Virchows Arch (1999) 435:422–427

ORIGINAL ARTICLE

Kosaku Nitta · Shigeru Horita · Kazuho Honda
Keiko Uchida · Teruo Watanabe · Hiroshi Nihei
Michio Nagata

Glomerular expression of cell-cycle-regulatory proteins in human crescentic glomerulonephritis

Received: 29 January 1999 / Accepted: 1 April 1999

Abstract To elucidate the mechanism underlying crescentic formation, we assessed the phenotypic characterization and cell-cycle protein expression in human crescentic glomerulonephritis (CRGN). Kidney tissue specimens taken from CRGN patients (10 patients with pauci-immune type rapidly progressive glomerulonephritis (RPGN), 2 patients with Henoch-Schönlein purpura nephritis, and 1 patient with IgA nephropathy) were examined immunohistochemically. Most of the cellular components of the crescents expressed cytokeratin, whereas few cells expressed PHM-5. CD68-positive cells were minor components of cellular crescents, indicating that the major principal cellular component of the crescents is made up of cells with the parietal glomerular epithelial cell (PEC) phenotype. Additionally, serial section analysis revealed that Ki-67-positive cells in the crescents were frequently cyclin-A positive and Bcl-2 positive, but seldom cyclin-B_1 positive. Moreover, the expression of cyclin-dependent kinase inhibitor $p27^{Kip1}$ was low in the cellular crescents, despite being exclusively positive in podocytes within the same section. We concluded that the major component of the cellular crescents is made up of PECs and that apparent expression of cyclins and Bcl-2 and restrained expression of $p27^{Kip1}$ may be synergistically associated with the development of cellular crescents in human CRGN.

Key words Cellular crescents · Cyclin · $p27^{Kip1}$ · Parietal glomerular epithelium · Proliferation · Crescentic glomerulonephritis

K. Nitta · S. Horita · K. Honda · K. Uchida · H. Nihei
Department of Medicine, Kidney Center,
Tokyo Women's Medical University, Tokyo, Japan

T. Watanabe · M. Nagata (✉)
Department of Pathology,
Institute of Basic and Clinical Medical Sciences,
University of Tsukuba,
Tsukuba, Ibaraki 305-8576, Japan
e-mail: nagatam@md.tsukuba.ac.jp, Tel./Fax: +81-298-53-3165

Introduction

Glomerular crescents are a central pathological feature of human rapidly progressive glomerulonephritis (RPGN). Crescentic glomerulonephritis (CRGN) is the counterpart of RPGN, in which glomeruli with cellular crescents result in glomerulosclerosis. It has been shown that the population of glomeruli with crescent formation in biopsy specimens correlates well with outcome [19, 36]. Elucidating the origin of the cellular components and the mechanism of cell proliferation in the cellular crescents is important for better understanding of the pathogenesis of crescent formation.

Classically, the cellular origin of crescents was believed on the basis of electron microscope studies [18], to be in the glomerular epithelial cells (GECs). In contrast, immunohistochemistry with recently established cellular markers for monocytes has indicated that monocytes/macrophages are the major component of crescents in human and experimental CRGN, as revealed by in vivo and in vitro studies [1, 8, 15, 34]. Cattell et al. [3] identified major involvement of parietal glomerular epithelial cells (PECs) in experimental crescentic glomerulonephritis in the rabbit. Yoshioka et al. [37] have used several antibodies against monocytes/macrophages for human crescentic glomerulonephritis and concluded that these inflammatory cells are minor factors in the cellular crescents. Several studies have shown that the predominant cellular component in glomerular crescents is epithelial in histogenesis, rather than having a monocytes/macrophage origin, as revealed by cytokeratin expression [7, 11, 17]. Candidates for the origin of the proliferating epithelial cells in the crescent include GECs and PECs. However, the expression of podocytic markers in glomeruli with cellular crescents has not been demonstrated.

GECs, generally referred to as podocytes, have been shown to be postmitotic cells that have a minimal capacity to proliferate [24]. Although the cell cycle of podocytes is quiescent in normal mature glomeruli, podocytes can become a component of crescents if they undergo

proliferation. PECs are not postmitotic cells and when stimulated may undergo proliferation and contribute to crescent formation. Thus, evaluation of the phenotype to determine the cellular background of crescent formation in human CRGN may provides important data that could help us to understand the cellular features of crescents.

