Podocytes Populate Cellular Crescents in a Murine Model of Inflammatory Glomerulonephritis

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Abstract. Cellular crescents are a defining histologic finding in many forms of inflammatory glomerulonephritis. Despite numerous studies, the origin of glomerular crescents remains unresolved. A genetic cell lineage-mapping study with a novel transgenic mouse model was performed to investigate whether visceral glomerular epithelial cells, termed podocytes, are precursors of cells that populate cellular crescents. The podocyte-specific 2.5P-Cre mouse line was crossed with the ROSA26 reporter line, resulting in irreversible constitutive expression of β-galactosidase in doubly transgenic 2.5P-Cre/ROSA26 mice. In these mice, crescentic glomerulonephritis was induced with a previously described rabbit anti-glomerular basement membrane antiserum nephritis approach. Interestingly, β-galactosidase-positive cells derived from podocytes adhered to the parietal basement membrane and populated glomerular crescents during the early phases of cellular crescent formation, accounting for at least one-fourth of the total cell mass. In cellular crescents, the proliferation marker Ki-67 was expressed in β-galactosidase-positive and β-galactosidase-negative cells, indicating that both cell types contributed to the formation of cellular crescents through proliferation *in situ*. Podocyte-specific antigens, including WT-1, synaptopodin, nephrin, and podocin, were not expressed by any cells in glomerular crescents, suggesting that podocytes underwent profound phenotypic changes in this nephritis model.

Cellular glomerular crescents are a pathognomonic histologic finding in inflammatory glomerulonephritis that can have many different causes and pathogenic mechanisms (1). The histogenesis and origin of cellular crescents, which are cap-like multilayered accumulations of proliferating cells, have remained controversial. Although early ultrastructural studies suggested that crescents are formed by proliferating epithelial cells (2,3), subsequent studies with newly developed antibodies against leukocytes identified the presence of macrophages in cellular crescents (4). However, it soon became evident that different cell populations, particularly cells expressing epithelial antigens such as cytokeratin, may inhabit the crescents under different conditions (4–7). In fact, the composition of cellular crescents changes during the progression of disease after the inciting glomerular injury. Boucher *et al.* (8) demonstrated that epithelial cells predominated in crescents of patients during the early phases of disease. Later phases were characterized by rupture of the basement membrane of Bowman's capsule and subsequent infiltration of cellular crescents, predominantly by macrophages. Therefore, the proportion of observed epithelial cells in cellular crescents varies among patients, depending on how far the glomerulonephritis has progressed in the affected glomeruli. The predominance of crescentic epithelial cells during the early phase of disease progression and of inflammatory cells during later phases of disease progression was supported by studies with various animal models of anti-glomerular basement membrane (GBM) glomerulonephritis. The animal models are characterized by synchronized progression of inflammation, allowing the study of crescent formation at defined time points. Similar to findings in human tissues, early cellular crescents in rodents were composed of epithelial cells. Rupture of the parietal basement membrane (PBM) was associated with fibrocellular infiltration of crescents, with a high incidence of macrophages/monocytes (9,10).

It was widely assumed that crescentic epithelial cells originated entirely from parietal epithelial cells and that podocytes were not involved in the formation of cellular crescents. In a recent study with a murine anti-GBM model, a more detailed description of the earliest steps of cellular crescent formation was provided (11). In that study, crescent formation was preceded by the formation of podocyte bridges between the glomerular tuft and Bowman's capsule. Podocytes extended processes that disrupted intercellular junctions between parietal epithelial cells and adhered to the PBM. This seemed to be the initiating event for cell proliferation on the capsular side and the formation of cellular crescents. Unlike bridging podocytes,

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all newly formed cells in glomerular crescents were negative for podocyte markers, leading the authors to conclude that crescentic epithelial cells were derived exclusively from parietal epithelial cells.

Our results confirm and build on the findings of Le Hir *et al.* (11) regarding early crescent formation in this model. With intercrossing of the podocyte-specific 2.5P-Cre mouse line with the ROSA26 reporter line, constitutive expression of β-galactosidase was irreversibly activated specifically in podocytes *in vivo*. Genetically tagged podocytes were traced in a murine anti-GBM nephritis model. We provide evidence that early cellular crescents were composed of cells derived in part from genetically tagged podocytes. In summary, a novel behavior of podocytes during the early phase of cellular crescent formation has been identified.

