, before and after 8 months therapy with the ACE inhibitor ramipril (titrating up the dose from 2.5 to 7.5 mg/day) associated with the immunosuppressant azathioprine (50 mg/day). All kidney biopsy specimens considered for the present study had been originally obtained for the diagnosis of renal disease. In addition to specimens from patients with proliferative and nonproliferative diseases, renal biopsies from an uninvolved portion of kidney collected from tumor nephrectomy specimens were obtained from 10 patients and used as controls.

Immunofluorescence and Confocal Microscopy

Archived kidney biopsies, snap frozen in liquid nitrogen and embedded in OCT compound, were used for immunofluorescence analysis. Double or triple immunostaining for CD24, CD133, CXCR4, SDF-1, nephrin, CD68, ED1, and AT₁ receptor expression was performed. Three-micron frozen sections were air dried, fixed with cold acetone, washed with PBS, and incubated with 1% bovine serum albumin to block nonspecific sites. The following primary antibodies were used: goat or mouse anti-CD24 (1:25; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-CD133 (1:50; Miltenyi Biotec, Bergisch Gladbach, Germany), rabbit anti-CXCR4 (1:50; Abcam, Cambridge, UK), rabbit anti-SDF-1 (1:200; Abcam), goat anti-nephrin (1:50; Santa Cruz Biotechnology), mouse anti-CD68 (1:100; Dako, Glostrup, Denmark), mouse anti-ED1 (1:100; Millipore, Billerica, MA) and rabbit anti-AT₁ receptor (1:25; Santa Cruz Biotechnology), followed by the specific fluorescein isothiocyanate or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were stained with DAPI, and the renal structure with lectins, either rhodamine lens culinaris agglutinin or fluorescein wheat germ agglutinin (Vector Laboratories, Burlingame, CA). Negative controls were obtained by omitting primary antibodies on adjacent sections. Fluorescence was examined by an inverted confocal laser scanning microscope (LS 510 Meta; Zeiss, Jena, Germany). For SDF-1 expression in patients with extracapillary glomerulonephritis and in normal kidneys, all glomeruli were acquired and subjected to semiquantitative analysis. Glomerular SDF-1 expression

was graded on a scale of 0 to 3 (0: no staining, 1: mild, 2: moderate, 3: strong diffuse). For AT₁ receptor expression in MWF and Wistar rats, all glomeruli were acquired and subjected to quantitative analysis. The number of AT₁ receptor⁺ cells was counted with respect to the Bowman's capsule length.

Immunoperoxidase

Human biopsies, which contained 5 to 30 glomeruli, were also analyzed for AT₁ receptor expression by immunoperoxidase staining. Duboscq-Brazil—fixed, 3- μ m paraffin-embedded kidney sections were deparaffinized, rehydrated, and incubated for 30 minutes with 0.3% H₂O₂ in methanol to quench endogenous peroxidase. Antigen retrieval was performed using microwave [twice for 5 minutes in citrate buffer 10 mmol/L (pH 6.0) at an operating frequency of 2450 MHz and 600W power output] and citrate buffer incubation (15 minutes at room temperature) to increase the reactivity of the antibody to antigen. After blocking with 1% bovine serum albumin, sections were incubated with rabbit anti-AT₁ receptor primary antibody (1:25; Santa Cruz Biotechnology), followed by a specific biotinylated secondary antibody (Jackson ImmunoResearch Laboratories) and diaminobenzidine (Merck, Darmstadt, Germany) substrate solution. Slides were finally counterstained with hematoxylin, dehydrated in graded alcohols, mounted with coverslips, and then observed by light microscopy (ApoTome Axio Imager Z2; Zeiss). Negative controls were obtained by omitting the primary antibody on adjacent sections.

