CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

The Role of Angiotensin II in Parietal Epithelial Cell Proliferation and Crescent Formation in Glomerular Diseases

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Crescentic glomerulonephritis (GN) is a devastating disease with rapidly progressive deterioration in kidney function, which, histologically, manifests as crescent formation in most glomeruli. We previously found that crescents derive from the aberrant proliferation and migration of parietal epithelial cells (PECs)/progenitor cells, and that the angiotensin (ang) II/ang II type-1 (AT₁) receptor pathway may participate, together with the stromal cell–derived factor-1 (SDF-1)/C-X-C chemokine receptor 4 axis, in the development of those lesions. Herein, we elucidated sequential events and cellular and molecular interactions occurring during crescentic lesion onset and evolution. By analyzing kidney biopsy specimens of patients with extracapillary GN, divided according to the grade of glomerular lesions, we found that the accumulation of macrophages expressing matrix metalloproteinase-12 started manifesting in glomeruli affected by early-stage lesions, whereas AT₁ receptor expression could not be detected. In glomeruli with advanced lesions, AT₁ receptor expression increased markedly, and the up-regulation of SDF-1, and its receptor C-X-C chemokine receptor 7, was documented on podocytes and PECs, respectively. *In vitro* studies were instrumental to demonstrating the role of ang II in inducing podocyte SDF-1 production, which ultimately activates PECs. The present findings support the possibility that angiotensin-converting enzyme inhibitor treatment might limit PEC activation and reduce the frequency and extension of crescents in extracapillary GN. (*Am J Pathol* 2017, 187: 2441–2450; <http://dx.doi.org/10.1016/j.ajpath.2017.07.004>)

Glomerular crescentic lesions are the result of a pathologic accumulation of proliferating cells between the Bowman capsule and the capillary tuft. Crescentic lesions are found in glomeruli of patients affected by rapidly progressive glomerulonephritis (GN), a clinical syndrome characterized by rapid loss of renal function, often accompanied by hypertension, macroscopic hematuria, and proteinuria.¹ Three types of crescentic GN are currently recognized: anti–glomerular basement membrane (GBM) GN, immune complex–mediated GN, and antineutrophil cytoplasm antibody–associated vasculitis, which is the most common form of crescentic GN, accounting for >60% of all cases.^{1–3} Identifying cellular components of crescents, and their pathogenic role, has attracted scientists' interest for

decades. Early studies revealed that macrophages play a crucial role in the pathogenesis of crescentic GN, stimulating self-recruitment and promoting the proliferation of epithelial cells through the release of proinflammatory cytokines.^{4,5} It later became clear that crescentic lesions are composed of a mixture of cell types, including parietal epithelial cells (PECs), myofibroblasts, and

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macrophages,^{6–8} with the latter predominating when a rupture of the Bowman capsule occurs.^{6,9,10} It has also been suggested that podocytes could contribute to crescent formation.^{11–15} However, given their terminally differentiated phenotype and the limited ability to migrate and proliferate,¹⁶ podocytes cannot be considered as major constituents of multilayered hyperplastic lesions.^{15,17} During the past decade, all evidence from human and animal experiments has pointed to PECs playing a key role in the pathogenesis of different glomerular diseases.^{18,19} In physiological conditions, a subset of PECs serves as a reservoir of renal progenitors, possibly contributing to podocyte turnover.²⁰ However, in the presence of extensive glomerular damage, these cells can respond with exuberant proliferation and/or migration from the Bowman capsule toward the glomerular tuft.^{15,21–25}

While looking for mediators responsible for the dysregulated response of renal progenitors in proliferative diseases, we previously suggested that the immune-related chemokine stromal cell–derived factor-1 (SDF-1)/C-X-C chemokine receptor (CXCR) 4 axis,²² known to be crucial for cell migration and proliferation,^{26,27} might play a role. In human and rat crescentic GN, marked SDF-1 and CXCR4 up-regulation in podocytes and PECs, respectively, suggested that SDF-1 produced by podocytes might trigger PEC/progenitor cell activation via CXCR4, leading to the formation of crescentic lesions.²² In accordance with evidence showing that angiotensin (ang) II can promote cell proliferation and migration through the ang II type-1 (AT₁) receptor,²⁸ we also found that AT₁ receptor expression was up-regulated in patients' parietal progenitor cells and within hyperplastic lesions,²² and treatment with an angiotensin-converting enzyme (ACE) inhibitor significantly reduced the frequency and extent of crescents in an experimental model of severe proliferative disease.²¹ Moreover, a patient with antineutrophil cytoplasm antibody–positive extracapillary GN who displayed regression of crescentic lesions and reduction of AT₁ receptor expression in renal progenitors in a repeated biopsy after ACE inhibitor treatment²² was also informative in this context.