Materials and Methods

Animals

Heterozygous ROSA26 mice (C57BL/6 background) were obtained from The Jackson Laboratory (Bar Harbor, ME) and crossed with 2.5P-Cre mice (F2N3, 87.5% C57BL/6J/12.5% SJL/J background) (12). Animals were maintained under specific pathogen-free conditions. The University of Michigan Committee on the Use and Care of Animals approved the protocol, in accordance with the principles and procedures outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Identification of Transgenic Mice

Transgenic mice were identified by using a PCR strategy with DNA recovered from tail biopsies, as described previously (12,13). The *ROSA26* transgene was identified with the primers LacZ.fwd (TTCACTGGCCGTCGTTTTACAACGTCGTGA) and LacZ.rev (ATGTGAGCGAGTAACAACCCGTCGGATTCT). The transgene coding for Cre recombinase was identified with the primers Cre.fwd (GCATAACCAGTGAAACAGCATTGCTG) and Cre.rev (GGACATGTTCAGGGATCGCCAGGCG).

Induction of Anti-GBM Glomerulonephritis

Anti-GBM nephritis was induced as described previously (11), with minor modifications. In brief, 8- to 16-wk-old mice were immunized with an intraperitoneal injection of 0.2 mg of rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in 0.2 ml of a 1:1 emulsion with complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). Six days later (day 0), glomerulonephritis was induced with an intravenous injection of 0.4 ml of a 1:5 dilution of rabbit anti-mouse GBM serum (14). Urinary protein concentrations and hematuria were evaluated on days 3 and 6 with dipsticks (Multistix 7; Miles, West Haven, CT).

Fixation and Tissue Processing

Mice were anaesthetized and briefly perfused intracardially with ice-cold PBS for 1 min, followed by 3% paraformaldehyde in PBS for 1 min. Kidneys were resected, and the renal cortex was cut into 1-mm^3 cubes with opposing razor blades. Tissue cubes were immersion-fixed, on a rotator, in ice-cold 3% paraformaldehyde in PBS for 25 min, followed by 30% sucrose for 15 min. Equal amounts of tissue cubes were directly subjected to enzymatic staining with X-gal, snap-frozen, or embedded in paraffin. Paraffin sections (4-μm thick) were stained with periodic acid-Schiff or Masson-trichrome stain.

Immunohistochemical Analyses

Serial sections (4 μm) of paraffin-embedded tissues were rehydrated in PBS and subjected to microwave heating (5 × 5 min at 600 W). Monoclonal rat anti-Ki-67 clone MIB-5 (1:50; Dako, Carpinteria, CA) and goat anti-β-galactosidase polyclonal antibody (1:500, product no. 4600-1409; Biogenesis, Brentwood, NH) were used as primary antibodies. Detection was performed with Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA), with peroxidase as the label and diaminobenzidine as the substrate.

β-Galactosidase Assays

Immersion-fixed tissue cubes were mounted (Tissue-Tek; Miles Inc., Iowa City, IA), and 4-μm cryosections were cut and enzymatically stained with X-gal for 4 to 10 h, as described previously (13). The sections were then postfixed in 4% paraformaldehyde for 30 min., washed in PBS, subjected to Jones methenamine silver staining with a fungus stain kit (product no. 9121; Newcomers Supply, Middleton, WI), according to the protocol provided by the manufacturer, briefly counterstained with eosin, dehydrated through grades of ethanol and xylene, and mounted.

Alternatively, immersion-fixed tissue cubes were washed briefly in PBS and incubated in X-gal staining solution at 37°C for approximately 2 h, with gentle agitation, until the tissue was macroscopically intensely stained. Tissue cubes were washed in PBS, dehydrated in methanol, and cleared in benzyl benzoate/benzyl alcohol (2:1, vol/vol).

Immunofluorescence Microscopy

Indirect immunofluorescence staining was performed with cryosections (4 μm) that had been postfixed in ice-cold acetone for 2 min, washed, blocked with 10% donkey serum, and incubated with the following antibodies: goat anti-β-galactosidase polyclonal antibody (1:50, product no. 4600-1409; Biogenesis), FITC-conjugated AffiniPure donkey anti-goat IgG (1:100, product no. 705-095-147; Jackson Immunoresearch Laboratories), monoclonal rat anti-F4/80 antigen (1:400, product no. MCA497R; Serotec, Duesseldorf, Germany) (15), and Cy3-conjugated AffiniPure goat anti-rat IgG (1:200, product no. 112-165-167; Jackson Immunoresearch Laboratories).