Cell proliferation is ultimately governed at the level of cell cycle regulatory proteins [27]. Progression of the cell cycle depends on the balance of positive and negative cell cycle regulatory proteins, namely cyclins and cyclin-dependent kinase inhibitors (CKIs) [25, 32]. The work done by Shankland et al. in nephron-reduced models [28], experimental mesangial proliferative glomerulonephritis [29] and mesangial cells in culture [30] suggests that the balance between positive and negative cell cycle regulatory proteins has an important role in mesangial cell proliferation. These observations led the authors to argue that a cell-cycle-dependent mechanism may be responsible for the cell proliferation that determines progressive glomerular injury. However, the epithelial cell cycle regulatory system and its role in progressive crescent formation have not been well characterized.

Recently, we [22] and other [4] have shown that CKIs, such as $p27^{Kip1}$ and $p57^{Kip2}$, are strongly expressed in podocytes at terminal differentiation. This suggests that CKIs are among the determinants of cell cycle stability in mature podocytes. Since vigorous cell proliferation is the nature of cellular crescents, we set up the hypothesis that alteration of cell cycle regulatory proteins might provide information on the mechanism responsible for crescent formation.

The aims of the present study were to determine the cellular phenotype of human glomerular crescents in vivo and to investigate the expression of cell cycle regulatory proteins in crescent formation. Our results suggested that cellular crescents are composed of PEC phenotypic cells that show up-regulation of cyclins concurrent with restrained expression of $p27^{Kip1}$.

Materials and methods

The CRGN patients were 10 patients with pauci-imune type RPGN, 2 patients with Henoch-Schönlein purpura nephritis, and 1 patient with IgA nephropathy. Kidney tissue specimens were obtained from the 13 CRGN patients by percutaneous needle or wedge biopsy, fixed in 10% buffered formaldehyde for 24 h and embedded in paraffin. Six renal specimens in which no morphological abnormalities were revealed on light and immunofluorescent microscopy were used as controls; these had been obtained from patients with microscopic haematuria or kidney transplants.

The tissues were stained with periodic acid-Schiff (PAS) and periodic acid-silver methenamine (PAM). The pathological features of the glomeruli were assessed by light microscopy. The biopsy specimens were evaluated for the number of glomeruli exhibiting different types of glomerular crescents. The percentage of glomeruli exhibiting cellular, fibrocellular, and fibrous crescents on serial sections was calculated as glomeruli with crescents/total glomeruli in all sections.

The monoclonal antibodies used in this study, together with the characteristics of the antigens which they recognize and appropriate references, are listed in Table 1. The specificities of these antibodies have been well characterized in previous studies. Consistent immunoreactivity of a monoclonal antibody against CD68, which recognizes macrophages/monocytes, for kidney tissue has already been demonstrated [21]. PHM-5 is a specific monoclonal antibody, which recognizes a podocyte antigen and has been characterized as previously described [9]. LSAB staining kit was purchased from Dako, Glostrup, Denmark. The specificity of the antibodies against cyclin A, cyclin B_1 and $p27^{Kip1}$ was previously demonstrated by Western blotting [22].

The biopsy specimens were evaluated on serial sections for immunohistochemical staining. In paraffin-embedded tissue sections, the streptavidin-biotin method was used, with a LSAB staining kit, as previously described [12, 20]. The sections were immersed in a glass container containing 10 mmol/l sodium citrate buffer pH 6.0) and processed in an autoclave for 10 min at 121°C. In the CD68 staining procedure, the sections were preincubated for 5 min with a proteinase K enzyme digestion kit in Tris-HCI solution (Dako). Incubation (4°C, overnight) with primary antibodies was followed by incubation for 30 min with biotinylated antibodies against mouse IgG (Dako), and colour was then developed by incubation with 3,3'-diaminobenzidine in phosphate-buffered saline (PBS) with 1% H_2O_2. Finally, some sections were counterstained with PAS reagent and PAM.

As negative and positive controls for immunostaining the normal kidney tissues described above were fixed by the same procedures as were applied to renal tissues and treated either with primary antibodies, normal mouse serum, or PBS alone. Positive staining with primary antibodies served as positive controls, while kidney tissues incubated with either irrelevant monoclonal antibodies or PBS alone were entirely negative for immunostaining as negative controls (data not shown).