MWF Rat Model of Crescentic Glomerulonephritis

Eighteen male MWF rats from our colony that develop renal damage characterized by extracapillary lesions and glomerulosclerosis²⁰ were divided into two groups as follows: group 1 ($n = 6$) received saline and was sacrificed at 60 weeks of age; group 2 ($n = 6$) received the ACE inhibitor lisinopril (80 mg/L in the drinking water) from 50 to 60 weeks of age. Sixty-week-old Wistar rats (Charles River S.p.A., Calco, Italy) were used as controls ($n = 6$). All rats were maintained in a room with constant temperature and light, having free access to water and food. Animal care and treatment were conducted according to the institutional guidelines that are compliant with national (Decreto Legislativo n116, Gazzetta Ufficiale suppl. 40, 18/2/1992, Circolare N.8, Gazzetta Ufficiale 14/7/1994) and international laws and policies (EEC Council Directive 86/609, OJL 358-1, 1987; Guide for the Care and Use of Laboratory Animals. NIH publication n. 85-23. Revised 1996). All animal studies were approved by the Institutional Animal Care and Use Committees of IRCCS - Istituto di Ricerche Farmacologiche Mario Negri. At sacrifice, kidneys were perfused with PBS under anesthesia, fixed in 4% paraformaldehyde, snap frozen in liquid nitrogen, and embedded in optimal cutting temperature compound.

Statistical Analysis

Results were expressed as means $\pm$ SD. Statistical analysis of AT₁ receptor quantification in experimental animals was performed using analysis of variance with the Bonferroni post hoc analysis for multiple comparisons. The Student's *t*-test was applied for SDF-1 quantification in patients. Statistical significance was defined as $P < 0.05$.

Results

Clinical and Histopathological Characteristics of Patient Populations

Two cohorts of patients affected by glomerular diseases associated with or without extracapillary proliferation at renal biopsy were evaluated, and their clinical and histopathological characteristics are outlined in Table 1. Demographic and clinical findings at the time of biopsy were similar among the different groups. The mean age of the patients was 53 years, and there was a slight male predominance. There were no differences in baseline systolic and diastolic blood pressure among groups, whereas proteinuria and serum creatinine levels were slightly lower in patients with IgA nephropathy. At histological examination, renal biopsies of patients with extracapillary glomerulonephritis, and to a lesser extent with IgA nephropathy, showed a high percentage of glomeruli with extracapillary proliferation. These proliferative lesions were absent in biopsies of patients with membranous or diabetic nephropathies.

CD133⁺CD24⁺ Renal Progenitor Cells Are Major Constituents of Hyperplastic Lesions

Consistent with our previous study,¹⁹ multiple layers of cells in the Bowman's space of tissue specimens from patients with extracapillary glomerulonephritis were mostly composed of CD133⁺CD24⁺ cells (Figure 1A). To further confirm that accumulation of progenitor cells in the glomerular tuft was a feature of proliferative diseases, we also analyzed biopsies of patients with IgA nephropathy, in which abundant CD133⁺CD24⁺ cells were consistently found in all segmental lesions between the Bowman's capsule and the glomerular capillary tuft (Figure 1B). Exuberant progenitor cell accumulation was confined to proliferative disorders, as it was not found in biopsies of patients with membranous nephropathy (Figure 1C) and diabetic nephropathy (Figure 1D). In these latter settings, the distribution of CD133⁺CD24⁺ cells along the Bowman's capsule was comparable to control kidneys (Figure 1E).

CXCR4 Is Overexpressed in Progenitor Cells within Extracapillary Lesions

In vitro, human renal progenitor cells express the chemokine receptor CXCR4, which has been related to the migratory

Table 1 Demographic, Clinical, and Histopathological Characteristics of Patient Populations