Sections were evaluated with a Leica DMIRB inverted microscope and a RT slider digital camera (type 2.3.1; Diagnostic Instruments, Los Angeles, CA). Images were collected with Spot software (Diagnostic Instruments Inc.) and prepared for presentation with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Results

Somatic Cre Recombination as a Tool to Genetically Tag Podocytes In Vivo

ROSA26 reporter mice were bred to mice of the 2.5P-Cre-transgenic mouse line, which mediates Cre recombination driven by a 2.5-kb human *NPHS2* (podocin) promoter fragment exclusively in podocytes (12). Cre excision of the floxed *neo* cassette allowed expression of β-galactosidase exclusively in podocytes of doubly transgenic mice (Figure 1). The expression of β-galactosidase was driven by the *ROSA* locus, which is transcriptionally active in a ubiquitous manner in all tissues (16). After Cre recombination, β-galactosidase expression became irreversibly active in podocytes, independent of podocin promoter activity.

Inflammatory glomerulonephritis was induced in doubly

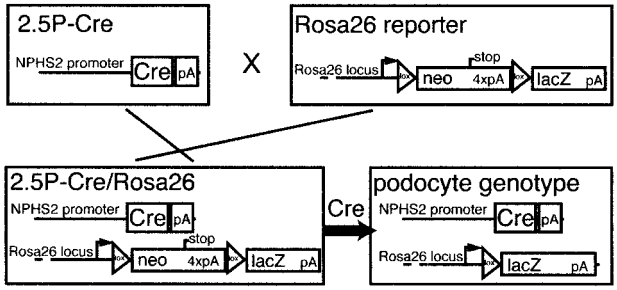

Figure 1. Schematic representation of irreversible genetic tagging of podocytes *in vivo*. Podocyte-specific 2.5P-Cre mice were crossed with ROSA26 reporter mice (top). In doubly transgenic offspring (bottom), transcription of the *LacZ* cassette (coding for β-galactosidase) occurred after Cre-mediated excision (arrow) of the floxed neomycin (*neo*) cassette exclusively in podocytes. In all remaining tissues, expression of β-galactosidase remained silent. lox, *loxP* site; pA, polyadenylation signal.

transgenic mice in a previously characterized anti-GBM nephritis model. Similar to previous reports, approximately 50% of injected mice were affected by renal disease, which was detected with proteinuria and hematuria assays 3 and 6 d after injection of anti-GBM antiserum (11,17). Mice affected by renal disease were euthanized during the early phase of crescent formation, 10 to 12 d after injection. Cellular crescents were observed with periodic acid-Schiff and Masson-trichrome histochemical staining in 10 to 50% of glomeruli of affected kidneys (Figure 2A).

Detection of β-Galactosidase-Positive Podocytes in Cellular Crescents

Kidneys of doubly transgenic mice were cut into 1-mm^3 cubes and immersion-fixed in paraformaldehyde, to preserve β-galactosidase activity. In contrast to perfusion fixation,