Although our previous findings^{21,22} have allowed us to lay the groundwork for understanding the formation of crescents, what is still missing in the literature is an accurate analysis of the sequential events occurring during the onset and progression of glomerular hyperplastic lesions. As multiple biopsies at different time points cannot be performed in humans, herein we sought to outline a step-by-step sequence of crescent evolution by performing a serial section analysis of glomeruli displaying morphologically different degrees of lesions, and to investigate the cellular and molecular mediators involved. For this purpose, we analyzed kidney biopsy specimens from patients with extracapillary GN to i) evaluate the presence and extent of breaks affecting both the GBM and Bowman capsule, which may lead to macrophage infiltration in the Bowman space; ii) define at what stage of crescentic lesion evolution the

accumulation of infiltrating macrophages expressing matrix metalloproteinase-12 (MMP-12) occurs, and how it was associated with AT₁ receptor up-regulation; and iii) investigate whether the glomerular expression of the chemokine SDF-1 and its receptors CXCR4 and CXCR7 was modulated. Then, functional co-culture *in vitro* studies allowed us to dissect cellular and molecular mechanisms underlying the cross talk between progenitor cells and podocytes, crucial to hyperplastic lesion onset and progression.

Materials and Methods

Patients

Ten patients with extracapillary GN from the archives of the Unit of Nephrology, Azienda Socio-Sanitaria Territoriale Papa Giovanni XXIII (Bergamo, Italy), were enrolled in the study. All kidney biopsy specimens had originally been obtained to diagnose renal disease. Written informed consent was obtained from all patients. Demographic, clinical, and hematochemical parameters at the time of biopsy were retrieved from the hospital database. In addition to specimens from patients with extracapillary GN, renal biopsy specimens from an uninvolved portion of kidney collected from tumor nephrectomy specimens were obtained from 10 patients and used as controls.

Quantification of Glomerular Crescents

Renal biopsy specimens of patients with extracapillary GN were fixed in Duboscq-Brazil and embedded in paraffin. Sections (2 μ m thick) were stained with periodic acid–Schiff reagent and observed by light microscopy (BH2-RFCA; Olympus, Melville, NY). All of the glomeruli present in renal biopsy specimens were examined for each patient, and the extent of crescents was expressed by giving a score from 0 to 4, related to the percentage of the glomerular tuft that was occupied by the lesions (0, no lesions; 1, lesions affecting <25%; 2, $\geq$ 25% to <50%; 3, $\geq$ 50% to <75%; 4, $\geq$ 75% to 100% of the glomerulus). The index of crescents, which considers both the number and the extent of the lesions, was calculated by using the following formula: Index = $(1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4) / n_0 + n_1 + n_2 + n_3 + n_4$, where n_x is the number of glomeruli for each percentage of crescent extension.

Evaluation of the Integrity of the GBM and Bowman Capsule

To detect basal membranes and to evaluate the integrity of the GBM and Bowman capsule, Duboscq-Brazil–fixed and paraffin-embedded kidney sections from patients with extracapillary GN and from normal kidneys were stained with silver methenamine reagents (Bio Optica, Milan, Italy) and observed by light microscopy (ApoTome AxioImager Z2; Zeiss, Jena, Germany). All of the glomeruli present in

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

Characteristics	Value (n = 10)
Age in years, range	20–81
Male:female ratio	2:8
Clinical parameters	
Systolic blood pressure, mmHg	135.4 ± 14.4
Diastolic blood pressure, mmHg	80.2 ± 8.4
Laboratory parameters	
Proteinuria, g/day	5.2 ± 2.2
Serum creatinine, mg/dL	3.4 ± 2.6
Hematuria, 1:0	9:1
Diagnosis, n	
Anti-GBM antibody glomerulonephritis (type I)	1*
ANCA-associated or pauci-immune vasculitis (type II)	4 [†]
Immune-complex–mediated glomerular disease (type III)	5 [‡]
Histopathological findings	
Index of crescents, score 0–4	2.0 ± 0.8
Glomeruli with extracapillary proliferation, %	56.0 ± 35.3
Glomeruli with global sclerosis, %	21.4 ± 20.3

Data are expressed as means ± SD.

*Anti-GBM antibodies with associated p-ANCA antibodies.

[†]p-ANCA, n = 3; c-ANCA, n = 1.

[‡]IgA nephropathy, n = 1; lupus nephritis, n = 3; immune complex–mediated glomerulonephritis secondary to systemic disease, n = 1.

ANCA, antineutrophil cytoplasm antibody; c, cytoplasmic; GBM, glomerular basement membrane; p, perinuclear.

renal biopsy specimens were examined for each patient. The extent of Bowman capsule rupture was expressed by giving a score from 0 to 4 related to the percentage of the capsule affected by gaps (0, intact Bowman capsule; 1, rupture affecting <25%; 2, 25% to 50%; 3, 50% to 75%; 4, 75% to 100% of the Bowman capsule).

Electron Microscopy Analysis

Biopsy specimens were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 4 hours at 4°C and washed in cacodylate buffer. Kidney fragments were then postfixed in 1% osmium tetroxide for 1 hour, dehydrated through ascending grades of alcohol, and embedded in Epon resin (Electron Microscopy Science, Hatfield, PA). Ultrathin sections (60 to 100 nm thick) were cut on an EM UC7 ultramicrotome (Leica Microsystems, Mannheim, Germany), stained with uranyl acetate and lead citrate, and examined with transmission electron microscopy (Morgagni 268D; Philips, Brno, Czech Republic).

Immunoperoxidase

CD68, MMP-12, and AT₁ receptor expression was analyzed by immunoperoxidase staining in human biopsy specimens. Duboscq-Brazil–fixed, paraffin-embedded

kidney sections were deparaffinized, rehydrated, and incubated for 5 minutes with peroxidized I (Biocare Medical, Concord, CA) to quench endogenous peroxidases. Antigen retrieval was performed using a Decloaking chamber (DCNxGen 220V; Biocare Medical) for 15 minutes at 110°C with DIVA decloaker buffer (Biocare Medical). After blocking for 5 minutes with Background Punisher (Biocare Medical), sections were incubated with mouse anti-CD68 (1:100; DakoCytomation, Glostrup, Denmark) or rabbit anti-AT₁ receptor primary antibodies (1:25; Santa Cruz Biotechnology, Dallas, TX), followed by MACH4 HRP-Polymer kit (Biocare Medical) and diaminobenzidine (Biocare Medical) substrate solution. For

Figure 1 Histological and ultrastructural analysis of Bowman capsule and glomerular basement membrane (GBM). **A: Top panel:** Silver methenamine–stained renal biopsy specimen from a patient with extracapillary glomerulonephritis (GN). **Bottom panel:** A break in the Bowman capsule (arrow) and several irregularities and wrinkling along the GBM. **B:** Uninvolved portion of renal tissue removed because of renal cell carcinoma, used as age-matched control, displays glomeruli with intact Bowman capsule and linear GBM. **Boxed areas in A and B** are shown below at higher magnification. **C:** Representative transmission electron micrograph showing a rupture in the GBM. **D:** At the ultrastructural level, red blood cells (RBCs; arrow) were found outside of glomerular capillaries in the proximity of multiple GBM discontinuities. **E–H:** Higher magnification of the **dashed boxes in D**, showing GBM discontinuities and gaps. Scale bars: 50 μ m (**A** and **B**, top panels); 20 μ m (**A** and **B**, bottom panels); 2000 nm (**C** and **D**); 500 nm (**E–G**); 1000 nm (**H**). EC, endothelial cell.

Figure 2 Infiltration of macrophages and matrix metalloproteinase (MMP)-12 accumulation in patients with extracapillary glomerulonephritis (GN). **A:** Representative immunoperoxidase staining showing a significant increase in CD68⁺ cells in the glomeruli and interstitium of patients with extracapillary GN compared to age-matched normal kidneys. **Arrow** shows crescentic lesion. **B:** Quantitative analysis of CD68⁺ macrophages in the interstitium, in the glomerulus, and in the periglomerular area. **C:** Representative double staining of CD68 (brown signal) and MMP-12 (red signal) in glomeruli of extracapillary GN patients with early- and advanced-stage histological lesions. **Dashed boxes** are shown at higher magnification in the **insets**. **Insets** show strong MMP-12 expression in the areas where a high number of CD68⁺ macrophages are also present. **Arrowheads** show MMP-12⁺CD68⁺ cells along the Bowman capsule. ***P* < 0.01 versus normal kidneys. Scale bars = 50 μ m (**A** and **C**).

CD68 and MMP-12 double staining, paraffin-embedded kidney sections were processed as described above, and co-incubated with mouse anti-CD68 and rabbit anti-MMP-12 (1:250; Proteintech Group Inc., Chicago, IL) primary antibodies, followed by MACH2 double stain 2 (Biocare Medical) polymer. Diaminobenzidine and Warp red (Biocare Medical) substrate solutions were used for CD68 and MMP-12, respectively. Slides were finally counterstained with Meyer hematoxylin and observed by light microscopy (ApoTome). Negative controls were obtained by omitting the primary antibodies on adjacent sections. CD68 expression was quantified as the number of positive cells in each glomerulus or in each field ($\times 40$), at both interstitial and periglomerular areas. MMP-12 and AT₁ receptor expression was evaluated in two serial sections for each patient. The positive area for each signal was quantified in all of the glomeruli that were found in kidney biopsy specimens, divided according to the grade of glomerular lesions (early, intermediate, or advanced, up to 20 glomeruli for each grade of lesion). Quantification was performed by using ImageJ version 1.50i (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>), and values were expressed as a percentage of positive area on total glomerular area.

Immunofluorescence and Confocal Microscopy

Archived kidney biopsy specimens, snap frozen in liquid nitrogen and embedded in OCT compound, were used for immunofluorescence analysis. Single or double immunostaining for SDF-1, CXCR4, CXCR7, and CD24 expression was performed. Frozen sections (2 μ m thick) were air dried and fixed with cold acetone. For SDF-1 detection, antigen retrieval was performed by boiling sections using a microwave (twice for 5 minutes in 10 mmol/L citrate buffer, pH 6.0, at an operating frequency of 2450 MHz and 600 W power output). After blocking nonspecific sites with 1%

bovine serum albumin, the following primary antibodies were used: rabbit anti-SDF-1 (1:200; Abcam, Cambridge, UK), rabbit anti-CXCR4 (1:50; Abcam), mouse anti-CXCR7 (1:5; R&D Systems, Minneapolis, MN), and goat anti-CD24 (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. Negative controls were obtained by omitting primary antibodies on adjacent sections. Fluorescence was examined by an inverted confocal laser scanning microscope (LSM510 Meta; Zeiss). CXCR4 and CXCR7 expression was quantified in all of the glomeruli that were found in patients' biopsy specimens and in normal kidneys (up to 10 glomeruli from each patient), by using ImageJ version 1.50i. Values are expressed as a percentage of positive area in the total glomerular area.

Cell Cultures

Podocytes

Conditionally immortalized human podocytes (kindly provided by Prof. M.A. Saleem, Children's Renal Unit and Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK) were cultured under growth-permissive conditions at 33°C in RPMI 1640 medium (Invitrogen Corp., Grand Island, NY), as already described.²⁹ To induce differentiation, podocytes were seeded on rat collagen type I (BD Bioscience, San Diego, CA) and maintained in nonpermissive conditions at 37°C for 12 days.

PEC Isolation

Human PECs were isolated, as previously described.^{20,30} Briefly, PECs were recovered from total renal cells and isolated from a normal kidney fragment from a young patient (3 years old) who underwent nephrectomy, using CD133-

immunomagnetic separation. Clones were generated from CD133⁺ cells by limiting dilution, and immunofluorescence and fluorescence-activated cell sorting analysis were used to identify clones positive for CD24 and CD106.

Immunofluorescence Studies *in Vitro*

To assess the expression of SDF-1 by podocytes and PECs, cells were incubated for 6 hours with 10^{-7} mol/L ang II. Then, cells were fixed in 2% paraformaldehyde (Electron Microscopy Science) and 4% sucrose for 15 minutes at 37°C, permeabilized with Triton 0.3% for 3 minutes at room temperature, and incubated with blocking solution for 1 hour. Podocytes and PECs were stained with rabbit anti-SDF-1 antibody, followed by a goat anti-rabbit Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Nuclei were counterstained with DAPI. The area occupied by the signal was quantified using ImageJ version 1.50i, and normalized for the number of DAPI-positive cells. Data were expressed as pixel²/cell.

Migration Assay

The ability of PECs to migrate toward injured podocytes was assessed in a co-culture transwell system (Costar; Sigma-Aldrich, St. Louis, MO). PECs were seeded in a transwell chamber on the upper side of an 8- μ m permeable membrane, whereas podocytes were co-cultured in the lower chamber. Podocytes and PECs were incubated for 24 hours with endothelial cell growth medium–MV medium (Lonza, Walkersville, MD) plus 2% fetal bovine serum Hyclone (Hyclone Laboratories, Logan, UT) in the presence or absence of 10^{-7} mol/L ang II, 10 μ g/mL ang II + SDF-1 neutralizing antibody, or 1 μ mol/L ang II + candesartan. At the end of the incubation, PECs at the upper side of the filter were mechanically removed. PECs that migrated to the lower side of the filter were fixed in 11% glutaraldehyde for 30 minutes at room temperature and then stained with hematoxylin and eosin. The number of cells that had migrated was counted in five to eight random fields for each filter. Data were expressed as the number of transmigrated PECs/high-power field (magnification, $\times 10$).

Statistical Analysis

Results were expressed as means $\pm$ SD. The *t*-test was applied for quantification of Bowman capsule rupture and CD68, CXCR4, and CXCR7 expression. Statistical analysis of MMP-12 and AT₁ receptor expression was performed using analysis of variance with Tukey post hoc analysis for multiple comparisons; for *in vitro* experiments, comparisons were made using the Bonferroni post hoc test. Statistical significance was defined as $P < 0.05$.

Results

Patient Population

Ten patients with extracapillary lesions (one patient affected by anti-GBM GN, four by antineutrophil cytoplasm antibody–associated vasculitis, and five by immune complex–mediated GN) were enrolled in the study. Clinical and histopathological characteristics of patients at the time of the biopsy are outlined in Table 1. Their mean age was 53.4 ± 20.9 years. Eight patients had renal impairment at onset, whereas two had initial normal serum creatinine levels, despite active sediment. All but one completely anuric patient had a high degree of proteinuria (nephrotic in four cases) and all had hematuria (one with macrohematuria). In terms of histology, most glomeruli had extracapillary lesions, whose index averaged 2.0, and a large number of the glomeruli exhibited global or segmental sclerosis.

Figure 3 Macrophage and matrix metalloproteinase (MMP)-12 accumulation is an early event in the development of crescentic lesions compared to angiotensin II type 1 receptor (AT₁ receptor) up-regulation. **A–F:** Representative images of serial sections labeled with CD68 (brown) and MMP-12 (red; **A**, **C**, and **E**), and AT₁ receptor (brown; **B**, **D**, and **F**), showing glomeruli with early-stage (**A** and **B**), intermediate-stage (**C** and **D**), and advanced-stage (**E** and **F**) lesions in patients with extracapillary glomerulonephritis. **Dashed boxes** are shown at higher magnification in the **insets**. **Insets** show areas with different degrees of glomerular lesions and protein expression. **G:** Quantitative analysis of MMP-12 and AT₁ receptor expression in the glomerular area, in patients' glomeruli with early-, intermediate-, and advanced-stage lesions. * $P < 0.05$ versus glomeruli with early-stage lesions; †† $P < 0.01$ versus glomeruli with intermediate-stage lesions. Scale bars = 50 μ m (**A–F**).

Figure 4 Stomatal cell-derived factor-1 (SDF-1) and C-X-C chemokine receptor (CXCR) 7 are up-regulated in patients with extracapillary glomerulonephritis (GN). **A:** Representative immunofluorescence staining for SDF-1 (red) showing increased glomerular expression in patients with extracapillary GN compared to age-matched normal kidney. **B:** The expression of the SDF-1 receptor CXCR4 (green) is up-regulated in patients with extracapillary GN as compared with age-matched normal kidney. **C:** SDF-1 receptor CXCR7 (red) is highly expressed in patients with extracapillary GN as compared with age-matched normal kidney. **D:** CXCR7 (red) colocalizes with progenitor cell marker CD24 (green) in patients with extracapillary GN, in particular in areas with crescentic lesions (**boxed area**) or along the Bowman capsule (**arrows**). CD24⁺ cells do not coexpress CXCR7 in age-matched normal kidney. DAPI (blue) stained nuclei. **E:** Quantitative analysis of CXCR4 and CXCR7 expression in the glomerular area. ***P* < 0.01 versus normal kidneys. Scale bars = 50 μ m (**A–D**).

Alterations of Bowman Capsule and GBM in Patients with Extracapillary GN

Most glomeruli ($59.25\% \pm 19.50\%$) in our patient population showed recurring breaks along the Bowman capsule, which were heterogeneous in size (score, 0 to 4: 0.94 ± 0.67 in patients with extracapillary GN versus 0.00 ± 0.00 in normal kidneys; *P* < 0.01) (Figure 1A). Several segmental irregularities and wrinkling were also visible along the GBM, with areas of disrupted continuity (Figure 1A). Conversely, glomeruli from normal kidneys displayed a linear GBM without evident signs of rupture or irregularities (Figure 1B). Through transmission electron microscopy analysis of affected kidney sections, GBM ruptures (Figure 1C) as well as GBM discontinuities and gaps (Figure 1, D–H) were detected, in association with red blood cells that had spilled from the glomerular capillaries, and were consequently found on the outer side of the glomerular filtration barrier (Figure 1D).

CD68⁺ Macrophage Accumulation Already Occurs in Early-Stage Crescentic Lesions

Compared to normal kidneys, there was a significant increase in CD68⁺ macrophages in the interstitium, glomerulus, and periglomerular area in patients with extracapillary GN (*P* < 0.01) (Figure 2, A and B). At the glomerular level, CD68⁺ cells were mainly localized in crescentic lesions (Figure 2A). In patients with extracapillary GN, the high infiltration of CD68⁺ macrophages was associated with increased expression of their own elastase MMP-12, an extracellular matrix-degrading enzyme that is capable of

degrading almost all of the components of glomerular membranes^{31,32} (Figure 2C). Double staining with CD68 and MMP-12 revealed that the accumulation of macrophages within the glomeruli was closely correlated with the release of macrophage elastase, paralleling the progression of the hyperplasia (Figure 2C). In particular, in glomeruli with lesions at early stages, CD68⁺MMP-12⁺ cells were present only along the GBM in focal areas of the glomerular tuft. In contrast, in glomeruli affected by advanced lesions, CD68⁺MMP-12⁺ cells were abundant both outside and inside the glomerulus, and accumulated mainly in crescents and along the Bowman capsule.

AT₁ Receptor Expression Is Up-Regulated Only in Advanced-Stage Crescentic Lesions

All glomeruli that were found in patients' biopsy specimens were divided according to the grade of lesions: those showing negligible proliferative lesions (Figure 3, A and B), those displaying intermediate lesions with synechiae and segmental extracapillary proliferation (Figure 3, C and D), and glomeruli with severe crescents (Figure 3, E and F). CD68, MMP-12, and AT₁ receptor staining, performed in two consecutive kidney sections, were detected faintly in glomeruli with negligible/early proliferative lesions (Figure 3, A, B, and G). In glomeruli with segmental/intermediate extracapillary proliferation, macrophage infiltration and their metalloproteinase increased in the periglomerular and glomerular area (*P* < 0.05) (Figure 3, C and G), whereas AT₁ receptor expression was still faint at this stage of the disease (Figure 3, D and G). The expression of AT₁ receptor increased abundantly in glomeruli displaying advanced crescentic lesions, in particular in areas of

Figure 5 Stromal cell–derived factor-1 (SDF-1) is produced by podocytes, but not by parietal epithelial cells (PECs), after stimulation with angiotensin II (Ang II), and plays a crucial role in PEC dysfunction. **A:** Representative immunofluorescence staining for SDF-1 (red) in human podocytes before and after ang II stimulation. DAPI (blue) stained nuclei. **B:** Quantification of SDF-1 staining in human podocytes showing an increase in chemokine expression after 6-hour stimulation with ang II. **C:** SDF-1 expression (red) in human PECs before and after ang II stimulation. DAPI (blue) stained nuclei. **D:** Histogram shows that SDF-1 expression does not increase after stimulation with ang II. **E:** Quantification of PEC migration at 24 hours in co-culture transwells from the upper chamber toward unstimulated or ang II–activated podocytes in the presence or absence of the anti–SDF-1 antibody or candesartan. Data were expressed as number of PECs that migrated from the upper chamber across the filter toward podocytes/high-power field. **F:** Representative images of transmigrated PECs stained with hematoxylin and eosin, toward untreated [control (CTR)], ang II–activated podocytes, ang II + antibody (Ab) anti–SDF-1, or ang II + candesartan treated podocytes. * $P < 0.05$, *** $P < 0.001$ versus CTR; ††† $P < 0.001$ versus ang II. Scale bars: 20 μ m (**A**); 50 μ m (**C**); 100 μ m (**F**).

hyperplasia in which MMP-12 was also detected ($P < 0.01$) (Figure 3, E–G).

SDF-1 and Its Receptors CXCR4 and CXCR7 Are Up-Regulated in Biopsy Specimens of Patients with Advanced Crescentic Lesions

Given the contribution of the immune-related chemokine SDF-1 and its receptor CXCR4 in proliferative diseases,²² herein we studied in depth the role of SDF-1 in mediating progenitor cell activation by evaluating the expression of the alternative SDF-1 receptor CXCR7, in biopsy specimens of patients with extracapillary GN. A previous study has shown that CXCR7 receptor is a critical mediator of the therapeutic homing of human PECs/progenitor cells in mouse injured renal tissue.³³ However, its expression in human biopsy specimens from patients with extracapillary lesions has not been reported yet. In line with our previous data,²² glomeruli with advanced crescentic lesions showed increased expression of the chemokine SDF-1 and of its receptor CXCR4 compared to normal kidneys (Figure 4, A and B). Concomitantly with CXCR4 receptor up-regulation ($P < 0.01$) (Figure 4, B and E), in the same biopsy specimens, increased expression of CXCR7 was observed ($P < 0.01$)

(Figure 4, C and E). Double staining with CXCR7 and the progenitor cell marker CD24 revealed that SDF-1 receptor CXCR7 was mainly expressed by renal progenitors in the Bowman capsule and within crescentic lesions (Figure 4D), similar to the pattern previously observed for CXCR4.²²

Ang II Promotes SDF-1 Production by Cultured Podocytes

To further confirm the cellular source of SDF-1 production observed in biopsy specimens of patients with extracapillary GN, and to analyze whether ang II was involved in SDF-1 stimulation, cultured podocytes and PECs/progenitor cells were exposed to 10⁻⁷ mol/L ang II for 6 hours and subjected to immunofluorescence analysis. A faint SDF-1 signal was observed in podocytes and PECs under basal conditions (Figure 5, A and C). Exposure to ang II significantly increased ($P < 0.05$) SDF-1 expression in podocytes (Figure 5, A and B), whereas the peptide failed to induce SDF-1 overexpression in cultured PECs, indicating the inability of these cells to produce the chemokine (Figure 5, C and D). These results confirmed data obtained from kidney biopsy specimens (Figure 4A).²²

Figure 6 **A:** The early phase of the disease is characterized by CD68⁺ macrophages (violet), which by releasing matrix metalloproteinase (MMP)-12 (pink) induce ruptures in Bowman capsule and glomerular basement membrane (GBM). Gaps in GBM allow red blood cells (red) to exit the glomerular capillaries and to reach parietal epithelial cells (PECs; blue). Activated PECs begin to proliferate. **B:** The late phase of the disease is characterized by macrophage-induced increase in angiotensin II (ang II; yellow), which via angiotensin II type 1 receptor (AT₁ receptor) activates podocytes (green) to produce the stromal cell-derived factor-1 (SDF-1) chemokine. SDF-1 binds C-X-C chemokine receptor (CXCR) 4 and CXCR7 on PECs, promoting their proliferation and migration toward the glomerular tuft, resulting in crescent formation. **C:** Schematic representation of the mechanisms involved in crescentic lesion formation in patients with extracapillary glomerulonephritis. ACEi, angiotensin-converting enzyme inhibitor.

Ang II Induces PEC/Progenitor Cell Transmigration via SDF-1 and AT₁ Receptor

To explore in depth the mechanisms through which ang II induced PEC activation, we studied PEC/progenitor cell migration using a transwell system, where a porous membrane physically separated PECs and podocytes, treated or not treated with ang II. The migration of PECs from the upper chamber across the membrane toward ang II-activated podocytes was significantly higher than that observed toward untreated podocytes (Figure 5, E and F). The functional blockade of podocyte-derived SDF-1 with a specific antibody significantly reduced PEC transmigration toward activated podocytes (Figure 5, E and F). Similarly, treatment with the AT₁ receptor antagonist candesartan significantly limited the number of

PECs that migrated toward ang II-treated podocytes (Figure 5, E and F).

Discussion

This study sought to describe in depth the sequential events and molecular interactions that occur during the onset and evolution of crescentic lesions. Serial section immunohistochemical analysis was performed on kidney biopsy specimens of rapidly progressive GN patients, followed by rigorously designed *in vitro* assays. These approaches allowed us to draw a step-by-step sequence from early abnormalities to aberrant PEC/progenitor cell proliferation and migration toward the glomerular tuft, and to suggest which mediators may be involved.

