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Mitotic cell cycle proteins increase in podocytes despite lack of proliferation

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Mitotic cell cycle proteins increase in podocytes despite lack of proliferation.

Background. Podocyte proliferation is an uncommon response to glomerular injury and its lack may underlie the development of glomerulosclerosis. However, whether podocytes have the capacity to enter and finish mitosis and cytokinesis is not known.

Methods. The expression of mitotic cell cycle proteins (phosphorylated Histone 3, Cdc2, cyclin B1 and B2) was examined by immunohistochemistry in kidneys of embryonal mice, transgenic HIV-mice, and rats with experimental membranous nephropathy (passive Heymann nephritis, PHN). Mitotic proteins also were measured by Western blot in glomerular protein from PHN-rats and the activity of mitotic cyclins was quantified by histone kinase assay.

Results. Mitotic proteins were increased in embryonal mouse glomeruli during the S- and comma-shaped stages and were absent at the capillary loop stage and in mature rodent glomeruli. There was an increase in podocyte expression of Cdc2, cyclin B1 and B2 and phosphorylated histone 3 in PHN rats, and in HIV transgenic mice.

Conclusions. Podocytes have the ability to increase cell cycle proteins required for mitosis. Without obvious differences in the expression of the major mitotic proteins in PHN- and HIV-nephropathy, a regulatory disturbance in cytokinesis might be responsible for the development of polynucleated cells and a lack of podocyte proliferation in experimental glomerular disease.

Podocytes (also called visceral glomerular epithelial cells) form the filtration barrier to proteins in the glomerulus [1]. Presumptive podocytes proliferate during glomerulogenesis. However, mature podocytes are terminally differentiated and quiescent [2]. The role of the lack of podocyte proliferation in the development of glomerulosclerosis also has been well documented. Kriz and others have shown that the apparent inability of podocytes to proliferate and replace cells lost following injury leads to progressive glomerulosclerosis [3]. In contrast, podocytes proliferate in collapsing focal segmental glomerulosclerosis (FSGS) and cellular FSGS, and this is associated with a rapid decline in renal function [4]. Taken together, these studies suggest that podocyte proliferation following injury is an important determinant of disease progression.

Cell proliferation is governed at the level of the cell cycle by specific cell cycle regulatory proteins. This requires that cyclin dependent kinases (Cdk) are activated by partner cyclins [5]. In contrast, proliferation is inhibited by Cdk-inhibitors, which bind to, and inactivate, cyclin-Cdk complexes [6]. We have previously shown that C5b-9 induced injury to podocytes in the passive Heymann nephritis (PHN) model of membranous nephropathy increases the expression of cyclin A-Cdk2 required for DNA synthesis. However, C5b-9 injury also was associated with a marked increase in the Cdk-inhibitors p21 and p27, which limited podocyte DNA synthesis by binding to cyclin A-Cdk2 complexes [7]. In the absence of specific CDK-inhibitors, podocyte proliferation was markedly increased in p21 null and p27 null mice following immune-mediated glomerular injury [8, 9]. These studies show that limiting DNA synthesis is one mechanism whereby podocyte proliferation is limited.

Studies have shown that once cells undergo DNA synthesis, they typically enter mitosis, followed by cytokinesis [10]. However, it still remains unclear why, despite DNA synthesis, podocytes do not proliferate in membranous nephropathy. Mitosis is regulated by the mitosis-promoting factor, which requires that Cdc2 (formerly called Cdk1) is activated by complexing with positive regulating subunits, cyclin B1 and/or cyclin B2 [11]. Cyclin B-cdc2 complexes in turn are negatively regulated by the kinases Myt1 and Wee1 [12, 13], and are positively regulated by the phosphatases Cdc25B and Cdc25C [14, 15]. Cyclins B1 and B2 levels increase at the end of the S-phase,

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and continue throughout the G_2 phase [16, 17]. Cyclin B1-Cdc2 complexes are imported into the nucleus shortly before the breakdown of the nuclear membrane, at the end of prophase [18]. In contrast, cyclin B2 remains cytoplasmic and has a role in Golgi apparatus division [19].

This study explored whether expression differences of specific M-phase cell cycle proteins were rate limiting in podocyte proliferation in the PHN model, characterized by the absence of podocyte proliferation. Comparisons were made with experimental HIV associated nephropathy, which is characterized by podocyte proliferation. Our results show that expression of specific mitotic cell cycle proteins is increased following injury in both models. These data suggest that the apparent lack of podocyte proliferation is not solely due to the inability to increase the expression of cell cycle regulatory proteins required for mitosis, but must involve other events.

METHODS

Experimental design

The expression of specific M-phase cell cycle regulatory proteins was examined during murine glomerulogenesis, in the PHN rat model of membranous nephropathy, and in human immunodeficiency virus (HIV)-transgenic mice.

Glomerulogenesis. Podocytes proliferate during glomerulogenesis and cease to proliferate once a mature phenotype is reached. To examine the expression of mitotic cell cycle proteins during glomerulogenesis, mouse embryonal kidneys were obtained at embryonic day 15 (E15), E18 and E21, and at birth, and fixed in paraffin for immunostaining.

PHN model. PHN was induced in male Sprague-Dawley rats (Simson, Gilroy, CA, USA) weighing 180 to 200 g by intraperitoneal injection of sheep antibody to Fx1A (5 mL/kg body wt) prepared as previously described [20]. Control animals were injected with normal sheep serum. PHN and control animals were sacrificed on days 3, 5, 10 and 30 ($N = 6$ per group at each time point). Previous studies have shown that giving basic fibroblast growth factor (bFGF) to PHN rats augments podocyte injury [21]. To determine the effects of bFGF on the expression of M-phase cell cycle proteins in rats with PHN, an additional six PHN rats were injected intravenously with human recombinant bFGF (5 μg/200 g animal; gift of M. Reidy, Ph.D., University of Washington, Seattle) on days 3 and 4 and sacrificed on day 5. A 24-hour urine collection was performed on control and PHN rats prior to sacrifice, and urine protein excretion was determined in control and PHN animals by the sulphsalicylic method as previously reported [22]. At sacrifice, renal biopsies were taken from control and PHN rats, and glomeruli were isolated by the sieving method as we have previously reported [20].