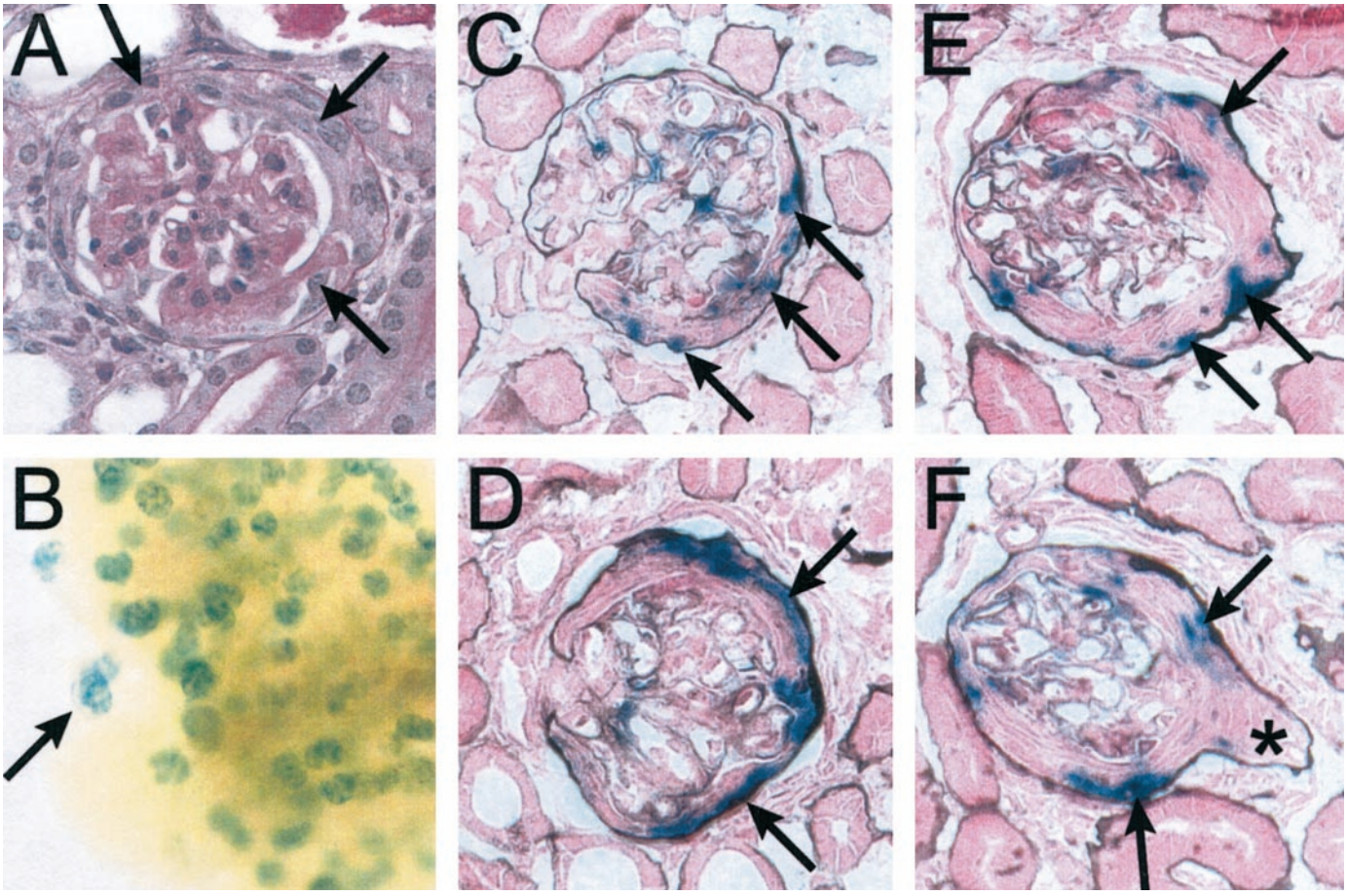

Figure 2. Detection of β-galactosidase-positive cells in cellular crescents with X-gal staining. (A) Successful induction of inflammatory glomerulonephritis with crescent formation in doubly transgenic mice in an anti-glomerular basement membrane (GBM) antiserum model. Arrows indicate an almost circumferential cellular crescent 10 d after injection of the anti-GBM antiserum. Periodic acid-Schiff staining. Magnification, ×400. (B) Glomerular β-galactosidase staining in cubes (1 mm^3) from the renal cortex of the same animal as in A, which had been immersion-fixed, stained with X-gal, and subsequently cleared. Glomerular β-galactosidase staining was preserved throughout the entire block of tissue. β-Galactosidase-positive crescentic caps were observed adjacent to multiple glomeruli (arrow). Magnification, ×50. (C to F) Four representative glomeruli derived from doubly transgenic mice, showing significant crescent formation 10 d after injection. X-gal, eosin, and silver staining. Magnification, ×400. Arrows indicate β-galactosidase-positive cells (blue staining) derived from podocytes. Of note, β-galactosidase-positive cells were located predominantly along the periphery of cellular crescents, along the basement membrane of Bowman's capsule. Complete obstruction of the urinary pole (*) was observed in one specimen (F).

which preserved β-galactosidase activity less efficiently in severely affected glomeruli (data not shown), immersion fixation resulted in homogeneous β-galactosidase staining of all glomeruli throughout the tissue specimens (Figure 2B).