	Proliferative		Nonproliferative	
	Extracapillary glomerulonephritis	IgA nephropathy	Membranous nephropathy	Diabetic nephropathy
Patients, <i>n</i>	9	9	7	11
Age, years	32–81	32–50	44–66	54–70
Male:female	3:6	7:1	4:3	9:1
SBP, mm Hg	135.6 ± 4.7	126.0 ± 8.1	136.8 ± 4.7	145.8 ± 5.4
DBP, mm Hg	80.0 ± 1.78	78.8 ± 5.3	80.8 ± 0.8	85.0 ± 2.1
Proteinuria, g/day	5.6 ± 1.4	1.2 ± 0.2	5.6 ± 1.3	4.1 ± 0.6
Serum creatinine, mg/dL	3.9 ± 0.9	1.8 ± 0.6	2.5 ± 0.6	1.2 ± 0.2
Glomeruli with extracapillary proliferation, %	60.9 ± 8.6	15.0 ± 5.8	0.0 ± 0.0	0.4 ± 0.0
Normal glomeruli, %	9.4 ± 5.5	16.1 ± 5.6	17.9 ± 4.6	30 ± 7.2
Glomeruli with global sclerosis, %	17.5 ± 4.3	32.8 ± 9.2	14.8 ± 7.9	15.0 ± 3.8
Glomeruli with segmental disorders, %	4.4 ± 3.0	14.4 ± 3.4	1.9 ± 1.9	27.2 ± 10.2
Other glomerular changes, %*	21.8 ± 4.2	28.2 ± 4.2	71.4 ± 4.5	49.5 ± 6.4

*Capillary loop thickening, mesangial hypercellularity and matrix expansion, and epithelial vacuolization.
DBP, diastolic blood pressure; SBP, systolic blood pressure.

property of these cells.²¹ We first evaluated the expression of CXCR4 in renal progenitor cells in proliferative glomerulonephritis and nonproliferative glomerular disorders. Biopsy immunostaining of patients with extracapillary glomerulonephritis and IgA nephropathy revealed high CXCR4 expression on the majority of CD24⁺ progenitor cells within hyperplastic lesions (Figure 2A). On the contrary, in biopsies from patients with membranous nephropathy or diabetes (Figure 2, B and C), the expression of CXCR4 was faint and mainly localized in the Bowman’s capsule, similar to the distribution in glomeruli from normal kidneys, where staining was restricted to a few CD24⁺ progenitor cells (Figure 2D).

Podocytes Express the CXCR4 Ligand, SDF-1

Concomitant with high expression of CXCR4 in renal progenitor cells, a significantly increased expression of the

CXCR4 ligand, SDF-1, was found in glomerular tuft of biopsies from patients with proliferative glomerulonephritis in respect to controls (Figure 3, A–C). Quantification of SDF-1 revealed that the difference was statistically significant (*P* < 0.05) (Figure 3D). By double immunostaining, we investigated the cell population that expressed SDF-1. Macrophages were found to infiltrate the hyperplastic lesions as revealed by the presence of CD68⁺ cells in hyperplastic lesions (Figure 3A). Double immunostaining of CD68 and SDF-1 revealed that macrophages were not the cellular source of SDF-1 (Figure 3A) as documented by the absence of signal colocalization. Instead, co-staining of nephrin and SDF-1 revealed that podocytes expressed SDF-1 and so, presumably, provide the ligand for CXCR4 in patients with proliferative glomerulonephritis (Figure 3B). A few parietal epithelial cells also expressed SDF-1 to a comparable extent in proliferative diseases and in controls (Figure 3, A–C).

Figure 1 CD133-CD24 expression in patients with proliferative and nonproliferative disorders. **A:** Double immunofluorescence staining for CD24 (green) and CD133 (red) revealed that in a glomerulus of a patient with extracapillary glomerulonephritis, the two antigens colocalized inside crescentic lesions. **B:** In IgA nephropathy, abundant CD133⁺CD24⁺ cells are found in the lesions between the Bowman’s capsule and the glomerular capillary tuft. **C–E:** In membranous nephropathy (**C**) and in diabetic nephropathy (**D**), CD133⁺CD24⁺ cells are confined to the Bowman’s capsule as observed in normal control kidneys (**E**). Scale bars: 50 μm.