HIV-associated nephropathy. We also studied HIV transgenic mice, characterized by podocyte proliferation as previously reported [23]. Transgenic mice bearing a GAG-Pol deleted genome and control wild-type mice were studied at weeks 6, 10 and 35 ($N = 4$). Renal biopsies were paraffin embedded for immunostaining.

Immunostaining

In PHN rats, detection of sheep IgG and C5b-9 in glomeruli was performed by direct (sheep IgG) and indirect (C5b-9) immunofluorescent staining on 4-μm frozen sections fixed in ether/alcohol as described elsewhere [20]. Sections were stained with fluorescein-conjugated rabbit anti-sheep IgG (Organon Teknika Corporation, West Chester, PA, USA) or biotinylated 2A1, a murine monoclonal antibody to rat C5b-9, followed by fluorescein-conjugated streptavidin (Amersham, Arlington Heights, IL, USA).

For cell cycle regulatory protein immunostaining, renal biopsies were fixed in either methyl Carnoy's solution or formalin, and embedded in paraffin and cut into 4-μm thick sections. Indirect immunoperoxidase immunostaining was performed on formalin fixed tissue using the following primary antibodies incubated overnight at 4°C: Cdc2 (mouse monoclonal, Clone A17.1.1, 1:600; Neomarkers, Fremont, CA, USA), cyclin B1 (Clone GNS11, 1:600, mouse monoclonal; Neomarkers), cyclin B2 (rabbit polyclonal, 1:1200; gift of Dr. Mark Carrington, Department of Biochemistry, Cambridge, UK) and phosphorylated histone 3 (rabbit polyclonal; 1:2000; methyl Carnoy's fixed tissue was used for this antibody; gift of Dr. Hidenasa Goto and Dr. Masaki Inagaki, Aichi Cancer Center Research Institute, Nagoya, Aichi, Japan). Controls for this step included omitting the primary antibody and substituting the primary antibody with pre-immune rabbit serum.

Tissue sections were boiled in citric acid (10 mmol/L; pH 6.0) for 10 minutes to unmask the antigens. Nonspecific background staining was reduced with the Avidin/Biotin blocking Kit (SP-2001; Vector Laboratories Inc., Burlingame, CA, USA) and Background Buster (Accurate Chemical & Scientific Corporation, Westbury, NY, USA). A biotinylated goat anti-rabbit secondary antibody (BA-1000; 1:500; Vector Laboratories Inc.) or an anti-mouse secondary antibody (BA-2001; 1:400; Vector Laboratories Inc.) was then added, and detection was performed using the ABC-Kit (Vector Laboratories Inc.). Black nuclear staining was detected using diaminobenzidine (Sigma Chemical Co.; St. Louis; MO; USA) with nickel as a chromagen.

Quantitation of immunostaining

The glomerular expression of each cell cycle protein was graded semi-quantitatively in a blinded fashion in PHN and control rats at each time point. Thirty glomeru-

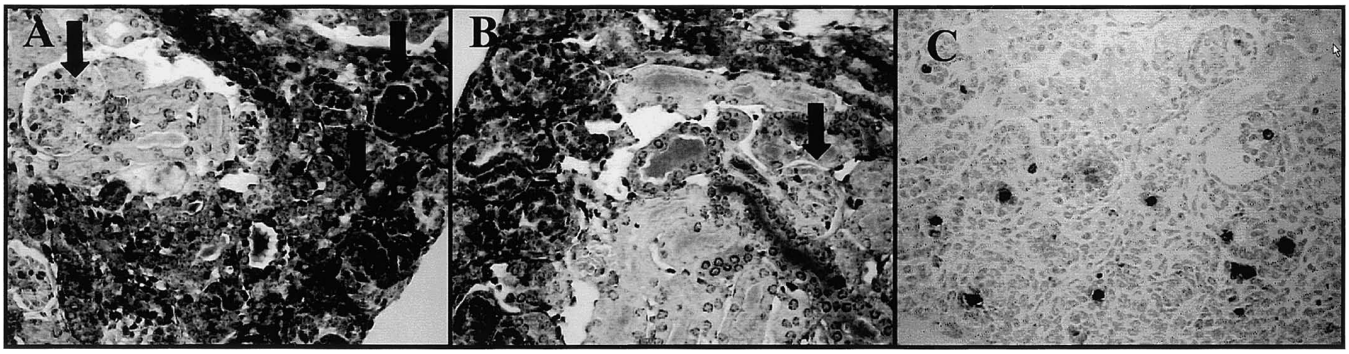

Fig. 1. Immunostaining of embryonal tissue (gestational day E21). (*A*) Cdc2 is increased in the comma- and S-shaped bodies. In more mature capillary loop glomeruli, staining is absent. (*B*) Staining is similar for cyclin B. (*C*) Phosphorylated histone-3 stains predominantly in immature glomeruli.

lar cross sections were evaluated in individual samples by counting the number of cells staining positive for each antigen. Mean values per time point were calculated and the results were expressed as the number of cells staining positive per 30 glomerular cross sections.

Western blot analysis

Glomeruli were isolated from the renal cortex of PHN and control rats by differential sieving as previously described [20]. Glomerular cells were disrupted by a combination of freezing/thawing and sonication. To extract glomerular protein, glomeruli were resuspended in a buffer containing 1% triton, 10% glycerol, 20 mmol/L HEPES, and 100 mmol/L NaCl with a mixture of protease inhibitors. After centrifugation at 14,000 rpm for ten minutes, protein concentration was measured by BCA protein assay (Pierce, Rockford, IL, USA). Glomerular protein extracts (20 to 50 μg) were seperated under reduced conditions on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Protein was transferred to polyvinyl difluoride PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting and blocked in 5% nonfat dried milk for 30 minutes before incubation with antibodies to cyclin B1 (1:200; Clone V152; Neomarkers), cyclin B2 (1:500; gift of Dr. Mark Carrington), Cdc2 (1:200; Clone A17.1.1), Cdc25B (1:1000; Clone 23; Transduction Laboratories, Lexington, KY, USA), Cdc25C (1:200; gift of Dr. James A. DeCaprio, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA), Myt1 (1:1000; Clone sc-6352; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Wee1 (1:200; Clone sc-325; Santa Cruz Biotechnology). An alkaline phosphatase-conjugated secondary antibody was used (Promega, Madison, WI, USA) with BCIP/NBT (Sigma) as the substrate. To insure equal protein loading, the levels of α-/β-tubulin were measured also (1:2000; Clone DM1A + DM1B; Neomarkers). Each Western blot was performed a total of three times.