Cryosections from doubly transgenic mice with severe anti-GBM nephritis were stained with X-gal for detection of genetically tagged podocytes and were counterstained with Jones methenamine silver stain (staining basement membranes black) and eosin (staining cytoplasm pink). Four representative glomeruli with cellular crescent formation are presented in Figure 2, C to F. Numerous cells with intense cytoplasmic β-galactosidase staining (blue) were observed in cellular crescents (Figure 2, C to F, arrows). In a quantitative evaluation of 50 cellular crescents (consecutively observed in 10 independent sections), 48 (96%) contained β-galactosidase-positive cells. In 26 cellular crescents (52%), approximately one-half of the cells or more were β-galactosidase-positive. Interestingly, β-galactosidase-positive cells were located predominantly along the periphery of cellular crescents, adjacent to the inner aspect of the PBM of Bowman's capsule. Similarly, the cellular composition of cellular crescents that were obstructing the urinary pole of affected glomeruli was heterogeneous (Figure 2F). At 10 to 12 d after injection, rupture of Bowman's capsule was observed in <5% of affected glomeruli during this early phase of crescent formation. In light-microscopic evaluations, β-galactosidase-positive cells were morphologically indistinguishable from β-galactosidase-negative cells.

For verification of our findings, the distribution of β-galactosidase-positive cells in cellular crescents in doubly transgenic mice was also examined with immunofluorescence staining (Figure 3). In glomeruli of mice with mild or no disease activity (as judged by the lack of proteinuria or hematuria), β-galactosidase-expressing podocytes (green) were distributed in a normal pattern around the capillary tuft (red) (Figure 3, A and A'). Numerous β-galactosidase-positive cells were detected outside the capillary tuft in cellular crescents of mice with severe disease activity (Figure 3, B and B'). Compared with enzymatic X-gal staining, the relative mass of β-galactosidase-positive cells was estimated to be slightly higher with immunofluorescence staining (one-fourth to one-half of the total cell mass). Consistent with the results obtained with enzymatic staining, β-galactosidase-positive cells were preferentially arranged in several layers along the periphery of cellular crescents. β-Galactosidase expression seemed to be reduced along the capillary tuft in severely affected glomeruli,