Figure 2 CXCR4 is overexpressed in progenitor cells within hyperplastic lesions in patients with proliferative diseases. **A:** In patients with extracapillary glomerulonephritis, CXCR4 (green) is expressed by the majority of CD24⁺ progenitor cells (red) within hyperplastic lesions. High magnification of CXCR4 and CD24 staining (inset) is shown in the right panels. **B and C:** In patients with membranous (**B**) and diabetic (**C**) nephropathies, CXCR4 expression is faint, and no cells in the Bowman's capsule coexpressed the CD24 progenitor marker. **D:** Representative photomicrograph of double staining for CXCR4 and CD24 in a normal human glomerulus showing that few cells in the Bowman's capsule co-expressed the two markers. High magnification of CXCR4 and CD24 staining in the Bowman's capsule (inset) is seen in the right panels. DAPI (blue) stained nuclei. Scale bars: 50 μ m; 25 μ m for high magnification (**A** and **D**, right panels).

AT₁ Receptor Overexpression on Progenitor Cells in Human Proliferative Disorders

Because activation of the Ang II/AT₁ receptor pathway may favor cell proliferation and migration,²² we then assessed the expression of the AT₁ receptor in progenitor cells in proliferative

disorders. In both patients with extracapillary glomerulonephritis and IgA nephropathy, the AT₁ receptor was abundantly expressed by progenitor cells along the Bowman's capsule as well as within cellular synechiae, the early morphological abnormalities preceding extracapillary lesion formation²⁰ (Figure 4A). Expression of the AT₁ receptor was maintained by progenitor cells forming crescentic lesions, as demonstrated by double immunofluorescence staining with the progenitor cell marker CD24 (Figure 4B). This was not the case in biopsy tissues from patients with membranous nephropathy and diabetes (Figure 4C) and in normal kidney (Figure 4D), where AT₁ receptor expression was only confined to a few cells in the Bowman's capsule (Figure 4, C and D). Colocalization of the AT₁ receptor with CD24 in normal kidney indicated that the rare parietal epithelial cells expressing this receptor were progenitor cells (Figure 4E).

Pattern of CXCR4 and SDF-1 Expression in Hyperplastic Lesions in the Rat

To evaluate whether parietal epithelial cell activation translated to more CXCR4 expression as in patients with proliferative glomerulonephritides, we studied the MWF rat model characterized by extracapillary proliferation and glomerulosclerosis.²⁰ Consistent with the human results, CXCR4 expression was markedly increased in the hyperplastic lesions of MWF rats (Figure 5A). The CXCR4 ligand SDF-1 was localized to podocytes in MWF rats as documented by double immunostaining for the chemokine and nephrin (Figure 5D). By contrast, SDF-1 staining showed no colocalization with ED1 macrophages (Figure 5G). MWF rats receiving ACE inhibitor treatment showed a normalization of the immunostaining for both CXCR4 and SDF-1 (Figure 5, B

Figure 3 SDF-1 is expressed by podocytes, but not macrophages, in proliferative glomerulonephritides. **A:** SDF-1 (red) is highly expressed in the glomerulus of patient with extracapillary glomerulonephritis and the double immunofluorescence staining of SDF-1 (red) and CD68 (green) does not show colocalization of the two antigens. **B and C:** SDF-1 (red) colocalized with the podocyte marker nephrin (green). DAPI (blue) stained nuclei. Scale bars: 50 μ m. **D:** SDF-1 expression is significantly increased in glomeruli of patients with extracapillary glomerulonephritis as compared with normal kidneys. * $P < 0.05$ versus normal kidneys.

Figure 4 AT_1 immunoexpression is up-regulated in proliferative diseases. **A:** Immunoperoxidase staining shows that in a patient with extracapillary glomerulonephritis, the cells forming the bridges between the Bowman's capsule and the capillary tuft abundantly express the AT_1 receptor (AT_1R ; arrows). **B:** Double immunofluorescence staining showing some $CD24^+$ progenitor cells (red) inside hyperplastic lesions expressing the AT_1 receptor (green, right panels, which show the high magnification of the inset). **C** and **D:** Representative photomicrographs of immunoperoxidase staining in nonproliferative diseases (**C**) and in a normal human glomerulus (**D**) showing AT_1 receptor expression in a few parietal cells of the Bowman's capsule (arrows). **E:** Colocalization of AT_1 receptor (green) and $CD24$ (red) expression shows that in normal human kidneys, the few parietal epithelial cells expressing the AT_1 receptor are progenitor cells (right panels, which show the high magnification of the inset). DAPI (blue) stained nuclei. Scale bars: 50 μm .

and E) and no more abundance of ED1 macrophages than in the normal kidney (Figure 5, H and I).