Histone kinase H1 assay

The activity of Cdc2 was measured by the histone H1 assay. Glomerular protein extract (50 μg) was immunoprecipitated with antibodies to cyclin B1 (1:10; Clone sc-752; Santa Cruz) or cyclin B2 (1:10; Clone sc-5238; Santa Cruz) for 30 minutes at 4°C. Fifty microliters of Protein G sepharose beads (Repligen, Cambridge, MA, USA) were added to each immunoprecipitation and incubated for 30 minutes at 4°C. Histone H1 (Boehringer Mannheim, Indianapolis, IN, USA), adenosine 5′-diphosphate (ATP; Pharmacia Biotech, Piscataway, NJ, USA) and [^{32}P] dATP (Dupont, Boston, MA, USA) were added for a 60 minute incubation at 37°C. Reduced sample buffer was then added and the mix was separated on a 15% SDS-PAGE gel. The gel was exposed to autoradiographic film (Amersham) overnight.

RESULTS

Cyclin B and Cdc2 increase transiently during glomerulogenesis

To determine the expression of M-phase cell cycle proteins during glomerular development, embryonic mouse kidneys were examined by immunohistochemistry. Figure 1 shows that immunostaining for cyclin B1 and Cdc2 was detected in renal vesicles and the comma- and S-shaped bodies on days E15, E18 and E21. M-phase cell cycle protein staining correlated with staining for phosphorylated histone 3, a marker of mitosis (Fig. 1) [24]. Immunostaining for cyclin B1, Cdc2 and phosphorylated histone 3 was detected in a diffuse cell type distribution at the comma- and S-shaped body level (Fig. 1). At the capillary loop stage, staining for cyclin B1, Cdc2 and phosphorylated histone 3 was occasionally detected in a glomerular endothelial and mesangial cell distribution, but not in a typical pattern of podocyte localization. These data show that the mitotic cell cycle proteins increase transiently in podocytes during glomerular development.

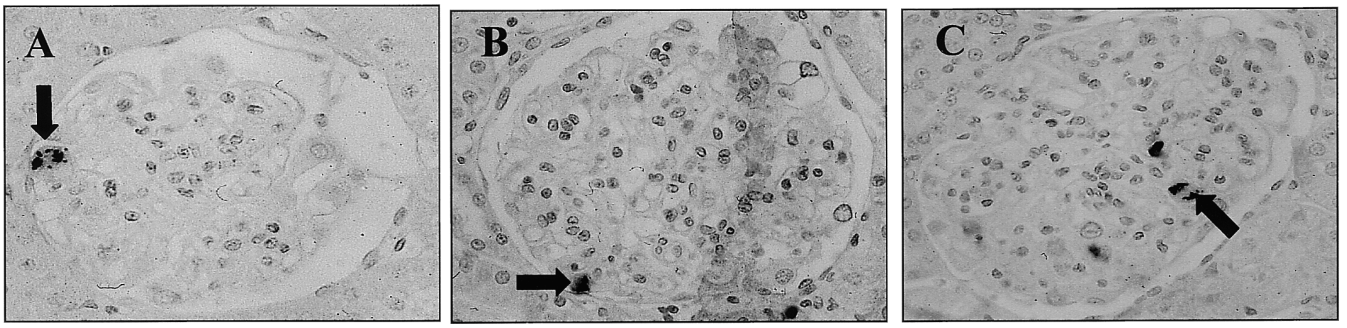

Fig. 2. Immunostaining for phosphorylated histone 3. Phosphorylated histone 3, a marker of mitosis, is increased in podocytes at day 5 of PHN rats during prometaphase (*A*), metaphase (*B*) and anaphase (*C*) (arrows indicate examples).

Table 1. Quantitation of mitotic cell cycle regulatory proteins

	Control d5	PHN d5	PHN d5 + bFGF	PHN d30
	number of positive cells/30 glomeruli			
Cdc2	19.75 ± 3.8	79.5 ± 8.4[a]	185.25 ± 14.3[b]	22.75 ± 2.7
Cyclin B1	1.7 ± 0.5	27 ± 2.8[a]	68 ± 4.9[b]	3.75 ± 0.8
Phosphorylated histone 3	1.35 ± 0.4	11 ± 1.6[a]	18.75 ± 2.5	2.5 ± 0.6

The number of glomerular cells staining positive for each cell cycle protein was quantitated in six animals at each time point.
[a] $P < 0.05$ compared to control animals
[b] $P < 0.01$ compared to control animals