Results

Since glomerular crescents were heterogeneous even in the same patient, we evaluated the percentage of glomeruli exhibiting cellular, fibrocellular, and fibrous crescents among all glomeruli in all the patients. The numbers of glomeruli containing different types of crescents were divided by the total number of glomeruli, and the aver-

Table 1 Characteristics of monoclonal antibodies used in this study

Designation	Class	Molecule recognized	Dilution	Commercial supplier	References
Anti-cytokeratin	IgG1/IgG2a (mixture)	Pan-cytokeratin	1:100	Sigma Chemical	[14]
PHM-5	IgG1	Podocyte antigen	1:50	Silenus Laboratories	[9]
PG-M1	IgG3	CD68	1:100	Dako	[5]
MIB-1	IgG1	Ki-67	1:100	Immunotech	[13]
Anti-Bcl-2	IgG1	GAAPAPGIFSSQPGC	1:100	Novo Castra Laboratories	[16]
H-432	IgG	Cyclin A	1:500	Santa Cruz Biotechnology	[2]
GNS1	IgG1	Cyclin B1	1:1000	Santa Cruz Biotechnology	[6]
Kip 1	IgG1	$P27^{Kip1}$	1:200	Transduction Laboratories	[26]

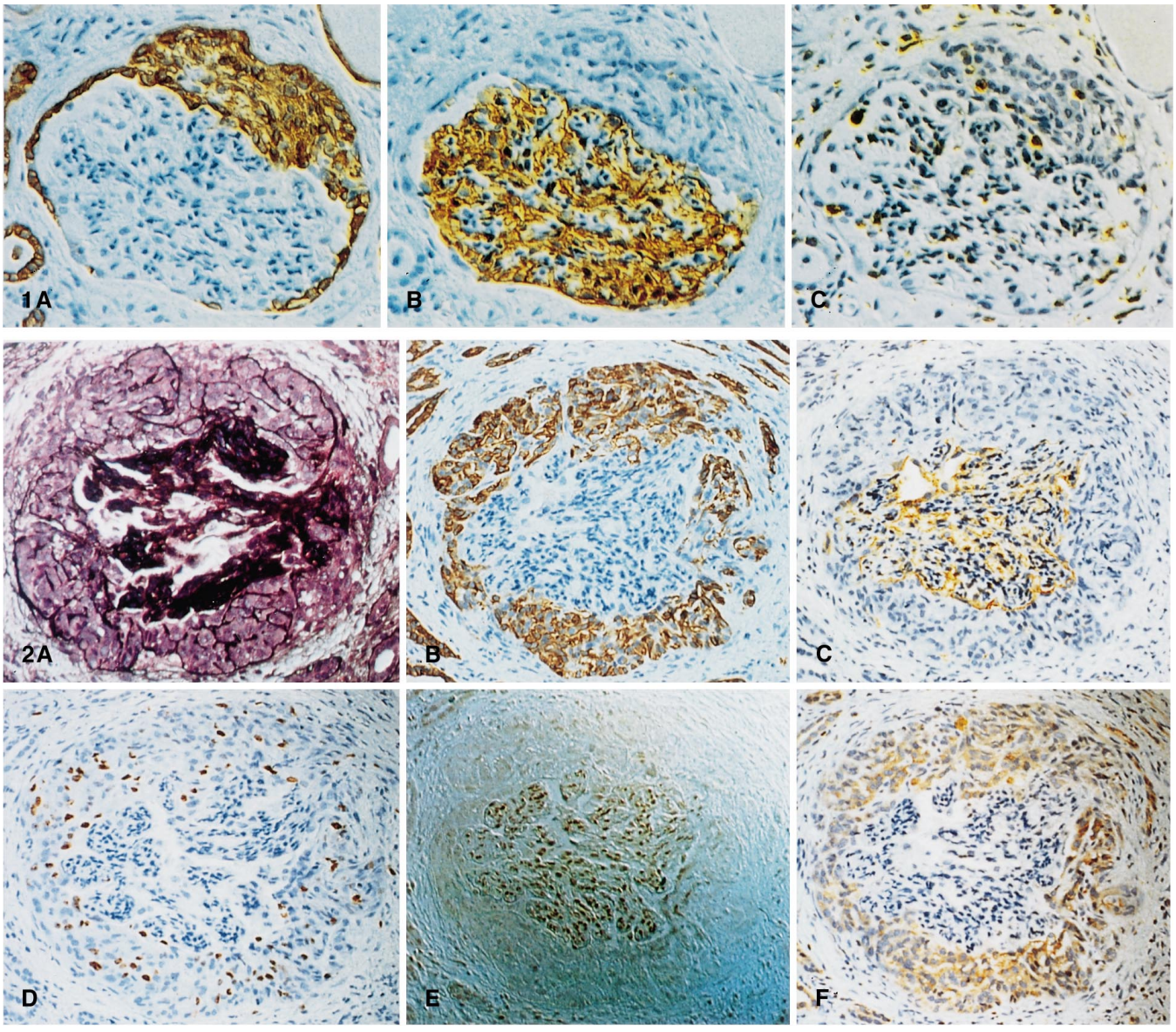

Fig. 1 Immunohistochemical localization of **A** cytokeratin, **B** PHM-5, and **C** CD68 in a glomerulus from a patient with crescentic glomerulonephritis. Cellular crescents expressed abundant cytokeratin, whereas PHM-5 immunoreactivity is abolished. Few cells in the cellular crescents expressed CD68. Magnification ×200

Fig. 2 **A** PAM staining and **B** expression of cytokeratin, **C** PHM-5, **D** Ki-67, **E** $p27^{Kip1}$ and **F** Bcl-2 in the circumferential cellular crescents of a patient with crescentic glomerulonephritis. Cellular crescents clearly express both cytokeratin and Bcl-2. Note the frequent expression of Ki-67, despite the absence of $p27^{Kip1}$. Podocytes in the collapsed tuft expressed $p27^{Kip1}$. Magnification ×200

age in each patient was calculated by two investigators (K.N. and M.N.). A total number of 114 glomeruli was observed in all the patients. Fibrous crescents were present in only 9.6% of the glomeruli (11/114). Cellular crescents appeared in 47.4% of the glomeruli (54/114), while fibrocellular crescents were seen in 31.6% (36/114) of the glomeruli. Thus, the specimens included the relatively early stage of crescent formation and were adequate for use in investigation of the cellular features of crescents.

As shown in Fig. 1, serial section analysis revealed that most of the cellular component of the crescents expressed cytokeratin. Only a small number of cells stained with PHM-5. Few cells expressed CD68. These results suggest that the major constituents were cells with the PEC phenotype and that macrophages/monocytes were minor components in cellular crescents. However, the contribution of the cells expressing CD68 may alter in different types of crescent in human CRGN.

The cellular component of the crescents frequently expressed Ki-67 concurrent with cytokeratin and Bcl-2 (Fig. 2). These results suggest that Bcl-2 expression in crescents may be one of the cellular responses of PECs to proliferation. $P27^{Kip1}$ expression was low in cytokeratin-positive cells. Compared with serial sections stained with PAM, CD68-positive cells tended to increase in fi-

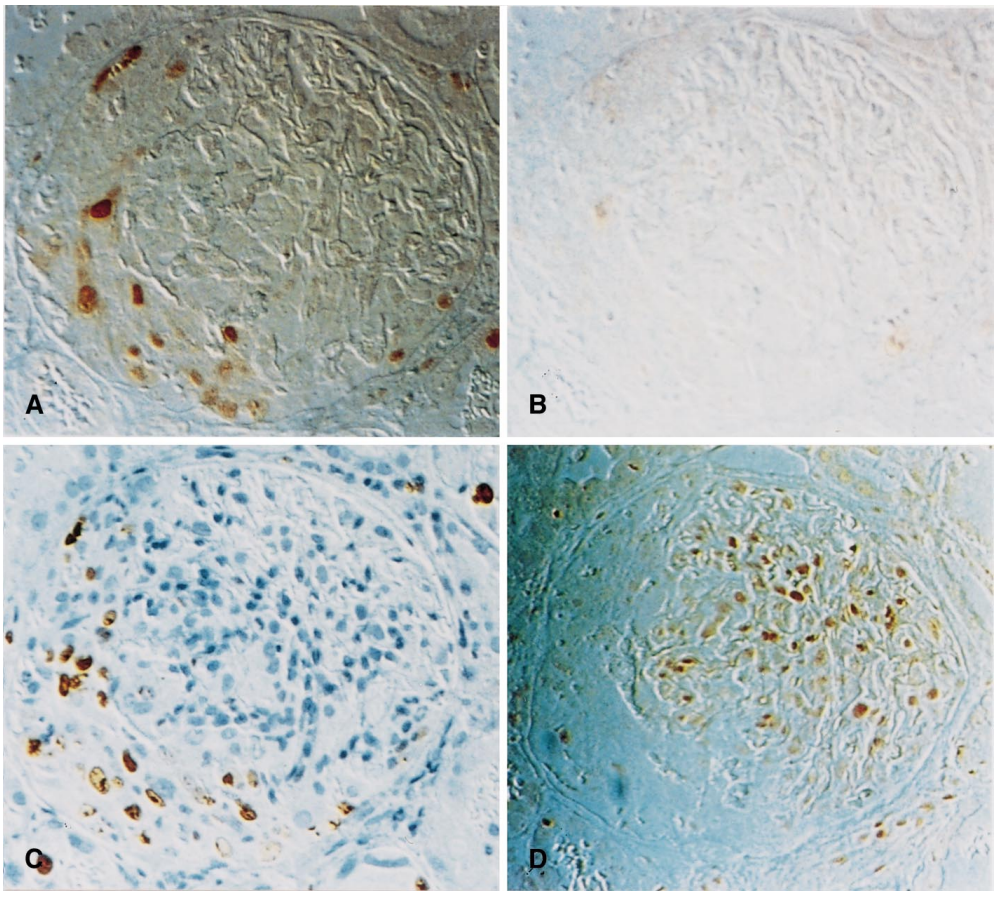

Fig. 3 Expression of **A** cyclin A, **B** cyclin B1, **C** Ki-67, and **D** $p27^{Kip1}$ in a cellular crescent. Ki-67-positive cells were frequently cyclin A positive. Cyclin B1 expression was less frequent. Note that the same cells expressed cyclin A, cyclin B1, and Ki-67. The negative cell-cycle regulator $p27^{Kip1}$ was markedly down-regulated in the cellular crescents. Magnification ×200

brocellular crescents, suggesting that the contribution of the cells expressing CD68 might change during crescent maturation in human CRGN.

As shown in Fig. 3, serial section analysis in our study clearly showed that the Ki-67-positive cells in the crescents were frequently cyclin A positive and seldom cyclin B_1 positive. We found few expression of $p27^{Kip1}$ in the cellular crescents. Compared with serial sections stained with PAM, the staining patterns of cell cycle regulatory proteins did not change significantly in the fibrocellular crescents. The frequent expression of Ki-67 and restrained expression of $p27^{Kip1}$ appeared in the cells with the PEC phenotype. However, collapsed glomerular tufts in glomeruli with cellular crescents showed clear $p27^{Kip1}$ expression in podocytes.

Discussion

The phenotype of cellular crescents was identified by using markers of macrophages/monocytes (CD68), epithelial cells (cytokeratin), and podocytes (PHM-5). Atkins et al. [1, 8] have emphasized that macrophages/monocytes are the major constituent of cellular crescents in human CRGN, as demonstrated by immunostaining and in vitro cell culture. In contrast, several studies [3, 7, 10, 17, 37] have shown cytokeratin expression in cellular composition in human CRGN. Our findings confirm the latter finding: cytokeratin expressing cells are the major constituent of cellular crescents.

In addition, we tested for expression of podocyte-specific marker, PHM-5, in the cellular crescents. The results showed that PHM-5 expression was markedly decreased in the crescents and associated with abundant expression of Ki-67 and cytokeratin. Moreover, CD68-positive cells were minor components in the cellular crescents. Thus, PECs, not podocytes or monocytes/macrophages, may be the major component of cellular crescents. However, we found few PHM-positive cells in the crescents. Since PHM-5 expression has been shown in detached podocytes in urine [10], it is possible that podocytes detached by severe inflammation form some components of cellular crescents.

Our study is the first to demonstrate the expression of cell cycle regulatory proteins in cellular crescents in glomeruli. Serial-section analysis clearly showed that Ki-67-positive cells in the crescents were frequently cyclin A-positive and less frequently cyclin B_1-positive, indicating that proliferation of epithelial cells is promoted by cyclins. Importantly, we found that $p27^{Kip1}$, a negative cell cycle regulatory protein, was sparsely expressed in the cellular crescents immunohistochemically, whereas podocytes in collapsed glomerular tufts in the same glomeruli showed strong $p27^{Kip1}$ expression. This last finding is supported by recent experimental work done by Shankland et al. [31]. In experimental membranous

nephropathy, these authors found abundant expression of $p27^{Kip1}$ and p21 in podocytes. The authors state that CKIs limit proliferative activity in podocytes. Thus, $p27^{Kip1}$ may protect podocytes from various proliferative stimuli, such as cytokines and complement activation. In contrast, we found that cytokeratin-positive components in the crescent were virtually negative for $p27^{Kip1}$.

Since $p27^{Kip1}$ was clearly low in PECs after nephrogenesis [4, 22], PECs probably have a low threshold for proliferation. In this regard, a recent report by Ophascharoensuk et al. [23] clearly showed more severe renal damage in $p27^{Kip1}$-deficient mice than in wild type mice with crescentic glomerulonephritis. Taken together, these findings suggest that different levels of $p27^{Kip1}$ in different kinds of glomerular cells determine the cellular reaction to glomerular damage. Furthermore, PEC proliferation has been shown to contribute to the development of tuft adhesion, and PECs may display a common pathogenic mechanism in various types of glomerular disorders based on their natural characteristics, a restrained expression of $p27^{Kip1}$. Further study is required to determine the cellular mechanism regulating $p27^{Kip1}$ expression in podocytes and PECs.

In contrast, we observed up-regulation of Bcl-2 in cellular crescents. Bcl-2 expression in crescents may be one of the cellular responses of PECs for proliferation. Apoptosis has been reported to occur in the repair process of cellular crescents [33] but not in developing cellular crescents. Wang et al. [35] have recently reported that overexpression of $p27^{Kip1}$ leads to apoptotic cell death in different types of cells, and that ectopic expression of Bcl-2 can protect HeLa cells from apoptosis mediated by $p27^{Kip1}$ overexpression. Therefore, up-regulation of Bcl-2 concurrent with low expression of $p27^{Kip1}$ in PECs may be a compensatory cellular mechanism to protect cells from apoptosis.

In conclusion, the present study has demonstrated for the first time that up-regulation of cyclins and Bcl-2 associated with low expression of $p27^{Kip1}$ in PECs synergistically promotes cellular crescent formation in human RPGN.

Acknowledgements This work was supported by a Grant for Progressive Kidney Disease from Ministry of Health and Welfare and Grant from the Ministry of Education, Culture and Science. The authors thank Mr. Hideki Nakayama and Miss Mayuko Kawashima for their skillful technical assistance.

References

1. Atkins RC, Glasgow EF, Holdworth SR, Thomson NM, Hancock WW (1980) The macrophage in human rapidly progressive glomerulonephritis. Kidney Int 17:515–527
2. Bandara LR, Adamczewski JP, Huntt LA, Thangue NB (1991) Cyclin A and the retinoblastoma gene product complex with a common transcription factor. Nature 352:249–251
3. Cattell V, Jamieson SW (1978) The origin of glomerular crescents in experimental nephrotoxic serum nephritis in the rabbit. Lab Invest 339:584–690
4. Combs HL, Shankland SJ, Setzer SV, Hudkins KL, Alpers CE (1998) Expression of the cyclin kinase inhibitor, p27Kip1, in developing and mature kidney. Kidney Int 53:892–896
5. Falini B, Flenghi L, Pileri S, Gambacorta M, Bigerna B, Durkop H, Eitelbach F, Thiele J, Pacini R, Cavaliere A (1993) PG-M1: a new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. Am J Pathol 142:1359–1372
6. Galaktinov K, Beach D (1991) Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell 67:1181–1194
7. Guettier C, Nochy D, Jacquot C, Mandet C, Camilleri JP, Bariety J (1986) Immunohistochemical demonstration of parietal epithelial cells and macrophages in human proliferative extracapillary lesions. Virchows Arch [A] 409:739–748
8. Hancock WW, Atkins RC (1984) Cellular composition of crescents in human rapidly progressive glomerulonephritis identified using monoclonal antibodies. Am J Nephrol 4:177–181
9. Hancock WW, Atkins RC (1983) Monoclonal antibodies to human glomerular cells. