Kidney International, Vol. 58 (2000), pp. 674–683

Differential expression of cyclin-dependent kinase inhibitors in human glomerular disease: Role in podocyte proliferation and maturation

Stuart J. Shankland, Frank Eitner, Kelly L. Hudkins, Tracy Goodpaster, Vivette D'Agati, and Charles E. Alpers

Department of Medicine, Division of Nephrology, and Department of Pathology, University of Washington School of Medicine, Seattle, Washington; and Department of Pathology, Columbia University, New York, New York, USA

Differential expression of cyclin-dependent kinase inhibitors in human glomerular disease: Role in podocyte proliferation and maturation.

Background. Normal human podocytes are terminally differentiated and quiescent cells. It is not known why podocytes fail to proliferate in response to most forms of injury. Proliferation is regulated by cell cycle proteins and their inhibitors. The Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors (p21, p27, p57) in general prevent proliferation by inhibiting cyclin-CDK complexes. In the current study, we determined the expression and possible role of specific CDK inhibitors in podocyte proliferation in human disease characterized by podocyte injury.

Methods. Immunostaining was performed for the CDK inhibitors p21, p27, and p57 and the proliferation marker Ki-67 on renal biopsies from patients with minimal change disease (MCD; $N = 6$), membranous glomerulopathy (MGN; $N = 19$), cellular variant of focal segmental glomerulosclerosis (FSGS; $N = 12$), collapsing glomerulopathy (CG; $N = 9$), and HIV-associated nephropathy (HIVAN; $N = 16$). Adult nephrectomy specimens without evidence of glomerular disease served as controls ($N = 9$).

Results. Normal quiescent podocytes express p27 and p57, but not p21. In diseases without podocyte proliferation (MCD, MGN), p21, p27, and p57 expression did not change. In contrast, there was a uniform decrease in p27 and p57 immunostaining in diseases with podocyte proliferation (cellular FSGS, CG, and HIVAN). This was accompanied by the de novo expression of p21 in podocytes.

Conclusions. Our results show that podocyte quiescence may require the presence of the CDK inhibitors p27 and p57. In human glomerular diseases, a decrease in p27 and p57 may be permissive for the altered proliferative podocyte phenotype. p21 may have a multifactorial role in podocyte cell cycle regulation.

Key words: cell cycle, kidney, proliferation, glomerular podocytes, visceral glomerular epithelial cell.

Received for publication October 4, 1999
and in revised form February 11, 2000
Accepted for publication March 4, 2000

The mature podocyte (also known as the visceral glomerular epithelial cell) is a terminally differentiated cell that serves specialized functions in the glomerulus [1]. During glomerulogenesis, immature or undifferentiated podocytes engage the cell cycle and proliferate [2]. However, upon acquiring a mature and differentiated phenotype, podocytes exit the cell cycle and cease proliferating so that mature podocytes exhibit a quiescent phenotype [3, 4]. The podocyte response to injury in glomerular disease includes flattening and effacement of foot processes, and in more severe injury, this is associated with podocyte detachment from the underlying glomerular basement membrane [1]. Recently, potential proliferative responses of mature podocytes to injury have been recognized and are a subject of considerable debate [5].

In glomerular diseases in which the podocyte is a primary target of injury, such as minimal change disease (MCD) and membranous nephropathy [6], it is generally accepted that podocytes do not re-engage the cell cycle and maintain their terminally differentiated phenotype. In some forms of focal segmental glomerulosclerosis (FSGS), podocytes detach from the underlying glomerular basement membrane following injury. Studies have shown that the inability of podocytes to proliferate and replace those lost in this injury process may result in a denuded glomerular basement membrane, which leads to the development of glomerulosclerosis [7–9].

More recently, it has been recognized that podocytes proliferate in response to certain forms of injury. The cellular variant of FSGS [10], collapsing glomerulopathy (CG) [11–13], and HIV-associated nephropathy (HIVAN) [11] are characterized by glomerular cell proliferation. The origin of the proliferating cells in CG has been somewhat controversial. Barisoni et al [11] and Bariety et al [14] showed a predominance of podocyte proliferation in idiopathic CG. In contrast, Nagata et al showed that parietal epithelial cells proliferate in CG [15]. It has even

been suggested that these cells may be monocytes arising from transformed podocytes [14].

Cell proliferation and, to some extent, cell differentiation are controlled at the level of the cell cycle by cell cycle-regulatory proteins. Proliferation requires that specific cyclin-dependent kinases (CDKs) are activated by a partner cyclin in each phase of the cell cycle [16]. Thus, CDK4 and CDK6 are activated by D-type cyclins in early G_1 phase of the cell cycle. CDK2 is activated by cyclin E in late G_1 and by cyclin A in the S phase of DNA synthesis, and cdc2 (formerly CDK1) is bound by cyclin B in mitosis [reviewed in 17, 18]. Cyclin-CDK complexes, in turn, are negatively regulated by CDK inhibitors [19]. The Cip/Kip family of CDK inhibitors, which include the individual proteins called $p21^{Cip1/WAF1}$ (p21) [20, 21], $p27^{Kip1}$ (p27) [22, 23], and $p57^{Kip2}$ (p57) [24], inhibits cyclin D-CDK4 and cyclin E-CDK2 in G_1 and cyclin A-CDK2 in S phase. Recent studies have shown that p21 also limits cyclin B-cdc2 in mitosis [25]. The CDK inhibitors p21, p27, and p57 are constitutively expressed in certain cell types, and there is a growing body of evidence that individual members of the Cip/Kip family of CDK inhibitors also have a critical role in the differentiation of certain cell types [24, 26–29].

The purpose of the current study was to examine which cell cycle-regulatory proteins contribute to the maintenance of a differentiated and quiescent podocyte phenotype in certain forms of glomerular disease and which contribute to processes of de-differentiation and proliferation in other forms of injury. We examined the expression of the Cip/Kip CDK inhibitors p21, p27, and p57 in different human glomerular diseases in which podocytes are the major target of injury. p27 and p57 are constitutively expressed in mature podocytes, but p21 is not (in agreement with previous studies by ourselves [27] and others [28]). A decrease in these levels of p27 and p57 and a corresponding de novo expression of p21 coincide with podocyte proliferation and an immature phenotype.

METHODS

Source of tissue

Cases of minimal change disease (MCD; $N = 6$), cellular FSGS ($N = 12$), collapsing glomerulopathy (CG; $N = 9$), and human immunodeficiency virus-associated nephropathy (HIVAN; $N = 16$) were selected retrospectively from the archives of the Renal Pathology Laboratory of Columbia University. All biopsies had been performed in the two-year period of 1998 to 1999. Cases of membranous glomerulopathy (MGN; $N = 20$) were selected retrospectively from the archives of the University of Washington Renal Pathology Laboratory. All patients were biopsied for investigation of nephrotic proteinuria, with or without associated renal insufficiency. The subgroups of cellular FSGS and CG were defined according to previously described morphologic and clinical features [11]. All patients with CG had negative HIV serologies. In all patients with HIVAN, diagnosis was confirmed by demonstration of serum antibodies to HIV-1 using enzyme-linked immunosorbent assay (ELISA) followed by Western blot. For control tissues without glomerulopathy, macroscopically normal-appearing renal tissues were obtained from nephrectomies ($N = 9$) performed for localized renal cell carcinoma, at sites away from the tumor. The number of glomeruli from each case was counted on light microscopy, and the number of injured glomeruli was recorded in focal glomerular diseases (FSGS, HIVAN, and CG).