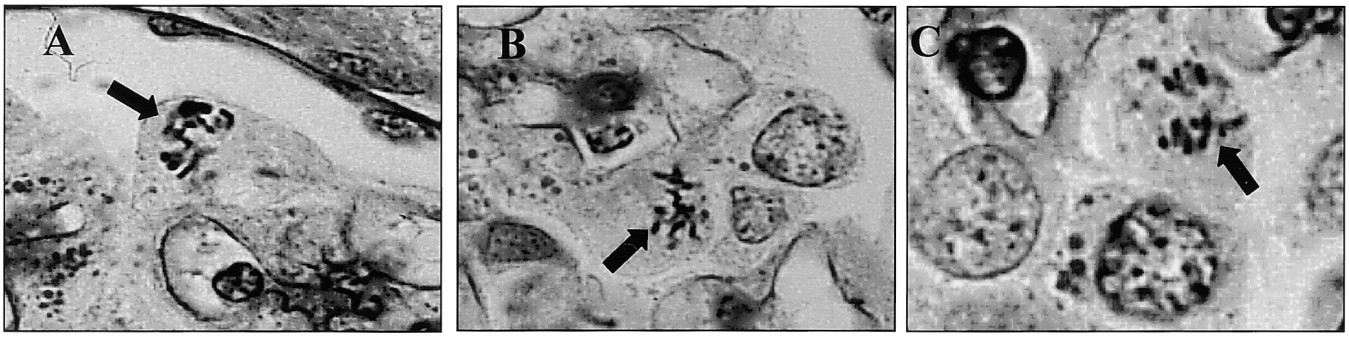

Fig. 3. Mitosis on PAS immunostaining. At day 5 of PHN, there was evidence of typical condensed mitotic chromosomes representing prometaphase (*A*), early metaphase (*B*) and anaphase (*C*) in podocytes (arrows indicate examples).

Podocyte mitosis increases in PHN rats

An antibody directed against phosphorylated histone 3 was used as a marker of mitosis [24]. Figure 2 shows an increase in staining for phosphorylated Histone 3 on PHN day 5, and this was in a typical podocyte distribution. Mitosis was absent in podocytes in control animals that were injected with sheep IgG. Administering the mitogen bFGF to PHN rats augmented podocyte staining for phosphorylated Histone 3 (Table 1). Podocytes undergoing mitosis were detected in PHN rats on days 5 and 10, with a peak at day 5. Mitotic figures were also seen in podocytes on PAS stained tissue sections in PHN rats (Fig. 3). In contrast, mitotic figures were absent in the podocytes in control animals.

M phase cyclins increase in the PHN model

Mitotic cell cycle regulatory proteins were measured by immunostaining and Western blot analysis in PHN and control rats. In control animals, glomerular immunostaining was not detected for cyclins B1 and B2 (Figs. 4 and 5 A, B). In contrast, immunostaining and Western blot analysis of glomerular protein showed that there was an increase in the protein levels for cyclin B1 (Figs. 4 C, D, and 7) and cyclin B2 (Figs. 5 C, D, and 7) at day 5 of PHN. Quantitation for immunostaining is shown in Table 1. Moreover, immunostaining for cyclins B1 and B2 was in a typical podocyte distribution (Figs. 4 and 5). In PHN animals injected with bFGF, the number of podocytes staining positive for the M-phase cell cycle proteins

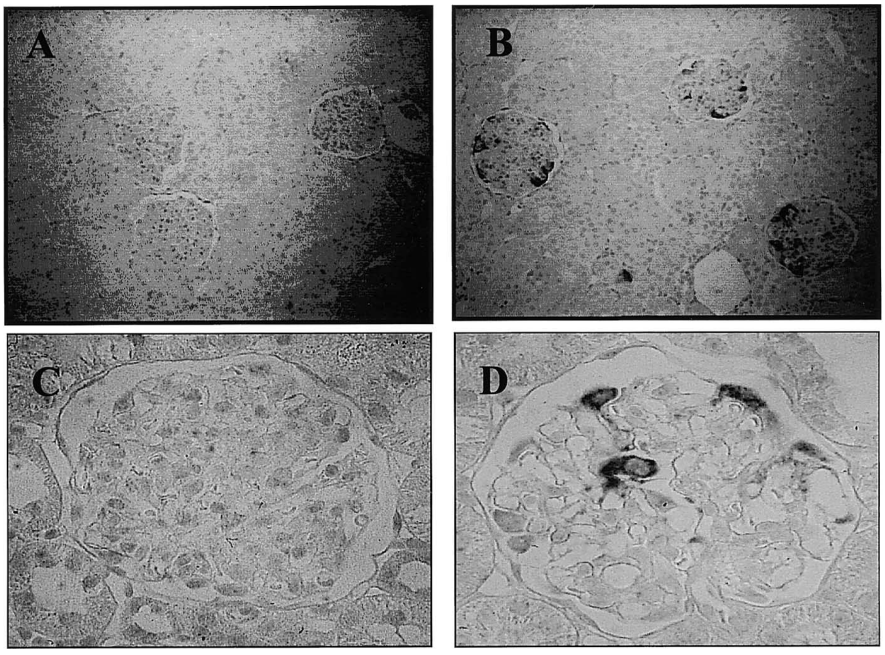

Fig. 4. Immunostaining for cyclin B1. (*A*, *C*) Immunostaining for cyclin B1 was not detected in control kidneys viewed by low power (A) and high power (C). There was an increase in cytoplasmic staining for cyclin B1 at day 5 PHN seen on low power magnification (*B*), and the increase seen on high power magnification was restricted to podocytes (*D*).

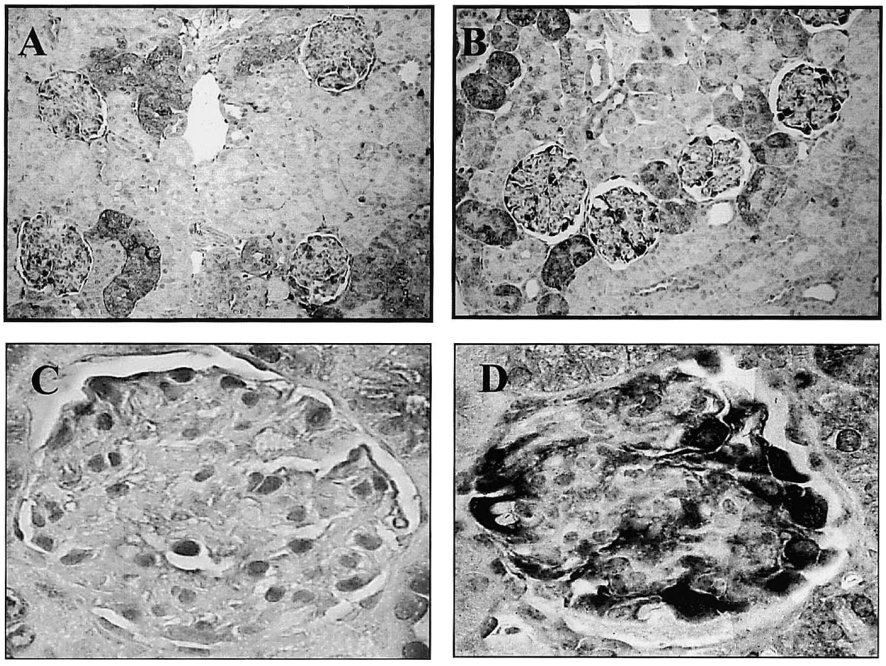

Fig. 5. Immunostaining for cyclin B2. In control rat kidneys, weak cyclin B2 staining was not detected when viewed under low (*A*) and high (*C*) power magnification. There was a marked increase in cyclin B2 staining at day 5 PHN in podocytes seen on low (*B*) and high (*D*) power magnification.

increased significantly compared to PHN animals not given bFGF (Table 1).