AT_1 Receptor Overexpression on Progenitor Cells in Rat Proliferative Disorders

We also assessed whether up-regulation and activation of the AT_1 receptor on progenitor cells of the Bowman's capsule contribute to their migration and proliferation into the glomerular hyperplastic lesions. In these animals, the AT_1 receptor was abundantly expressed by glomerular cells including parietal epithelial cells (Figure 6, A and D), as well as in the area of hyperplastic lesions (Figure 6A), very reminiscent of the expression profile in patients with proliferative disorders. Consistent with our previous findings,²⁰ MWF rats given the ACE inhibitor lisinopril showed negligible renal progenitor cell proliferation and migration. This treatment was associated with a significant reduction in the number of progenitor cells in the Bowman's capsule expressing the AT_1 receptor compared with untreated animals (Figure 6, B–D), suggesting a key role of the AT_1 receptor in progenitor cell-mediated extracapillary proliferation.

ACE Inhibitor Therapy Limits Progenitor Cell Proliferation by Restoring AT_1 Receptor Expression in a Patient with Extracapillary Glomerulonephritis

To provide evidence that up-regulation of AT_1 receptor contributes to the formation of glomerular hyperplastic

lesions in humans as well, we took advantage of the case of a 69-year-old patient with antineutrophil cytoplasm antibody-positive crescentic glomerulonephritis (already included in the cohort of patients studied here) who underwent a repeated renal biopsy after 8-month treatment with an ACE inhibitor (Figure 7A). At the first biopsy, 73.7% of the glomeruli were affected by florid extracapillary proliferation, whereas the remnant glomeruli were totally sclerotic, and no one glomerulus was intact and free of lesions (Figure 7, B and C). Because of high proteinuria (2.60 g/day) and severe renal insufficiency (serum creatinine, 6.99 mg/dL), the patient received standard immunosuppressive therapy with steroids and cyclophosphamide. After 7 months of treatment, proteinuria worsened (3.70 g/day), despite mild amelioration of renal function (serum creatinine, 4.37 mg/dL) (Figure 7A). Thus, therapy was modified and increasing doses of the ACE inhibitor ramipril (from 2.5 to 7.5 mg/day) in association with azathioprine were started. Eight months later, both proteinuria and serum creatinine levels were markedly reduced to 1.34 g/day and 1.74 mg/dL, respectively (Figure 7A). At this time, a second biopsy revealed that all glomeruli in the tissue specimen were

Figure 5 CXCR4 and SDF-1 up-regulation is normalized in MWF rats receiving the ACE inhibitor. **A:** CXCR4 (red) is markedly overexpressed by cells within hyperplastic lesions (box) in the MWF rat glomerulus. **B:** ACE inhibitor treatment normalizes CXCR4 expression to a level comparable with controls (**C**). **D:** SDF-1 (red) is up-regulated in the MWF rat glomerulus and colocalized with nephrin (arrows point to double-labeled podocytes stained yellow). Representative photomicrographs of double immunostaining for SDF-1 (red) and nephrin (green) (**D–F**) and SDF-1 (red) and ED1 (green) (**G–I**) show lack of association. ACE inhibitor reduces the abnormal expression of SDF-1 (red) (**E** and **H**). DAPI (blue) stained nuclei, and renal structures were labeled with fluorescein wheat germ agglutinin (WGA; green). Scale bars: 25 μm .