Antibodies used

The following primary antibodies were used: (*1*) $p21^{Cip1/Waf1}$ (p21), a mouse monoclonal antibody (clone SX118; Pharmingen, San Diego, CA, USA) was raised against a purified recombinant human p21-GST fusion protein [30]. This antibody reacts with amino acids 145 to 165 of human p21 and has been characterized by immunostaining and Western blot analysis. (*2*) $p27^{Kip1}$ (p27) is a goat polyclonal antibody (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) that maps to an epitope to the carboxy terminus of p27 and has been characterized by Western blot analysis [31, 32]. In the current study, staining was abolished by preabsorbing the antibody with immunizing peptide (Santa Cruz Biotechnology). (*3*) $p57^{Kip2}$ (p57; C-20 Santa Cruz Biotechnology) is a rabbit polyclonal antibody raised against a peptide corresponding to amino acids 286 to 305 of the carboxy terminus of human p57. This antibody has been previously characterized by immunoblotting and immunohistochemistry [28]. In the current study, immunostaining was abolished by preabsorbing the antibody with the immunizing peptide (Santa Cruz Biotechnology). (*4*) Ki-67, clone MIB-1, is a murine monoclonal antibody (Coulter/Immunotech, Miami, FL, USA) that reacts with the proliferation-associated Ki-67 antigen and has been previously shown to correlate with DNA synthesis after antigen retrieval.

Immunostaining

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections following protocols that we have previously reported [27]. Four-micron thick sections of tissue samples were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Sections were pretreated by steam heating for 20 minutes in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Non-specific antibody binding was blocked by incubation for 30 minutes in 10% normal

Table 1. Characteristics of biopsies studied

Disease	*N* patients	*N* total glomeruli	Average *N* glomeruli/case	% of glomeruli with podocyte pathology/biopsy[a]
MCD	6	67	11.17	0 (0.67)
MGN	19	247	26	0 (0/247)
Cellular FSGS	12	196	16.33	56.12 (110/196)
Collapsing GN	9	117	13.0	66.66 (78/117)
HIVAN	15	203	13.53	71.43 (145/203)

Uniform patterns in disease in which only a percentage of glomeruli demonstrated characteristic histopathologic changes. Histologically uninvolved glomeruli followed the patterns of CDK-inhibitors and Ki-67 expression observed in cases of MCD and MGN. The patterns in glomeruli with collapsing and sometimes sclerosing lesions with characteristic podocyte changes also demonstrated uniform patterns of altered CDK-inhibitor expression, as illustrated in Figures 4 and 5.

[a] Assessment by light microscopy

mouse serum (for monoclonal antibodies) or 10% normal rabbit serum (for polyclonal antibodies). The tissue sections were then incubated for one hour at room temperature (p27, p57, Ki-67) or overnight at 4°C (p21) with the primary antibody diluted in phosphate-buffered saline (PBS) plus 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA). After PBS washes, tissue sections were incubated sequentially with a biotinylated secondary antibody, the ABC-Elite Reagent (Vector Laboratories) and with 3.3′-diaminobenzidine with nickel chloride enhancement. A methyl green nuclear counter stain was used. Tissue from placenta and colon were used as positive controls for the antibodies to p21 and p57 [33]. Human fetal kidney was used as a positive control for p27 and p57, as previously described [27, 28].

Negative controls for immunostaining consisted of substituting the primary antibody with an irrelevant isotype-matched murine monoclonal antibody (Dako, Carpinteria, CA, USA) or nonimmune rabbit serum (Dako). Jones' silver methamine staining was performed on histologic sections from all cases using conventional procedures.

RESULTS

Ki-67 immunostaining separates different types of glomerular diseases

Table 1 shows the total number of glomeruli examined in each glomerular disease, the average number of glomeruli studied in individual cases, and the percentage of glomeruli with disease in the focal diseases. We stress that the immunostaining patterns described later in this article were homogeneous for all glomeruli in the cases of MCD and membranous glomerulonephritis and were homogeneous in those glomeruli or segments of glomeruli exhibiting the characteristic pathology of cellular FSGS, CG, and HIVAN, as illustrated in Figures 1–5.

To determine the presence of DNA synthesis and entry into S phase of the cell cycle, tissue sections were stained with an antibody to the proliferation antigen Ki-67. Ki-67 immunostaining was absent in normal podocytes (Fig. 1E) and was also not detected in MCD (results not shown) and membranous nephropathy (Fig. 3E). In contrast, there was a marked increase in glomerular immunostaining for Ki-67 in the cellular variant of FSGS (Fig. 4E), CG, and HIVAN (Fig. 5E). In glomeruli in which the features of collapse or of cellular FSGS were segmentally distributed, the expression of the Ki-67 antigen was also segmentally distributed and corresponded to the histologic alterations. Our results showed that Ki-67 staining divided diseases of the podocyte into those in which the podocyte maintained a differentiated and quiescent phenotype (MCD, membranous nephropathy) and those diseases associated with a de-differentiated and proliferative phenotype (cellular variant of FSGS, CG, and HIVAN).

Differential expression of CDK inhibitors in normal human glomeruli

Immunostaining for p57 was abundant and restricted to normal podocytes and was not present in glomerular mesangial and endothelial cells (Fig. 1B). The CDK inhibitor p27 is constitutively expressed in normal human glomeruli (Fig. 1C). Although positive nuclear staining was rarely detected in all three resident glomerular cell types in normal kidneys, immunostaining for p27 was abundant in podocytes (Fig. 1C).

Immunostaining for p21 was not detected in normal glomeruli (Fig. 1D). Positive controls included concurrent p21 immunostaining in tubulointerstitial cells and in the smooth muscle cells in small arteries in the same tissue sections and demonstration of p21 expression in simultaneously processed, and stained tissue sections from placenta where p21 expression have been well defined [30, 33].