Protein levels for Cdc2, the catalytic partner of B-type cyclins, were expressed in low abundance in podocytes in control animals (Figs. 6 A, B, and 7). However, there was a marked increase in Cdc2 protein expression on day 5 of PHN (Figs. 6 C, D, and 7), and the increase was significantly augmented by the administration of bFGF to PHN rats (Fig. 7 and Table 1). Staining for the B cyclins and Cdc2 was predominantly cytoplasmic; and only on occasion, positive nuclear staining for cyclin B1 and Cdc2 was noted also.

The increase in Cdc2 in PHN was transient, as Cdc2 was barely detected at day 10 (results not shown) and day 30 (Fig. 7) of PHN. Multiple bands were seen for Cdc2 and cyclin B1 on Western blot analysis, suggesting different phosphorylation states of the proteins. An increase in Cdc2 protein does not imply an increase in kinase activ-

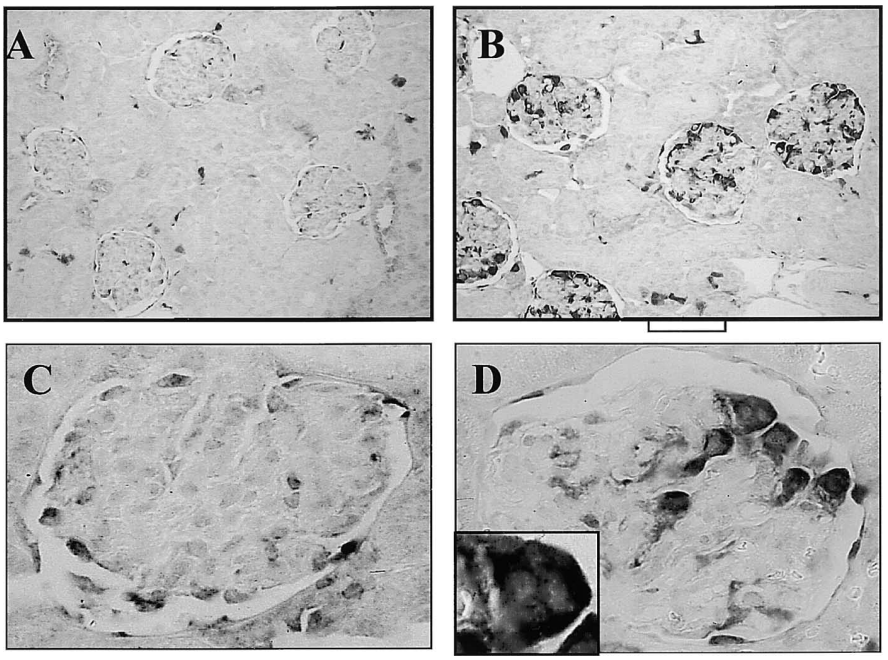

Fig. 6. Immunostaining for Cdc2. (*A*, *C*) Cdc2 staining was detected in low levels in podocytes of control animals. (*B*, *D*) There was a marked increase in Cdc2 staining at day 5 PHN. Staining was predominantly cytoplasmic and in podocytes. The inset shows increased Cdc2 staining in a polyploid podocyte, with nuclei of different sizes.

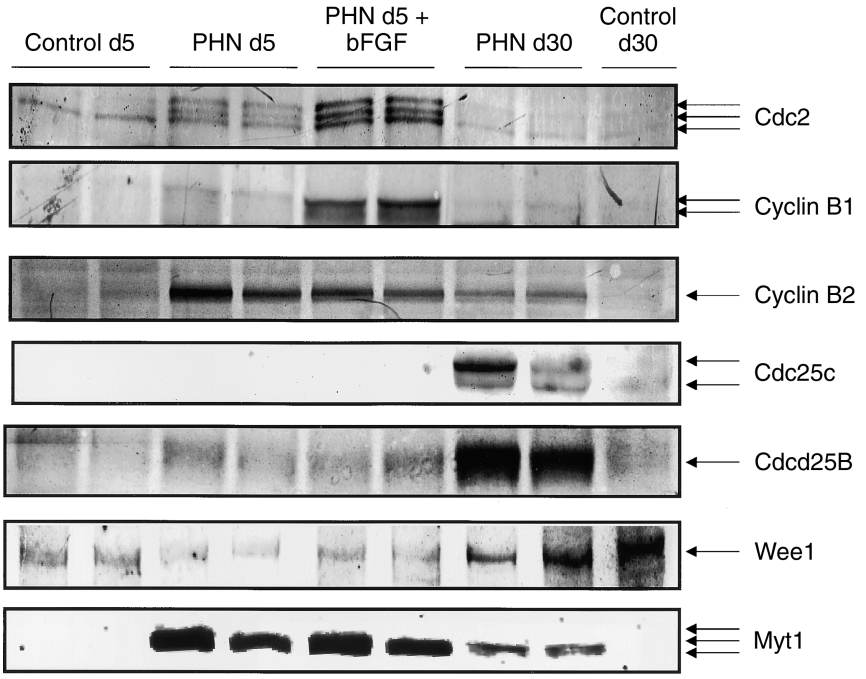

Fig. 7. Western blot analysis for mitotic cell cycle regulatory proteins. Protein was extracted from isolated glomeruli from individual control and PHN animals, and immunoblotted with antibodies to specific mitotic cell cycle-related proteins. Protein levels for Cdc2, cyclin B1 and cyclin B2 increased at PHN day 5, which was augmented by administering bFGF to PHN rats. There were multiple phosphorylated Cdc2 isoforms, representing active cdc2. Protein levels for Cdc2 and cyclin B1 normalized by day 30 PHN, whereas cyclin B2 protein levels remained moderately increased at day 30 PHN. Cdc25B and Cdc25C were up-regulated delayed with reaching their maximum at day 30. While Wee1 protein levels were decreased at day 5, Myt1 was present only in lower amounts in control animals and increased markedly in PHN animals on day 5.