Figure 6 AT₁ receptor overexpression in the cells of the Bowman's capsule in MWF rats is reduced by ACE inhibitor treatment. **A:** In MWF rats given saline, the AT₁ receptor (green) is expressed by the majority of parietal epithelial cells (arrows) as well as by cells within hyperplastic lesions (arrowheads), which possibly represent renal progenitor cells. **B:** ACE inhibitor treatment reduces the expression of the AT₁ receptor on parietal epithelial cells (arrows). **C:** In Wistar rats used as controls, few cells in the Bowman's capsule expressed the AT₁ receptor (arrow). DAPI (blue) stained nuclei, and renal structures were labeled with rhodamine lens culinaris agglutinin (red). Scale bars: 25 μ m. **D:** The number of cells expressing the AT₁ receptor along the Bowman's capsule is significantly higher in MWF rats given saline as compared with controls. AT₁ receptor overexpression on parietal epithelial cells, which were assumed to be progenitors, is reduced by ACE inhibitor treatment. ** $P < 0.01$ versus Wistar rats; †† $P < 0.01$ versus MWF rats receiving saline.

totally free of crescents, suggesting the remission of crescentic lesions observed in the first biopsy (Figure 7, B–D). Overall, 38.5% of glomeruli were normal, whereas 46.1% showed global sclerosis.

Double immunostaining of the first biopsy revealed that the AT₁ receptor was highly expressed and colocalized on CD24⁺ progenitor cells in the area of hyperplasia (Figure 7E). In the second biopsy, AT₁ receptor staining was markedly reduced on CD24⁺ progenitor cells (Figure 7F), which were no longer present within the extracapillary space, but only localized along the Bowman's capsule (Figure 7G), as previously documented in patients with nonproliferative diseases as well as in normal kidneys. Of note, CXCR4 expression, which in the first biopsy colocalized with the progenitor cell marker CD24 in the majority of cells in crescentic lesions (Figure 2A), was only faintly expressed after ACE inhibitor treatment (Figure 7H); immunostaining being comparable to that previously observed in patients with nonproliferative nephropathies and in the normal kidney (Figures 2, B to D).

Discussion

Stemming from our recent evidence in the MWF rat model of renal disease²⁰ and our initial study in patients with crescentic glomerulonephritis,¹⁹ here we confirmed that parietal epithelial cells expressing progenitor cell markers CD133⁺CD24⁺ proliferate and accumulate into the multilayered cellular

lesions in patients with glomerulonephritides characterized by extracapillary proliferation which include extracapillary glomerulonephritis and IgA nephropathy. Moreover, in the present study, we demonstrated for the first time that the dysregulated proliferation of CD133⁺CD24⁺ progenitor cells is a prominent feature of glomerulonephritides with extracapillary proliferation, but not of membranous or diabetic nephropathies. In these latter disorders, few CD133⁺CD24⁺ progenitor cells lined the inner surface of the Bowman's capsule in a manner similar to healthy human glomeruli. These findings would suggest that the abnormal proliferation of renal progenitors occurs preferentially in extracapillary glomerulonephritides, as a consequence of dysregulation of precursor cells in response to inflammatory injury and release of mediators from the glomerular tuft.

In a search of mediators responsible for the abnormal behavior of renal progenitor cells in the setting of proliferative diseases, we hypothesized a role for the chemokine SDF-1/CXCR4 axis, due to its property of promoting cell migration and proliferation.^{23,24} Indeed, evidence is available that the expression of CXCR4 on cancer cells correlates with the metastatic potential of multiple tumors,²⁵ and that the interaction of CXCR4 with its ligand SDF-1 is the principal effector of hematopoietic stem cell mobilization from the bone marrow.²³ Consistent with this observation are data showing that mice lacking CXCR4 or SDF-1 have a lethal defect in the colonization of bone marrow by transplanted hematopoietic stem cells.²⁴ In addition, normal human CD133⁺CD24⁺ progenitor cells have been shown to express the CXCR4 receptor.²¹ Based on these studies, we investigated the possible involvement of CXCR4 in the abnormal migration of CD133⁺CD24⁺ progenitors in patients with glomerular proliferative diseases. Although in normal human glomeruli, only a few cells in the Bowman's capsule were positive for CXCR4, in proliferative disorders, the expression of CXCR4 was markedly up-regulated, especially in cells forming the hyperplastic lesions. Finding that such cells coexpress the stem cell marker CD24 confirms the progenitor nature of most cells in these lesions. By contrast, the faint expression of CXCR4 in cells coexpressing CD24 in membranous and diabetic nephropathies indicates that progenitor cells have little or no propensity to invade the Bowman's space in these conditions. A possible explanation of the increased CXCR4 expression on progenitor cells exclusively in proliferative diseases is offered by the inflammatory nature of these glomerular disorders. In crescentic glomerulonephritis, the general dogma is that immune complex localization in glomerular capillary wall and mesangium activates multiple humoral and cellular mediator systems including the recruitment to the glomerular tuft of strongly phlogogenic neutrophils and monocytes/macrophages.^{26,27} The activated cells infiltrating the glomerular tuft release soluble cytokines and chemokines that enter the Bowman's space,^{28–30} eventually contributing to up-regulation of adhesion molecules and chemokine receptors on parietal progenitor cells.^{5,8,9} In our setting, monocytes infiltrating the glomerular