Expression of CDK inhibitors in nonproliferative glomerular diseases (MCD and MGN)

Podocyte expression of p27 and p57 was well preserved in all glomeruli in MCD (Fig. 2) and MGN (Fig. 3). The glomerular expression of p21 was undetectable in these diseases (Figs. 2 and 3). These results show that the glomerular expression of the CDK inhibitors p21,

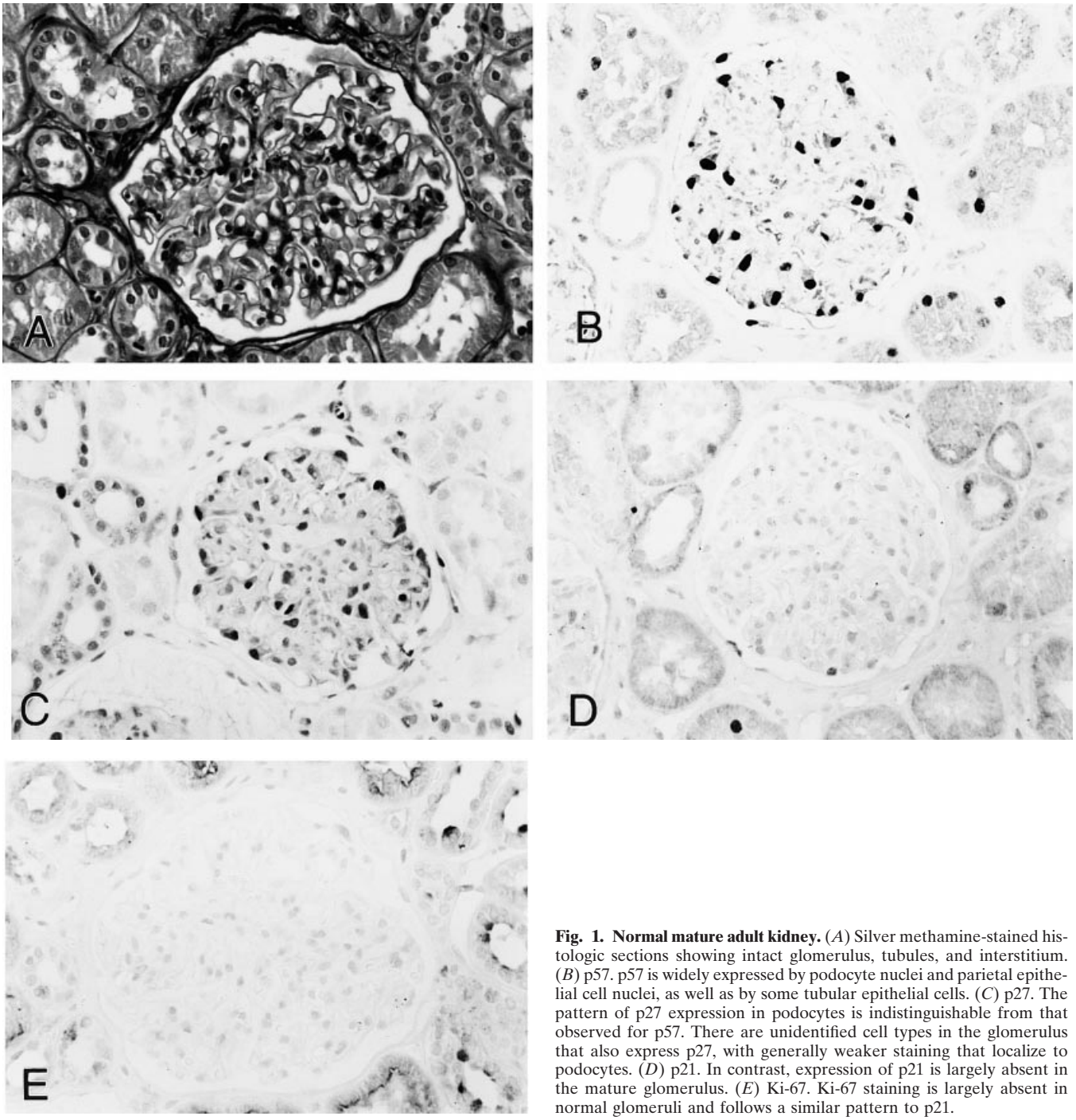

Fig. 1. Normal mature adult kidney. (*A*) Silver methamine-stained histologic sections showing intact glomerulus, tubules, and interstitium. (*B*) p57. p57 is widely expressed by podocyte nuclei and parietal epithelial cell nuclei, as well as by some tubular epithelial cells. (*C*) p27. The pattern of p27 expression in podocytes is indistinguishable from that observed for p57. There are unidentified cell types in the glomerulus that also express p27, with generally weaker staining that localize to podocytes. (*D*) p21. In contrast, expression of p21 is largely absent in the mature glomerulus. (*E*) Ki-67. Ki-67 staining is largely absent in normal glomeruli and follows a similar pattern to p21.

p27, and p57 was indistinguishable from normal adult nephrectomy specimens that did not have glomerular disease (Fig. 1).

Expression of CDK inhibitors in proliferative glomerular diseases (cellular FSGS, CG, and HIVAN)

Changes in p57 staining were also associated with podocyte proliferation (Figs. 4 and 5). There was a general decrease in intensity of p57 staining in glomeruli in which proliferation was not pronounced (results not shown). In areas of proliferation, there was a segmental absence of podocyte immunostaining for p57. The absence was not due to cell loss, as nuclei were easily detected by the methyl green counterstain. In many glomeruli in which serial sections were available, there was a concordant decrease/absence of p27 and p57 immunostaining in