ity. Accordingly, Cdc2 kinase activity was measured on glomerular protein lysates immunoprecipitated with cyclins B1 and B2, and the results are shown in Figure 8. Densitometric measurements showed an increase in Cdc2 kinase activity in PHN animals at day 5 when glomerular protein was immunoprecipitated with an antibody to cyclin B2. The increase in Cdc2 activity was further augmented by the administration of bFGF to PHN rats (Table 2). In contrast, when glomerular protein was immunoprecipitated with an antibody to cyclin B1, there was no significant increase in Cdc2 activity. However, giving bFGF to PHN rats was associated with a slight increase in Cdc2 activity.

Changes in M phase regulatory proteins in PHN

The activity of Cdc2 is tightly regulated by numerous proteins, including the complexing with B-type cyclins. However, the cyclin B-Cdc2 complex is further regulated

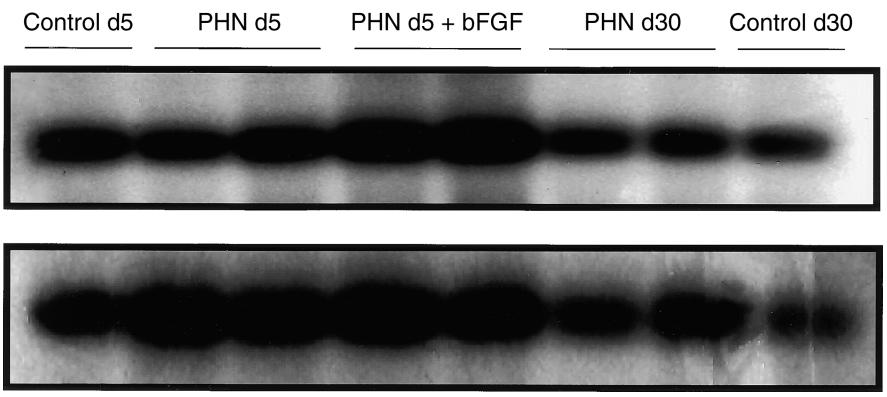

Fig. 8. Cdc2 activity in PHN. Cdc2 activity was measured by histone 1 kinase assay on glomerular protein lysates from control and PHN rats. (*A*) When protein extracts were immunoprecipitated with an antibody to cyclin B1, there was an increase in Cdc2 activity in PHN rats given bFGF. (*B*) When glomerular protein was immunoprecipitated with an antibody to cyclin B2, Cdc2 activity was increased at day 5 in PHN rats and PHN rats given bFGF.

Table 2. Quantitation of histone kinase assay

	Control d5	PHN d5	PHN d5	PHN d5 + bFGF	PHN d5 + bFGF	PHN d30	PHN d30	Control d30
Cyclin B1	10187	8853	11509	14110	15617	6942	6689	4899
Cyclin B2	7611	12431	10674	12797	11110	5651	6882	2769

Quantitation was performed by densitometric measurement using the software NIH image. Results are expressed in square pixel.

Table 3. Quantitation of Western blot analysis

	Cdc2	Cyclin B1	Cyclin B2	Cdc25C	Cdc25B	Wee 1	Myt 1
C d5	3736	2437	17374	0	6469	16285	0
C d5	3950	2222	17010	0	4514	14271	0
PHN d5	5406	5670	41282	0	10116	6159	27480
PHN d5	5144	3469	33493	0	7015	4211	17673
PHN d5 + bFGF	9030	8215	34294	0	8765	11889	25128
PHN d5 + bFGF	10079	8745	28157	0	10669	8582	19900
PHN d30	4741	2988	24429	28360	26924	14608	9458
PHN d30	4776	3834	25950	17045	23627	38162	8733
C d30	3774	2902	15945	4404	8485	45304	0

Quantitation was performed by densitometric measurement using the software NIH image. Results are expressed in square pixel.

by specific activating phosphatases (Cdc25B and Cdc25C) and inhibitory kinases (Wee1 and Myt1). Figure 7 shows that Cdc25B and Cdc25C were present in low abundance in protein extracted from isolated glomeruli. Interestingly, the expected increase of Cdc25B and Cdc25C protein levels was delayed until day 30 of PHN, and did not coincide with the increase in protein levels for cyclin B-Cdc2 complexes.

Figure 7 shows that Wee1 is present in normal glomerular protein. Wee1 levels decreased in PHN rats and in PHN rats given bFGF. Wee1 expression normalized by day 30, as determined by densitometric measures (Table 3).

In contrast to Wee1, Myt-1 was only detected in trace amounts in normal glomeruli in Western blot. The protein levels for Myt1 increased transiently on day 5 of PHN (Fig. 7), and returned to baseline levels on day 30 (Table 3).

M-phase cell cycle proteins increase in HIV transgenic mice

Previous studies have shown that podocytes dedifferentiate and proliferate in transgenic HIV mice, a finding analogous to that seen during glomerulogenesis. Our results showed an increase in immunostaining for Histone 3 (not shown), cyclin B1 and cyclin B2 (Fig. 9) in six-week-old HIV-transgenic mice, and the staining localized to podocytes. There was also a coincidental increase in podocyte immunostaining for Cdc2 in HIV-transgenic mice at 6 weeks (Fig. 10). The intracellular distribution of B cyclins and Cdc2 in podocytes in HIV-transgenic mice was also predominantly cytoplasmic, and was indistinguishable from PHN animals at day 5. Immunostaining for B-type cyclins and Cdc2 was not detected at later timepoints in HIV-transgenic mice, coincident with the cessation in podocyte proliferation in the chronic phase of disease (Figs. 9 C, F, and 10C).