Figure 7 ACE inhibitor (ACEi) therapy reduces progenitor cell migration associated with a normalization of AT₁ receptor (AT₁R) expression in a patient with extracapillary glomerulonephritis. **A:** Study design and clinical findings of a patient with severe antineutrophil cytoplasm antibody–positive crescentic glomerulonephritis, who underwent renal biopsies before and after ACE inhibitor treatment. **B:** Histogram reporting the percentage of glomeruli with different kinds of glomerular lesions observed in renal biopsies obtained before and after ACE inhibitor treatment. **C:** Periodic acid-Schiff–stained biopsy of the patient before ACE inhibitor treatment reveals that the majority of glomeruli were affected by extracapillary proliferation. A representative photomicrograph showing a glomerulus with extracapillary proliferation is illustrated in the high-magnification inset. Scale bars: 100 μ m. **D:** After ACE inhibitor treatment, all glomeruli of the biopsy were totally free of crescentic lesions, as shown in the representative glomerulus in the high-magnification inset. Scale bars: 100 μ m. **E:** The AT₁ receptor (green) was overexpressed in CD24⁺ progenitor cells (red) in the area of crescent formation in the patient (arrows). Scale bar = 20 μ m. **F:** After ACE inhibitor treatment, AT₁ receptor (green) expression was faint, and no cells in the Bowman's capsule coexpressed the CD24 (red) progenitor marker. **G:** The ACE inhibitor also reduces accumulation of CD24⁺ (green) and CD133⁺ (red) progenitor cells that were confined to the Bowman's capsule, as previously observed in normal kidney. **H:** Double immunostaining for CXCR4 (green) and CD24 (red) shows that the ACE inhibitor also reduced the expression of CXCR4 in comparison to patients not receiving the treatment (Figure 2A). Scale bars: 50 μ m.

tuft³¹ did not express SDF-1, whereas podocytes activated by the inflammatory microenvironment³² produced the chemokine providing the ligand for CXCR4 receptors up-regulated on CD133⁺CD24⁺ progenitor cells, ultimately allowing their

migration and proliferation. These observations contribute to the knowledge of how podocyte activation could affect the behavior of parietal CD133⁺CD24⁺ progenitors. Finding a similar pattern of expression of CXCR4 in activated parietal epithelial

cells and SDF-1 in podocytes in MWF rats further substantiates our belief that the production of a chemokine (SDF-1) of podocyte origin acts as trigger for the activation of progenitor cells in the Bowman's capsule leading to hyperplasia.