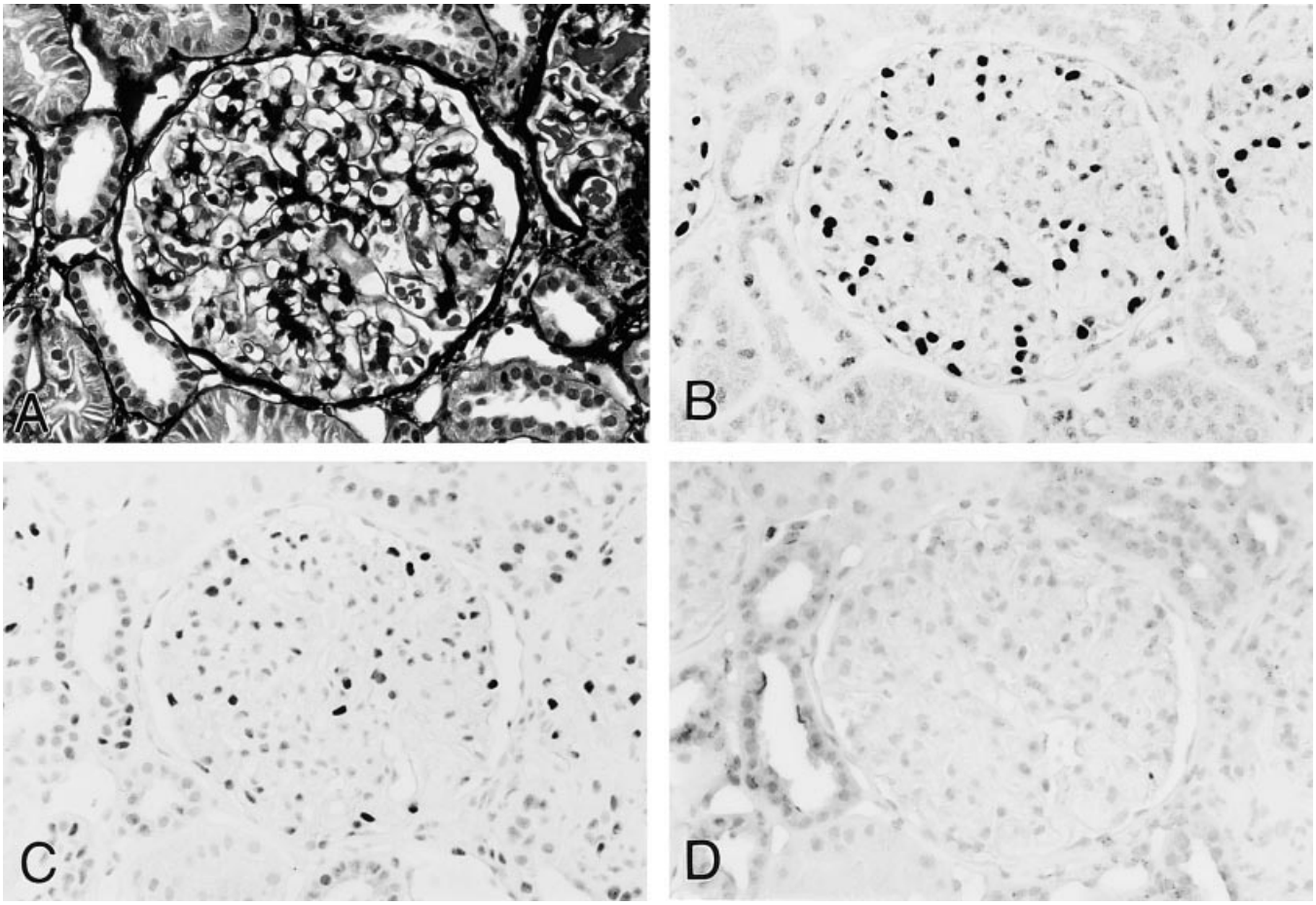

Fig. 2. Serial sections of a representative glomerulus from a patient with minimal change disease (MCD). (*A*) Silver methamine-stained glomerulus showing a histologically normal tuft architecture, tubules, and interstitium. (*B*) p57. The pattern of p57 expression is unchanged from the mature normal glomerulus without disease. (*C*) p27. The pattern of p27 expression is unchanged from the normal mature glomerulus without disease. (*D*) p21. There is an absence of p21 expression in this glomerulus that is unchanged from the normal mature glomerulus without disease.

identical segmental areas of proliferation in cellular FSGS, CG, and HIVAN (Figs. 4 and 5).

In contrast to the nonproliferative glomerular diseases, there was a marked decrease in staining intensity for p27 in the majority of glomeruli in patients with the cellular variant of FSGS (Fig. 4C), CG, and HIVAN (Fig. 5C), including glomeruli without histologically identifiable lesions. Moreover, p27 immunostaining was not detected in segmental areas of podocyte proliferation. Careful inspection of biopsies treated with nuclear counterstained methyl green showed that the decrease or absence of p27 immunostaining was not due to the loss of podocyte number, but rather due to an absolute decrease in nuclear staining.

There was a marked increase in p21 immunostaining in the cellular variant of FSGS (Fig. 4D), CG, and HIVAN (diseases characterized by podocyte proliferation; Fig. 5D). In these cases, the de novo staining for p21 was in a segmental pattern, which spatially coincided with the segmental decrease/absence of p27 and p57 immunostaining. Nuclear immunostaining for p21 was detected in podocytes and in mesangial and glomerular endothelial cells.

DISCUSSION

The mature podocyte is a terminally differentiated cell that expresses specific differentiation markers, including GLEPP-1, WT-1, C3b receptor, podocalyxin, and synaptopodin [reviewed in 34]. In contrast to immature podocytes, which proliferate during glomerular development in utero, differentiated podocytes have a quiescent phenotype [2]. The maintenance of a differentiated phenotype is required for podocytes to perform their specialized functions [1]. The current study provides important evidence that human podocyte differentiation and proliferation in vivo may depend on the levels of specific CDK inhibitors. Our results show that the CDK inhibitors p27 and p57, but not p21, are constitutively expressed in normal differentiated podocytes, and persistent expres-