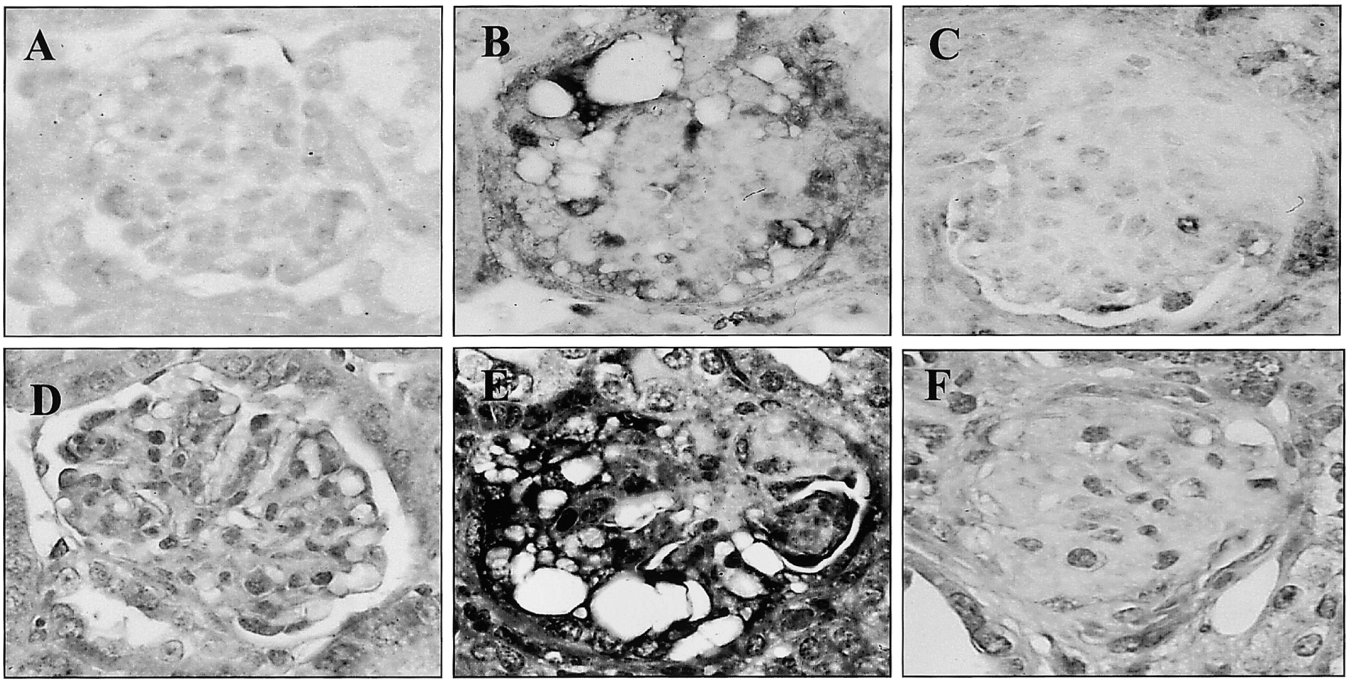

Fig. 9. Immunostaining for cyclins B1 and B2 in HIV transgenic mice. (*A*) Cyclin B1 was absent in the normal mouse glomerulus. (*B*) Cyclin B1 staining increased in 6-week-old HIV-transgenic mice, but decreased by 12 weeks (*C*). (*D*) Cyclin B2 is absent in normal mouse glomeruli, but increased in 6-week-old HIV transgenic mice (*E*). (*F*) Cyclin B2 is not detected in 12-week-old transgenic mice.

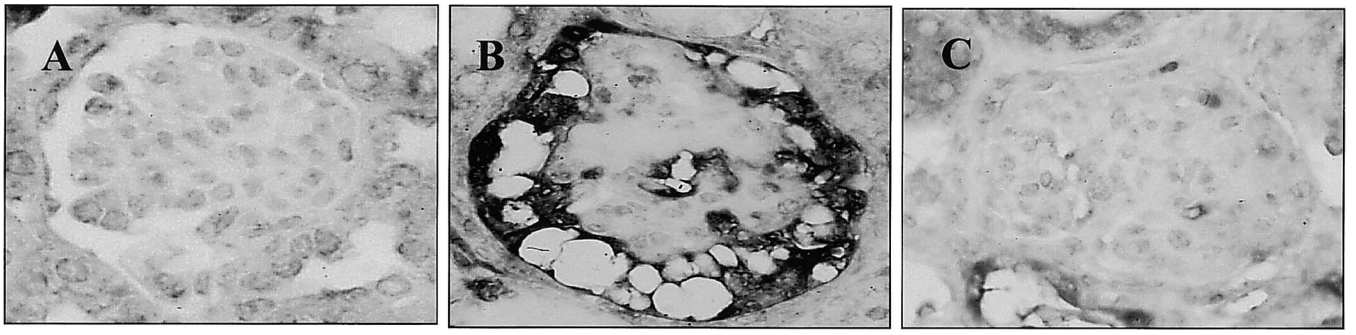

Fig. 10. Immunostaining for Cdc2 in HIV transgenic mice. (*A*) Cdc2 staining was not detected in normal mouse glomeruli. (*B*) Cdc2 staining increased in 6-week-old HIV transgenic mice, and was not detected in 12-week-old HIV transgenic mice (*C*).

DISCUSSION

Mature podocytes are terminally differentiated and quiescent cells [25]. However, in disease, the proliferative response to injury determines the nature of the glomerular lesion following injury. Accordingly, podocyte diseases can be classified into those characterized by proliferation and those where proliferation is absent. In classic FSGS, membranous nephropathy and miminal change disease, podocytes do not proliferate [3]. In contrast, podocytes proliferate in HIV-nephropathy [26], cellular FSGS and collapsing FSGS [27]. Thus, the mechanisms regulating podocyte proliferation are of major interest.