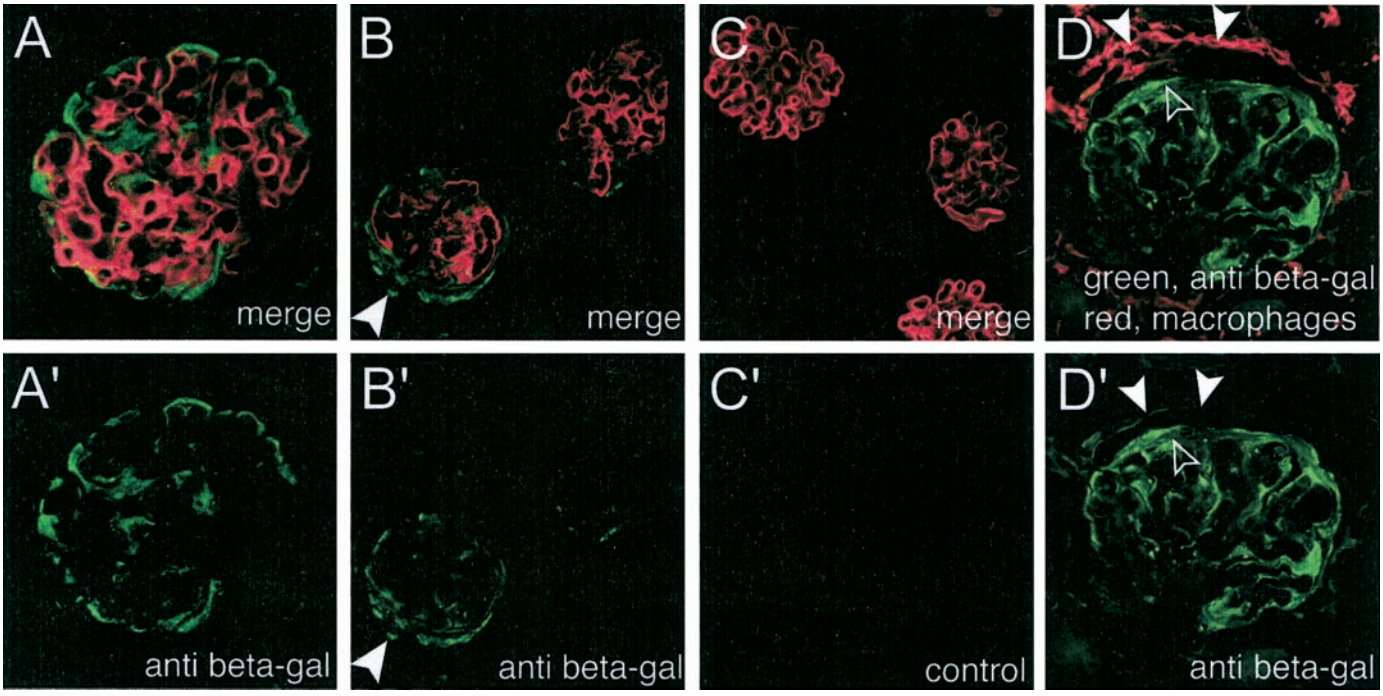

Figure 3. Detection of β-galactosidase-positive cells in cellular crescents with immunofluorescence microscopy. (A and A') β-Galactosidase staining of genetically tagged podocytes along the glomerular capillary tuft was observed in animals with mild or no renal disease (A and A', green, anti-β-galactosidase; A, red, anti-rabbit Ig, revealing the anti-GBM antiserum deposited in glomerular capillaries). Magnification, ×400. (B and B') β-Galactosidase-positive cells located in cellular crescents in several layers outside the capillary tuft were observed in animals with severe renal disease (B and B', green, anti-β-galactosidase; B, red, anti-rabbit Ig). Magnification, ×150. (C and C') In control experiments omitting the primary anti β-galactosidase antiserum, no β-galactosidase staining was observed in kidney sections from any experimental animal (C and C', green, control goat serum; C, red, anti-rabbit Ig). Magnification, ×150. (D and D') Cellular crescents did not contain macrophages or monocytes. F4/80-positive cells were enriched exclusively outside Bowman's capsule (closed arrowheads), in close proximity to cellular crescents (open arrowheads). No F4/80-positive cells were detected in cellular crescents until 11 to 13 d after injection (D and D', green, anti-β-galactosidase; D, red, anti-F4/80). Magnification, ×400.

relative to unaffected glomeruli, with both enzymatic and immunofluorescence staining.

As previously demonstrated by others (11), none of the cells in cellular crescents expressed podocyte markers, including WT-1, podocin, synaptopodin, CD2ap, and nephrin (data not shown). Similarly, F4/80-positive cells, *i.e.*, resident or interstitial cells of the macrophage/monocyte lineage (15), were enriched outside Bowman's capsule but did not infiltrate cellular crescents at this early stage of crescent formation (Figure 3, D and D').

To demonstrate the presence of podocyte bridges during the earliest phase of the disease, before crescent formation (11), paraffin-embedded kidney sections were immunostained for β-galactosidase expression. The anti-GBM model used in this study was focal in nature, so that earlier stages of the disease could also be observed in the same animals euthanized 10 d after injection. Indeed, multiple β-galactosidase-positive cells adhering to both the GBM and the PBM could be observed in each of 10 independent sections, although the tissues were not perfusion-fixed to preserve the urinary space (Figure 4A).

To investigate whether β-galactosidase-positive cells contributed to the formation of cellular crescents through proliferation rather than migration, 4-μm serial sections of kidneys from doubly transgenic mice were alternately stained with immunohistochemical stains for β-galactosidase and the nuclear proliferation marker Ki-67 (Figure 4, B and B'). Ki-67-positive cells were identified throughout the crescents, *i.e.*, peripherally as well as toward the urinary space. Double-positive cells were predominantly observed in peripheral locations, suggesting that podocyte-derived cells proliferate in cellular crescents. As in previous studies (18), many β-galactosidase-negative cells (presumably parietal epithelial cells) also expressed Ki-67, indicating that these cells proliferate in cellular crescents.

Discussion

In this study, a transgenic approach of irreversible genetic tagging was used to trace podocytes in a murine anti-GBM nephritis model *in vivo*. We provide evidence that cells derived from podocytes populate cellular crescents, and we propose that podocytes reach the parietal epithelium of Bowman's capsule during the early phase of crescent formation through migration from the GBM (Figure 5).