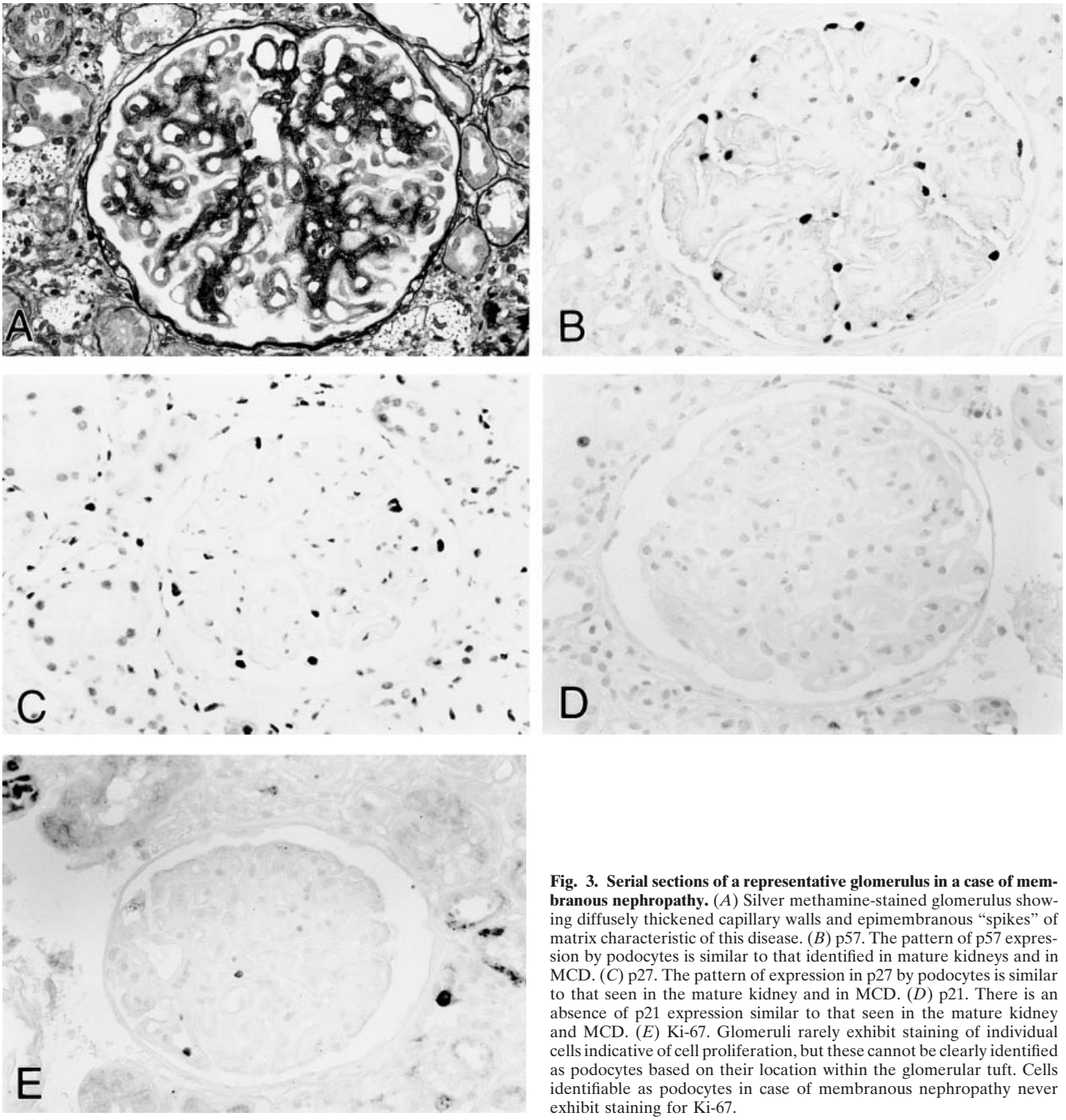

Fig. 3. Serial sections of a representative glomerulus in a case of membranous nephropathy. (*A*) Silver methamine-stained glomerulus showing diffusely thickened capillary walls and epimembranous "spikes" of matrix characteristic of this disease. (*B*) p57. The pattern of p57 expression by podocytes is similar to that identified in mature kidneys and in MCD. (*C*) p27. The pattern of expression in p27 by podocytes is similar to that seen in the mature kidney and in MCD. (*D*) p21. There is an absence of p21 expression similar to that seen in the mature kidney and MCD. (*E*) Ki-67. Glomeruli rarely exhibit staining of individual cells indicative of cell proliferation, but these cannot be clearly identified as podocytes based on their location within the glomerular tuft. Cells identifiable as podocytes in case of membranous nephropathy never exhibit staining for Ki-67.

sion of these molecules following injury is associated with the persistence of a quiescent (nonproliferative) phenotype. In contrast, glomerular diseases associated with a decrease in podocyte expression of p27 and p57 lose the mature phenotype and acquire a proliferative and immature phenotype.

In the current study, it was found that individual proteins of the Cip/Kip family of CDK inhibitors are differentially expressed in normal human podocytes. Both p27 and p57 are constitutively expressed in mature podocytes, where the CDK inhibitor p21 is not detected. These findings are consistent with previous studies by our group and others showing that podocyte differentiation and exit from the cell cycle during glomerulogenesis coincides with the de novo expression of p27 and p57, but not p21 [27, 28].

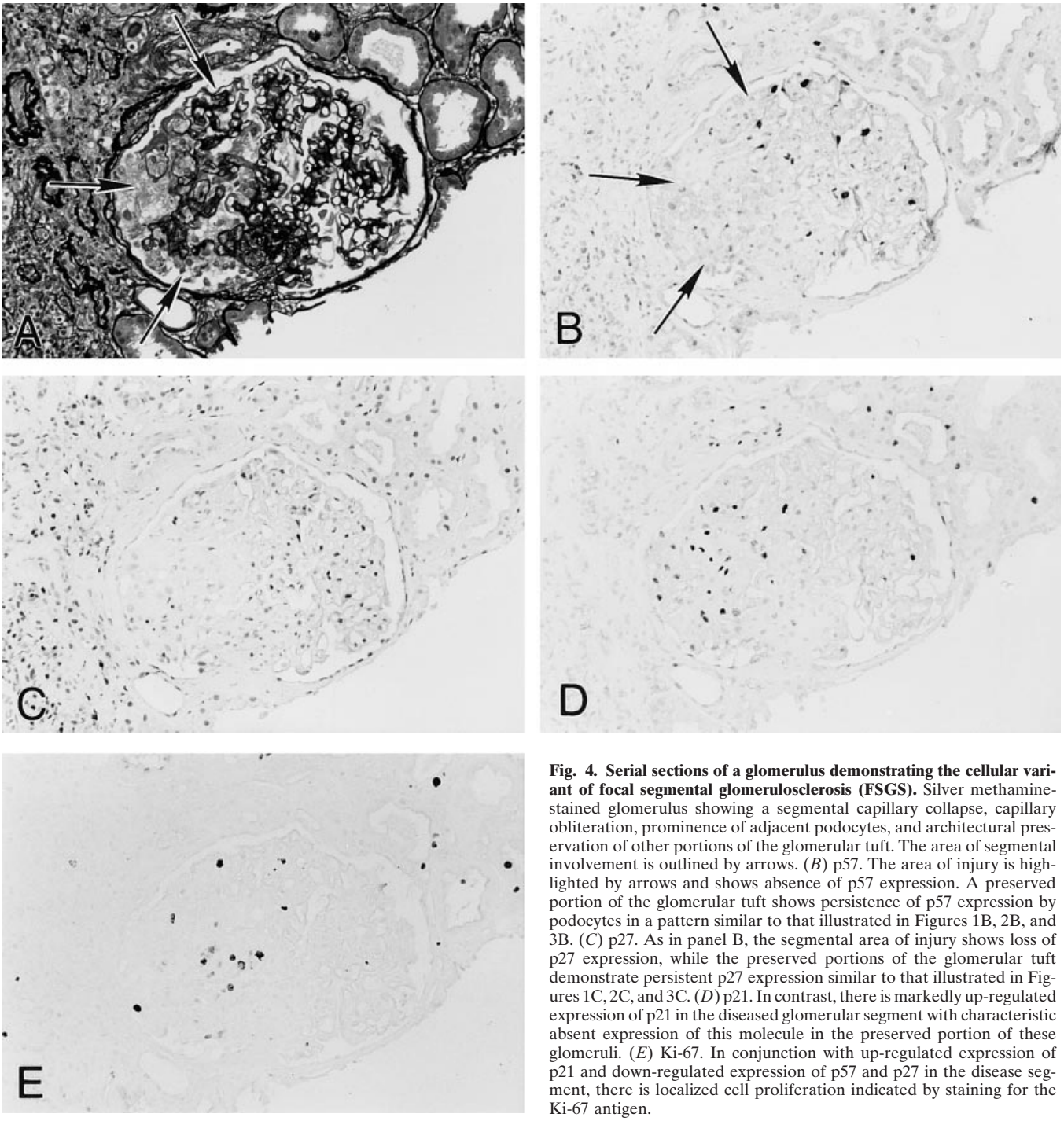

Fig. 4. Serial sections of a glomerulus demonstrating the cellular variant of focal segmental glomerulosclerosis (FSGS). Silver methamine-stained glomerulus showing a segmental capillary collapse, capillary obliteration, prominence of adjacent podocytes, and architectural preservation of other portions of the glomerular tuft. The area of segmental involvement is outlined by arrows. (*B*) p57. The area of injury is highlighted by arrows and shows absence of p57 expression. A preserved portion of the glomerular tuft shows persistence of p57 expression by podocytes in a pattern similar to that illustrated in Figures 1B, 2B, and 3B. (*C*) p27. As in panel B, the segmental area of injury shows loss of p27 expression, while the preserved portions of the glomerular tuft demonstrate persistent p27 expression similar to that illustrated in Figures 1C, 2C, and 3C. (*D*) p21. In contrast, there is markedly up-regulated expression of p21 in the diseased glomerular segment with characteristic absent expression of this molecule in the preserved portion of these glomeruli. (*E*) Ki-67. In conjunction with up-regulated expression of p21 and down-regulated expression of p57 and p27 in the disease segment, there is localized cell proliferation indicated by staining for the Ki-67 antigen.

Podocytes maintain a differentiated and nonproliferative phenotype in MCD and membranous nephropathy. In contrast, podocytes re-enter the cell cycle and acquire a proliferative phenotype in the cellular variant of FSGS [10], idiopathic CG [11], and HIVAN [11] resembling an immature phenotype. Moreover, Barisoni et al have shown that podocyte proliferation is associated with a loss of the differentiated markers such as WT-1 in certain forms of glomerular disease, including HIVAN [11]. A second major finding in this study was that the immunostaining for the CDK inhibitors p27 and p57 did not decrease in MCD. Furthermore, p27 was not altered in membranous nephropathy, although there was a decrease in the intensity of p57 immunostaining in podocytes in some biopsies exhibiting membranous nephropathy. In contrast, there were two patterns of p27 and p57

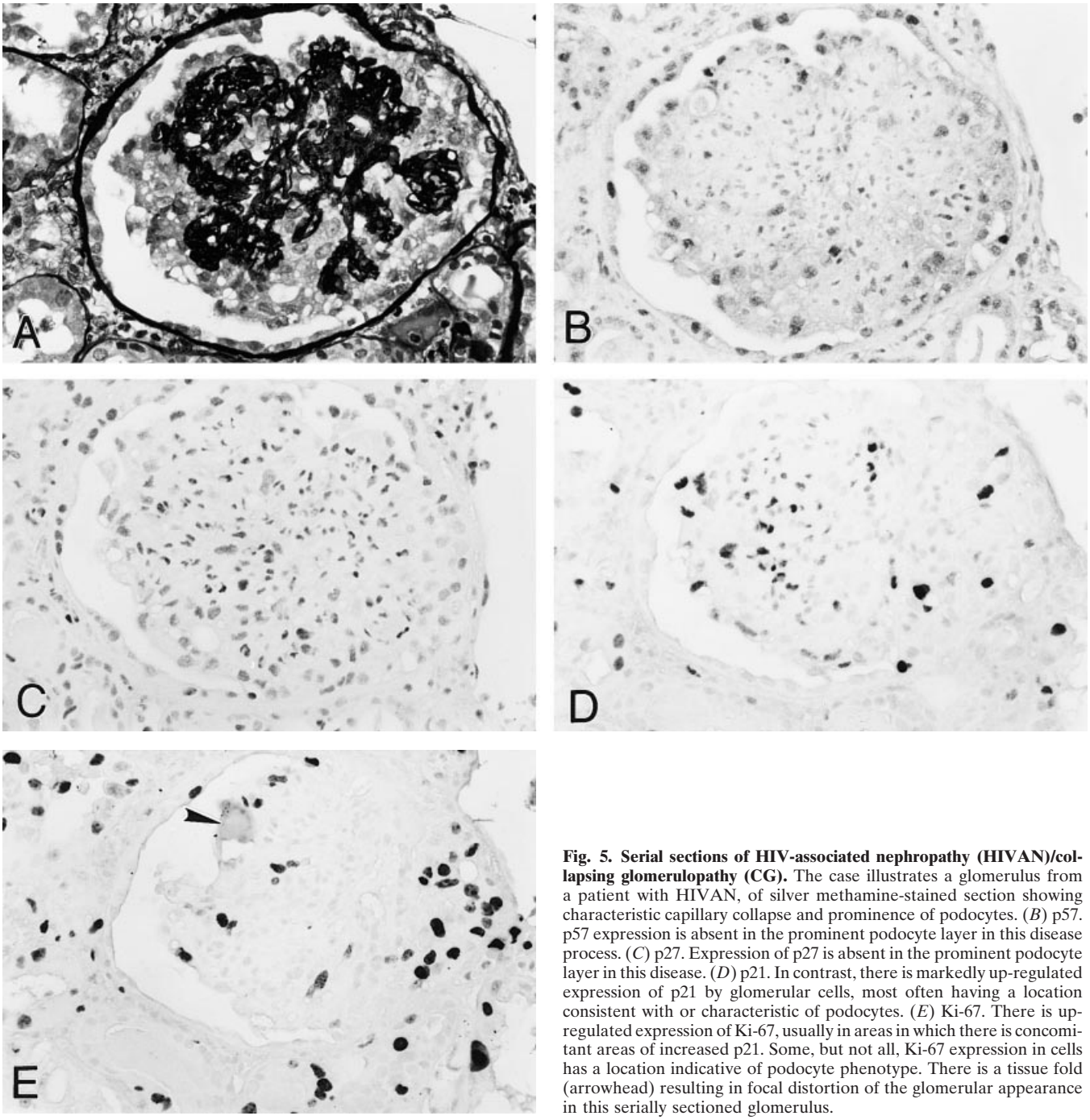

Fig. 5. Serial sections of HIV-associated nephropathy (HIVAN)/collapsing glomerulopathy (CG). The case illustrates a glomerulus from a patient with HIVAN, of silver methamine-stained section showing characteristic capillary collapse and prominence of podocytes. (*B*) p57. p57 expression is absent in the prominent podocyte layer in this disease process. (*C*) p27. Expression of p27 is absent in the prominent podocyte layer in this disease. (*D*) p21. In contrast, there is markedly up-regulated expression of p21 by glomerular cells, most often having a location consistent with or characteristic of podocytes. (*E*) Ki-67. There is up-regulated expression of Ki-67, usually in areas in which there is concomitant areas of increased p21. Some, but not all, Ki-67 expression in cells has a location indicative of podocyte phenotype. There is a tissue fold (arrowhead) resulting in focal distortion of the glomerular appearance in this serially sectioned glomerulus.

expression noted in cellular FSGS, CG, and HIVAN. First, there was a segmental absence of immunostaining for both p27 and p57 in a distribution that was nearly identical to that of Ki-67 expression in areas of podocyte DNA synthesis in individual glomeruli. Second, histologically "normal" glomeruli present in cases of cellular FSGS, CG, and HIVAN demonstrated a general decrease in staining intensity for p27 and p57 in the podocytes. The decrease in p27 and p57 immunostaining was not due to a loss of cell number because podocyte nuclei were present and easily identified in the conventionally stained histologic preparations. These findings support a paradigm in which the reduction in p27 and p57 precedes the development of histologically identifiable glomerular lesions.