Additionally, local production of Ang II, the key peptide of the renin-angiotensin system, is increased in proteinuric glomerulonephritis.³³ Phlogogenic cells can release enzymes that generate Ang II, including ACE in monocytes/macrophages^{34,35} and cathepsin G from neutrophils.³⁶ The local accumulation of Ang II activates AT₁ receptors in different cell types further sustaining the inflammatory environment via the production of reactive oxygen species, cytokines, and adhesion molecules.³⁷ There is also evidence that Ang II is capable of up-regulating AT₁ receptor expression in a dose- and time-dependent manner in macrophages.³⁸ Likewise in hypertensive rats, chronic Ang II infusion increases AT₁ receptor mRNA levels in rostral ventrolateral medulla.³⁹ Because Ang II promotes cell migration and proliferation via AT₁ receptor,²² we sought to assess whether CD133⁺CD24⁺ progenitor cells contribute to the progression of hyperplastic lesions as a response to the excessive expression of this receptor on their surface. One major observation in this article was the presence of high numbers of progenitor cells located between the Bowman's capsule and the capillary tuft expressing AT₁ receptors in patients with proliferative disorders. By contrast, only rare cells of the Bowman's capsule demonstrated AT₁ receptor immunoreactivity in patients with membranous and diabetic nephropathies, similar to normal human glomeruli. To our knowledge, this is the first demonstration of AT₁ receptor expression on progenitor cells and their up-regulation in the setting of hyperplastic lesion formation, highly suggestive of a contribution of the Ang II/AT₁ receptor pathway to the abnormal behavior of renal progenitors in proliferative diseases. Consistent with this possibility are the present findings in the MWF rats with hyperplastic glomerular lesions, where the majority of cells lining the Bowman's capsule as well as those constituting the lesions were highly expressive of the AT₁ receptor, a phenomenon markedly reduced in animals treated with an ACE inhibitor that also restored the normal Bowman's capsule architecture. Our *in vivo* findings in rats are consistent with *in vitro* studies in monocytes and endothelial cells in which the ACE inhibitor ramipril suppressed Ang II/AT₁ receptor intracellular proinflammatory signaling, mainly by down-regulating AT₁ receptor expression.⁴⁰

Significantly, we found a patient with extracapillary glomerulonephritis in which the ACE inhibitor ramipril normalized the up-regulated expression of the AT₁ receptor on CD133⁺ CD24⁺ renal progenitor cells concomitant with regression of glomerular hyperplasia, further suggesting that modulation of AT₁ receptor expression on renal precursor cells also has a major impact on the development of glomerulonephritides in man. Moreover, a possible link between AT₁ receptor and CXCR4 expression comes from the observation of a reduced accumulation of progenitor cells in

the Bowman's space with negligible CXCR4 expression on their surface in the biopsy specimen collected after ACE inhibitor treatment, as well as in MWF rats treated with lisinopril. This relationship is further supported by findings that renin-angiotensin system inhibition with ACE inhibitors or AT₁ receptor blockers attenuates CXCR4 mRNA expression and protein levels in the left atria in patients with chronic atrial fibrillation and mitral valve disease.⁴¹ As a caveat, although in the human study the reduction of proteinuria, the amelioration of renal function, and the dramatic improvement in renal histology were only observed after administration of ACE inhibitors, we cannot exclude a carryover effect of the immunosuppressive regimen to the favorable outcome.

In summary, we have documented that i) CD133⁺CD24⁺ progenitor cells are mostly responsible for the extracapillary lesions in patients with proliferative glomerular disease, but remain scarce in nonproliferative nephropathies; ii) both CXCR4 and AT₁ receptor expression is up-regulated on renal progenitor cells that proliferate and migrate into the Bowman's capsule; iii) activated podocytes, but not infiltrating macrophages, express the CXCR4 ligand SDF-1; iv) ACE-inhibition therapy modulates renal progenitor cell proliferation and migration, restoring the normal glomerular architecture associated with both a down-regulation of AT₁ receptor and CXCR4 on progenitor cells and SDF-1 on podocytes; and v) similar phenotypic changes occur in patients with proliferative glomerulonephritis and MWF rats with extracapillary lesions.

Taken together, these results provide new insights into the pathogenesis of hyperplasia in proliferative glomerulonephritis and allow us to advocate Ang II blockers as a novel therapeutic option. Targeting the Ang II/AT₁ receptor/CXCR4 pathogenic pathways might be beneficial in severe glomerular proliferative disorders that commonly lead to rapid loss of renal function requiring dialysis or transplantation.

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