The findings of this study expand the observations reported by Le Hir *et al.* (11). In that study, podocytes were observed to adhere to the GBM of the capillary tuft, as well as to the PBM of Bowman's capsule, at an early time point in the progression of crescentic glomerulonephritis, before the formation of cellular crescents (8 d after injection). The bridging podocytes remained positive for podocyte-specific marker proteins. In this study, we demonstrated that these cells were β-galactosidase-positive, indicating their origin from podocytes. Podocyte bridges connecting the PBM to the glomerular tuft, as an event preceding cellular crescent formation, have been observed in the rat Thy-1 model (19). Because crescentic cells expand within the space beneath the parietal epithelium and the PBM and because podocyte-derived cells adhered to the PBM at

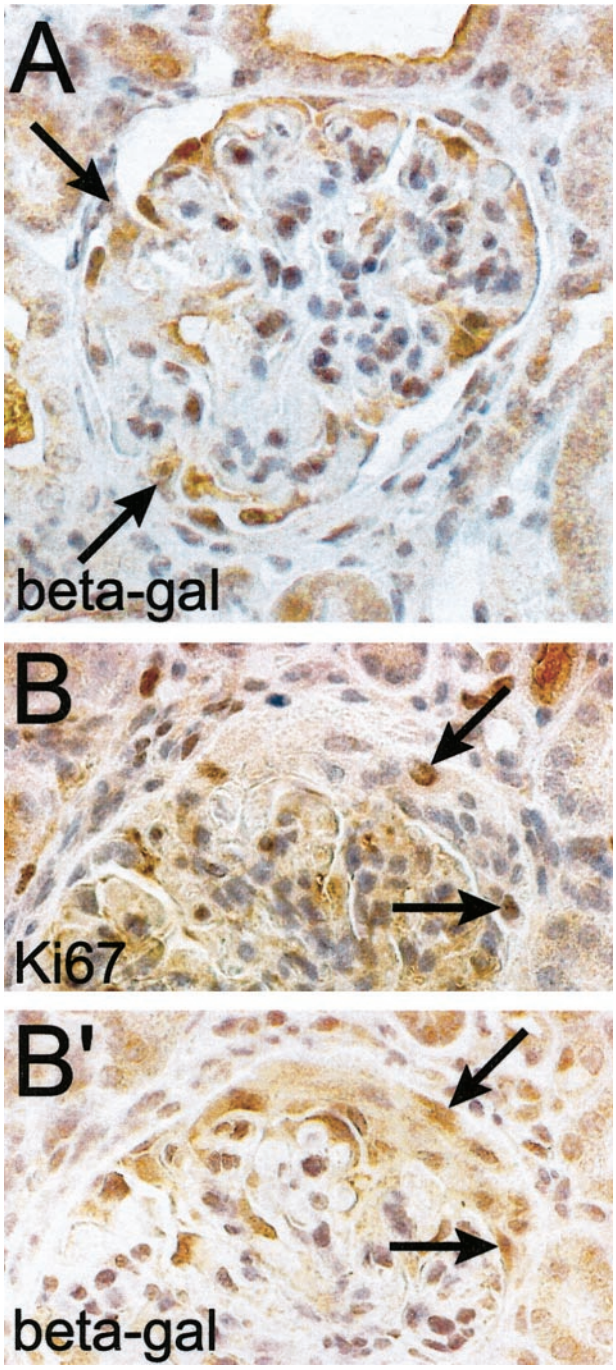

Figure 4. Podocyte-derived cell adherence to the parietal basement membrane (PBM) and expression of Ki-67 in cellular crescents. (A) Immunohistochemical staining for β-galactosidase expression, demonstrating β-galactosidase-positive cells adhering to the GBM and PBM, which is considered to be the initial step in the formation of cellular crescents (arrows). Hematoxylin counterstain. Magnification, ×500. Tubular background staining was observed because of heavy proteinuria. (B and B') Immunohistochemical staining for expression of nuclear proliferation marker Ki-67 (B) and cytoplasmic β-galactosidase (B') in consecutive 4-μm sections of kidneys from doubly transgenic mice (10 d after injection). Arrows indicate immunoreactivity (brown) for both markers in cells of a cellular crescent. Magnification, ×600.