Experimental studies also have shown a role for p27 in podocyte proliferation. A lack of podocyte proliferation in the passive Heymann nephritis model of experi-

mental membranous nephropathy coincides with increased p27 levels in injured podocytes [35]. It has been shown that p27 binds to and inhibits CDK2 activity in experimental membranous nephropathy, thereby limiting podocyte proliferation [35]. Immune-mediated glomerulonephritis in p27 null mice is associated with increased glomerular cell proliferation compared with controls, and the proliferating cells in nephritic p27 null mice were typically podocytes [36]. Taken together, the current study shows that p27 levels may have a critical role in podocyte differentiation and proliferation in human glomerular disease. The persistence/maintenance of p27 expression in MCD and membranous nephropathy was associated with and may be responsible for a differentiated and quiescent podocyte phenotype, whereas a decrease in p27 levels in the cellular variant of FSGS, collapsing GN, and HIVAN is closely correlated with podocyte proliferation and may be necessary for these processes to develop.

The CDK inhibitor p57 also regulates cell proliferation and differentiation [37]. p57 is typically expressed in differentiated and postmitotic nonrenal cells [24], and we (unpublished data) and others have shown that there is de novo expression of p57 in podocytes during glomerulogenesis that coincides with p27 expression and with podocyte acquisition of a terminally differentiated phenotype. In the current study, we show that a decrease or absence of p57 immunostaining was associated with an immature and proliferative podocyte phenotype in human disease. However, a decrease in p57 immunostaining alone, suggestive of diminished but not absent levels of p57 peptide, was apparent in membranous nephropathy without a concomitant decrease in p27 levels. This constellation of findings was not sufficient for demonstrable proliferation. Taken together, our results indicate that the inhibitory threshold imposed by both p27 and p57 needs to be overcome in order for podocytes to proliferate and acquire an immature phenotype.

A third and surprising major finding in the current study was that the pattern of expression for the CDK inhibitor p21 was different from p27 and p57. Immunostaining for p21 was absent in normal glomeruli and did not increase in MCD and membranous nephropathy. In contrast to p27 and p57, p21 expression increased during podocyte proliferation in the cellular variant of FSGS, collapsing GN, and HIVAN. We can only speculate on the role of p21 in human glomerular disease characterized by podocyte injury. First, increased p21 may serve a protective role to limit further podocyte proliferation when the CDK inhibitors p27 and p57 are decreased. Evidence for this is suggested by recent experimental studies. In anti-Thy1 glomerulonephritis, an increase in p21 levels coincides with the resolution of mesangial cell proliferation [38]. In experimental diabetic nephropathy [39], increased p21 levels are associated with a lack of mesangial cell proliferation. The absence of podocyte proliferation in experimental membranous nephropathy (passive Heymann nephritis model) also coincides with a marked increase in p21 expression, and lowering p21 levels with the mitogenic growth factor basic fibroblast growth factor in rats with passive Heymann nephritis are associated with low-grade podocyte proliferation [35]. Finally, we have recently shown that podocyte proliferation is markedly increased in experimental glomerulonephritis in mice made genetically deficient in p21 (p21$-/-$) compared with nephritic p21$+/+$ mice [40].

Second and mechanistically more confusing is that recent studies have shown that small increases in p21 levels may serve to facilitate proliferation by providing a "scaffold-like" function for the assembly of cyclins, CDKs, and proliferating cell nuclear antigen [41]. Thus, one may speculate that the increase in p21 in certain podocyte diseases facilitates proliferation in the absence of other CDK inhibitors such as p27 and p57. Third, Nagata et al has shown that p21 is transiently increased during podocyte development so that the increased expression could be a manifestation of an immature phenotype, independent of its regulatory role for cell proliferation [28].

In summary, the results of the current study show a role for specific cell cycle proteins in glomerular disease characterized by podocyte injury. In diseases characterized by podocyte proliferation, the decreased expression of specific CDK inhibitors is similar to their pattern of expression during early podocyte development. The corollary of this is also true in that we show that the presence of the CDK inhibitors p27 and p57 is associated with the persistence of a differentiated and quiescent podocyte phenotype, and is consistent with the general notion that the principal function of these molecules is to inhibit cell proliferation. Recent evidence that p21, often considered to function as a suppressor of cell proliferation like p27 and p57, has more complex biologic functions that include facilitation of cell proliferation, is further supported by our demonstration that p21 expression in podocytes is associated with a pro-proliferative antiquiescent effect. The potential diagnostic implications of this study is that determining the expression of specific cell cycle proteins may assist in differentiating the different forms of podocyte diseases.

Reprint requests to Stuart J. Shankland, M.D., Division of Nephrology, University of Washington Medical Center, Box 356521, 1959 NE Pacific Avenue, Seattle, Washington 98195-6521, USA.
E-mail: stuartjs@u.washington.edu

ACKNOWLEDGMENTS

This study was made possible by the National Institutes of Health grants to S.J.S. (DK-52121, DK-51096, DK-34198). S.J.S. and C.E.A. are also supported by the George M. O'Brien Kidney Research Center Award (DK-47659).

APPENDIX

Abbreviations used in this article are: CDK, cyclin dependent kinase; CG, collapsing glomerulopathy; FSGS, focal segmental glomerulosclerosis; HIVAN, HIV associated nephropathy; MCD, minimal change disease; MGN, membranous glomerulopathy; p21, $p21^{Cip1/WAF1}$; p27, $p27^{Kip1}$; p57, $p57^{Kip2}$.