A failure in an increase of podocyte number in disease may be due to a lack of proliferation, and/or increased cell loss due to apoptosis or cell detachment. Although we have previously shown that podocyte proliferation is limited in part because of limited DNA synthesis [20], the question in our current study was whether abnormalities in mitosis also limit podocyte proliferation. Our results show that following injury in vivo, podocytes increase the expression and activity of specific cell cycle regulatory proteins necessary for mitosis. These results suggest that compared to HIV nephropathy, the apparent lack of podocyte proliferation in experimental membranous nephropathy is not due to differences to HIV-nephropathy in the expression of M-phase cell cycle proteins required for mitosis.

Previous studies have shown that many of the responses to glomerular injury are reminscent of the changes observed during glomerulogenesis [28]. Accordingly, we began this study by determining the expression of cell cycle regulatory proteins that govern podocyte mitosis during development. Our results showed that proliferation of immature and presumptive podocytes during the comma- and S-shaped stages of glomerulogenesis was associated with increased expression of B1-type cyclin and its partner, Cdc2. In contrast, staining for M-phase cell cycle proteins was not detected in glomeruli of mature and quiescent podocytes, and immunostaining for M-phase cell cycle proteins was not detected in normal adult mouse and rat kidneys. These findings are consistent with those of Nagata and colleagues [2] and are not unexpected, as immature podocytes proliferate during glomerulogenesis.

We and others have previously shown that immune-mediated injury to podocytes in the PHN model of experimental membranous nephropathy is associated with DNA synthesis, albeit low grade [20]. This was accompanied by an increase in cyclin A-Cdk2, cell cycle regulatory proteins essential for DNA synthesis. This suggested that podocytes have the ability to enter the cell cycle upon immune-mediated injury. However, DNA synthesis was limited in part because of the binding of the Cdk-inhibitors p21 and p27 to cyclin A-Cdk2 [20]. Although DNA synthesis was detected in PHN rats, podocyte number did not increase. Our previous and current studies show that apoptosis is not detected by Tunel or Hoechst staining and, therefore, is an unlikely explanation for the lack of increase in podocyte number [20].

Accordingly, in the current study we asked if the lack of podocyte proliferation was due to an abnormality in cell cycle proteins that govern mitosis. Our results show that complement-mediated podocyte injury in experimental membranous nephropathy is associated with increases in protein levels for cyclins B1 and B2, and their catalytic partner, Cdc2. Moreover, we also show that B-type cyclins and Cdc2 increase in podocytes in HIV transgenic mice, a model characterized by podocyte proliferation. Taken together, these results show that following injury, podocytes are able to increase the levels of specific M-phase cell cycle regulatory proteins necessary for mitosis. However, despite the increase in M-phase cell cycle regulatory proteins, podocyte number is not increased in experimental membranous nephropathy. Thus, one has to entertain that the lack of podocyte proliferation is due to cell loss due to detachment, or an inability in cytoplasmic cell division during cytokinesis.

Previous studies by our group and others have shown the presence of bi- and multinucleated cells in PHN. However, polyploidy has not been documented in HIV nephropathy, and we also were unable to show podocyte polyploidy in the current study. This suggests that there is something fundamentally specific to the response of podocytes to C5b-9 mediated injury in PHN. However, the presence of bi- and/or multinucleated podocytes in experimental membranous nephropathy suggests that podocytes are able to both initiate and progress through mitosis, and supports the idea of an abnormality in mitotic exit and/or cytokinesis and/or a regulatory disturbance between mitosis and cytokinesis. We did not examine the mechanisms regulating cytokinesis in the current study, which has been predominantly confined to the study of yeast, *Drosophila* and *C. elegans*. Future studies are needed also in podocytes.

Nevertheless, the regulation of mitotic cell cycle proteins in PHN has some unexpected results. Activitation of the cyclin B-Cdc2 complex typically requires dephosphorylation by Cdc25 phosphatases [14, 15]. Our current study shows that Cdc25B and C are increased in podocytes in experimental membranous nephropathy. Interestingly, the increase in protein levels for Cdc25B and Cdc25C did not coincide with the increase in cyclin B or Cdc2 levels on day 5. The mechanism underlying this is not clear. Thus, one interpretation is that the delay in increase in these activating proteins may explain the relative lack of active Cdc2 kinase.

Studies have shown that Cdc2 is deactivated by inhibitory Wee1 and Myt1 kinases [29]. Our results demonstrate that protein levels for Wee1 decreased in PHN rats. The decrease in Wee1 would favor a shift from phosphorylated (inactive) Cdc2 to dephosphorylated (active) Cdc2, coinciding with the observed up-regulation of Cdc25B and C, favoring podocyte entry into mitosis. Interestingly, Myt1 was increased on day 5. This is similar to in vitro studies showing that Myt1 is absent in resting cells and is increased after entering the cell cycle to prevent a too early activation of Cdc2 already in the G_2-phase [30]. As the activity of Myt1 is not only regulated on the protein level, but also by multiple phosphorylations, a final statement about the enzymatic activity of Myt1 at the different timepoints with the used methods is not possible.

In summary, we show that podocytes are able to increase and activate specific cell cycle proteins required for mitosis in a proliferative and a non-proliferative model of podocyte disease. Also, the results show condensed chromosomes in podocytes, indicating that podocytes are able to initiate and pass through the different phases of mitosis. These data are consistent with the notion that following immune-mediated injury in experimental membranous nephropathy, podocytes are able to enter the cell cycle from their normally quiescent state, undergo limited DNA synthesis in S-phase and finally enter mitosis. Thus, the relative lack of podocyte proliferation is not solely a consequence of the inability of podocytes to enter mitosis. Although mitosis (nuclear division) is typically followed by cytokinesis (cytoplasmic division), the presence of bi- or multinucleated cells also suggests that there may be an abnormality in the exit from mitosis, and/or cytokinesis. Future studies are needed to delineate this hypothesis.

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