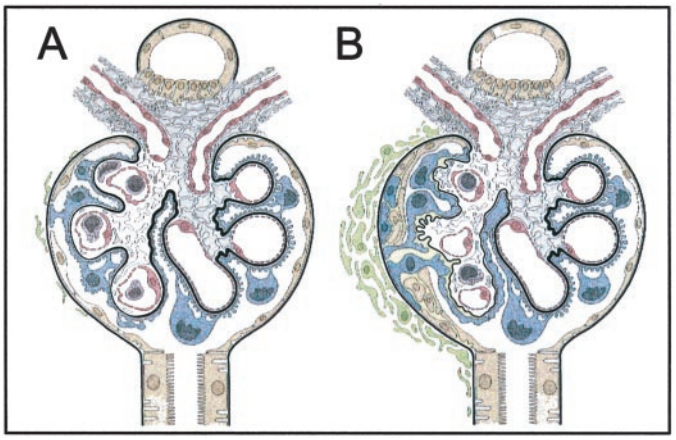

Figure 5. Schematic drawings of the development of a crescent. The morphologic features of a healthy glomerulus are depicted on the right half of the glomerulus, and alterations are indicated on the left half. (A) During the early phase of the disease, podocytes (blue) project multiple filopodial protrusions (microvillous transformation). A cell process of a podocyte intrudes between two parietal epithelial cells (brown) and establishes a bridge between the GBM and the PBM (basement membranes are shown as black lines). (B) Subsequently, β-galactosidase-positive cells derived from podocytes (blue) loose contact with the GBM and are located close to the PBM. Parietal epithelial cells are preferentially distributed toward the urinary space. As suggested by Ki-67 expression, both cell populations may contribute to the formation of cellular crescents by proliferation. Outside the former PBM, an interstitial infiltration (green) develops. Modified from reference 11.

earlier time points, it is not surprising that podocyte-derived cells were preferentially localized in the periphery of cellular crescents, close to the PBM, at later times of crescent formation. It is reasonable to speculate that these cells were derived from bridging podocytes that had lost their contacts with the GBM of the capillary tuft and adhered solely to the basement membrane of Bowman's capsule.

Interestingly, crescentic cells derived from podocytes did not express any podocyte-specific markers. Indeed, antigens characteristic for podocytes, including WT-1, synaptopodin, GLEPP-1, podocin, and others, were never observed to be expressed in cells of cellular crescents in human patients or in any animal models (5,8,11,20). Therefore, a possible contribution of podocytes to the formation of cellular crescents has been generally dismissed. However, the results of this study suggest that podocytes are capable of profound changes in morphologic features (*i.e.*, appearing as simple polygonal cells) and protein expression profiles in crescentic nephritis. The lack of expression of the marker protein podocin in cellular crescents suggests that the 2.5-kb *NPHS2* (podocin) promoter fragment driving Cre expression was not active in any cells in cellular crescents. Therefore, it is unlikely that cells other than those derived from mature podocytes were labeled with Cre recombination in this model of experimental crescentic nephritis.

The origin of the remaining epithelial cells of cellular crescents in the early phase of crescent formation cannot be determined with certainty. In the synchronized anti-GBM nephritis model investigated in this study, cells of the monocyte/macrophage lineage, lymphocytes, polymorphonuclear cells, and fibroblasts were not present during the early phases of crescent formation (11,14,17). Because specific marker antigens for parietal epithelial cells have not yet been identified, we speculate that, in the anti-GBM model used in this study, the remaining, β-galactosidase-negative subpopulation of cells in cellular crescents was derived primarily from parietal epithelial cells.

A number of earlier studies using immunohistochemical staining and *in vivo* labeling suggested that early cellular crescents arise from proliferating epithelial cells intrinsic to glomeruli (10,21). To date, proliferation of podocytes has not been clearly demonstrated. However, podocytes in adults are known to have a limited potential for cell proliferation (22,23). In collapsing FSGS, dysregulated podocytes lose the expression of specific markers and seem to proliferate in a manner reminiscent of localized benign neoplastic growth (24). In a transgenic mouse model, genetic reduction of the expression of WT-1, a transcription factor expressed exclusively in podocytes in the kidneys of adult mice, was sufficient to induce cell proliferation in dysregulated podocytes and resulted in the formation of cellular crescents (25). In this study, expression of Ki-67, a nuclear proliferation marker expressed by proliferating crescentic cells (18), was detected in both β-galactosidase-positive cells (*i.e.*, cells derived from podocytes) and β-galactosidase-negative cells (parietal epithelial cells) in cellular crescents in serial sections. The relatively high proportion of podocytes in cellular crescents suggests that podocytes contributed to the formation of cellular crescents primarily through proliferation, rather than migration from the capillary tuft.

Acknowledgments

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