REFERENCES

1. Mundel P, Kriz W: Structure and function of podocytes: An update. *Anat Embryol* 192:385–397, 1995
2. Saxen L: *Organogenesis of the Kidney*. Cambridge, Cambridge University Press, 1997
3. Pabst R, Sterzel RB: Cell renewal of glomerular cell types in normal rats: An autoradiographic analysis. *Kidney Int* 24:626–631, 1983
4. Nadasdy T, Laszik Z, Blick KE, Johnson LD, Silva FG: Proliferative activity of intrinsic cell populations in the normal kidney. *J Am Soc Nephrol* 4:2032–2039, 1994
5. Kriz W: Progressive renal failure: Inability of podocytes to replicate and the consequences for development of glomerulosclerosis. *Nephrol Dial Transplant* 11:1738–1742, 1996
6. Couser WG, Abrass CK: Pathogenesis of membranous nephropathy. *Annu Rev Med* 39:517–530, 1988
7. Kriz W: Progressive renal failure-inability of podocytes to replicate and the consequences for development of glomerulosclerosis. *Nephrol Dial Transplant* 11:1738–1742, 1996
8. Kriz W, Kretzler M, Nagata M, Provoost AP, Shirato I, Uiker S, Sakai T, Lemley KV: A frequent pathway to glomerulosclerosis: Deterioration of tuft architecture-podocyte damage-segmental sclerosis. *Kidney Blood Press Res* 19:245–253, 1996
9. Kriz W, Gretz N, Lemley KV: Progression of glomerular diseases: Is the podocyte the culprit? *Kidney Int* 54:687–697, 1998
10. D'Agati V: The many masks of focal segmental glomerulosclerosis. *Kidney Int* 46:1223–1241, 1994
11. Barisoni L, Kriz W, Mundel P, D'Agati V: The dysregulated podocyte phenotype: A novel concept in the pathogenesis of collapsing idiopathic focal segmental glomerulosclerosis and HIV-associated nephropathy. *J Am Soc Nephrol* 10:51–61, 1999
12. Valeri A, Barisoni L, Appel GB, Seigle R, D'Agati V: Idiopathic collapsing focal glomerulosclerosis: A clinicopathologic study. *Kidney Int* 50:1734–1746, 1996
13. Detwiler RK, Falk RJ, Hogan SL, Jennette JC: Collapsing glomerulopathy: A clinically and pathologically distinct variant of focal segmental glomerulosclerosis. *Kidney Int* 45:1416–1424, 1994
14. Bariety J, Nochy D, Mandet C, Jacquot C, Glotz D, Meyrier A: Podocytes undergo phenotypic changes and express macrophagic-associated markers in idiopathic collapsing glomerulopathy. *Kidney Int* 53:918–925, 1998
15. Nagata M, Hattori M, Hamano Y, Ito K, Saitoh K, Watanabe T: Origin and phenotypic features of hyperplastic epithelial cells in collapsing glomerulopathy. *Am J Kidney Dis* 6:962–969, 1998
16. Morgan DO: Principles of CDK regulation. *Nature* 374:131–134, 1995
17. Lees E: Cyclin dependent kinase regulation. *Current Opin Cell Biol* 7:773–780, 1995
18. Sherr CJ: Mammalian G1 cyclins. *Cell* 73:1059–1065, 1993
19. Sherr CJ, Roberts JM: Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* 9:1149–1163, 1995
20. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B: p21WAF1, a potential mediator of p53 tumor suppression. *Cell* 75:817–825, 1993
21. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge J: The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805–816, 1993
22. Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee M, Massague J, Crabtree GR, Roberts JM: Interleukin-2-mediated elimination of the p27kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* 372:570–573, 1994
23. Polyak K, Lee M-H, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, Massague J: Cloning of p27kip1, a cyclin-dependent kinase inhibitor and potential mediator of extracellular antimitogenic signals. *Cell* 78:59–66, 1994
24. Zhang P, Liegeois NJ, Wong C, Finegold M, Hou H, Thompson JC, Silverman A, Harper JW, Depinho RA, Elledge SJ: Altered cell differentiation and proliferation in mice lacking p57Kip2 indicates a role in Beckwith–Wiedermann syndrome. *Nature* 387:151–158, 1997
25. Barboule N, Lafon C, Chadebech P, Vidal S, Valette A: Involvement of p21 in the PKC-induced regulation of the G2/M cell cycle transition. *FEBS Lett* 444:32–37, 1999
26. Jacks T, Ra W: The expanding role of cell cycle regulators. *Science* 280:1035–1036, 1998
27. Coombs HL, Shankland SJ, Setzer SV, Hudkins KL, Alpers CE: Expression of the cyclin kinase inhibitor, $p27^{kip1}$, in developing and mature human kidney. *Kidney Int* 53:892–896, 1998
28. Nagata M, Nakayama K, Terada Y, Hoshi S, Watanabe T: Cell cycle regulation and differentiation in the human podocyte lineage. *Am J Pathol* 153:1511–1520, 1998
29. Casaccia-Bonnefil P, Tikoo R, Kiyokawa H, Friedrich V, Chao MV, Koff A: Oligodendrocyte precursor differentiation is perturbed in the absence of the cyclin-dependent kinase inhibitor p27Kip1. *Genes Dev* 11:2335–2346, 1997
30. Fredersdorf S, Milne AW, Hall PA, Lu X: Characterization of a panel of novel anti-p21 (WAF1/Cip1) monoclonal antibodies and immunochemical analysis of p21 (WAF1/Cip1) expression in normal human tissues. *Am J Pathol* 148:825–835, 1996
31. Schnier JB, Nishi K, Goodrich DW, Bradvury EM: G1 arrest and down-regulation of cyclin E/cyclin-dependent kinase 2 by the protein kinase inhibitor staurosporine are dependent on the retinoblastoma protein in the bladder carcinoma cell line. *Proc Natl Acad Sci USA* 93:5941–5946, 1996
32. Harvat BL, Seth P, Jettin AM: The role of p27kip1 in gamma interferon-mediated growth arrest of mammary epithelial cells and related defects in mammary carcinoma cells. *Oncogene* 14:2111–2122, 1997
33. Mateo MS, Saez AI, Sanchez-Beato M, Garcia P, Sanchez-Verde L, Martinez JC, Orradre JL, Piris MA: Expression of p21WAF1/CIP1 in fetal and adult tissues: Simultaneous analysis with Ki-67n and p53. *J Clin Pathol* 50:645–653, 1997
34. Adler S: Glomerular epithelial cells, in *Immunologic Renal Diseases*, edited by Nielson EG, Couser WG, Philadelphia, Lippincott-Raven, 1997, pp 1–13
35. Shankland SJ, Floege J, Thomas SE, Nangaku M, Hugo C, Pippin J, Henne K, Hockenberry DM, Johnson RJ, Couser WG: Cyclin kinase inhibitors are increased during experimental membranous nephropathy: Potential role in limiting glomerular epithelial cell proliferation in vivo. *Kidney Int* 52:404–413, 1997
36. Ophascharoensuk V, Fero ML, Hughes J, Roberts JM, Shankland SJ: The cyclin-kinase inhibitor $p27^{Kip1}$ safegaurds against inflammatory injury. *Nat Med* 4:575–580, 1998
37. Zhang P, Wong C, Depinho RA, Harper JW, Elledge SJ: Cooperation between the CDK inhibitors p27 (Kip1) and p57 (Kip2) in the control of tissue growth and development. *Gen Dev* 12:3126–3167, 1998
38. Shankland SJ, Hugo C, Coats SR, Nangaku M, Pichler RH, Gordon KL, Pippin J, Roberts JM, Couser WG, Johnson RJ: Changes in cell cycle protein expression during experimental mesangial proliferative glomerulonephritis. *Kidney Int* 50:1230–1239, 1996
39. Kuan CJ, Al-Douahji M, Shankland SJ: The cyclin kinase inhibitor $p21^{WAF1, CIP1}$ is increased in experimental diabetic nephropathy: Potential role in glomerular hypertrophy. *J Am Soc Nephrol* 9:986–993, 1998
40. Kim Y-G, Pippin JW, Johnson RJ, Alpers CE, Couser WG, Shankland SJ: The cyclin kinase inhibitor p21Cip1/WAF1 limits visceral glomerular epithelial cell proliferation in experimental glomerulonephritis. *Kidney Int* 55:2349–2361, 1999
41. Parry D, Mahoney D, Wills K, Lees E: Cyclin D-CDK subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. *Mol Cell Biol* 19:1775–1783, 1999