We have observed that macrophages, possibly recruited by neutrophils,³⁴ accumulated in the interstitium, in the periglomerular area, and in the tuft since the earliest stages of glomerular injury, and their infiltration progressively increased inside the glomeruli, paralleling the progression of hyperplasia. Interstitial macrophages produced a growing amount of MMP-12 elastase, which, coherently with data obtained in a rat model of anti-GBM GN,³² was already expressed before the onset of severe glomerular lesions. The proteolytic enzyme MMP-12 may contribute to focal ruptures of Bowman capsule, which are typically found in patients with extracapillary GN,² and have been confirmed in our patients through transmission electron microscopy observations. Interestingly, red blood cells found on the outer side of the glomerular capillaries in correspondence with GBM discontinuities or breaks are evidence of an abnormal leakage of blood from glomerular capillaries, which is probably the result of MMP-12-driven glomerular membrane lysis, and which clinically translates into patients experiencing hematuria.² It has been shown that exposure of cultured human PECs to blood serum promotes their activation and the acquisition of a proliferative and mitogenic phenotype, suggesting a possible relationship between blood leakage from the glomerular capillaries and crescent formation.³⁵ Thus, our *in vivo* observations, combined with previous *in vitro* findings,³⁵ can explain how, in patients with extracapillary GN, macrophage-driven lysis of glomerular membranes could lead to the contact between blood and PECs/progenitor cells, which, in turn, begin to proliferate, possibly contributing to crescent formation.

On the basis of our previous data showing that renal progenitors of the Bowman capsule constitutively express AT₁ receptor, the expression of which was markedly augmented in crescentic lesions of experimental and human extracapillary GN,²² herein we perform an unprecedented analysis to investigate the sequence of events linking the infiltration of inflammatory cells to the activation of the renin-angiotensin system during the evolution of crescentic lesions. Although MMP-12 produced by macrophages was expressed in the early stage of glomerular injury, AT₁ receptor, presumably expressed by

progenitor cells,²² could only be detected in advanced lesions, suggesting that the involvement of the renin-angiotensin system was a later phenomenon in the development of crescents with regard to MMP-12–expressing macrophage infiltration. Notably, AT₁ receptor was localized in glomerular areas in which MMP-12 was detected, providing new insights into the possible role of infiltrating macrophages in promoting PEC proliferation and migration. Thus, it is conceivable that crescent formation is primarily promoted by infiltrating macrophages, which may initially induce capillary blood leakage through MMP-12–driven GBM lysis, and, once accumulated in the glomerulus, are activated to produce ang II.³⁶ Ang II proinflammatory activities via AT₁ receptor³⁷ can, in turn, further trigger phlogogenic cell infiltration, ultimately resulting in a vicious cycle that worsens disease progression.

In vitro experiments then provided further clarification regarding the role of ang II and SDF-1 in regulating podocyte and PEC cross talk during hyperplastic lesion onset and evolution. Podocytes were the only glomerular source of SDF-1, whose production was directly stimulated by ang II through AT₁ receptor. Moreover, podocyte-produced SDF-1 played a pivotal role in mediating ang II–dependent PEC activation. These observations further corroborated and explained *in vivo* findings showing increased expression of SDF-1 in patients' podocytes and the relative receptors CXCR4 and CXCR7 in parietal progenitor cells and in crescentic lesions. To the best of our knowledge, this is the first piece of evidence showing how the ang II/AT₁ receptor/SDF-1 pathway orchestrates the pathological cross talk between podocytes and PECs in proliferative diseases.

In summary, these data indicate that PEC proliferation and migration in extracapillary GN can conceivably be triggered by multiple independent, but interconnected, pathological pathways. On one hand, macrophages may induce PEC activation by promoting blood spillage through the GBM, and producing ang II, which via AT₁ receptor stimulates podocytes to express SDF-1. On the other hand, excessive production of ang II stimulates podocytes to produce SDF-1, in turn promoting parietal progenitor activation via CXCR4 and CXCR7 receptors (Figure 6).

The understanding of the cross talk between these pathways, which have never been described and characterized in detail, provides crucial new insights into the pathogenesis of extracapillary GN. The present findings help to explain a previous preliminary study in rats and in a patient with advanced nephropathy, showing that ACE inhibitor treatment limits PEC activation and reduces the frequency and extension of crescents, paralleling the normalization of SDF-1/CXCR4 expression.^{19,21,22}

Thus, we provide a rationale for future studies that aim to investigate whether ACE inhibitor therapy given on top of immunosuppression may be able to limit or prevent crescent formation in extracapillary GN more effectively than immunosuppressive therapy alone. Treatment with ACE inhibitor may help to inhibit both the vicious cycle of macrophage infiltration and the ang II–dependent SDF-1

pathway, eventually translating into accelerated renal function recovery and, conceivably, reducing the need for immunosuppressive therapy and the related serious adverse events.

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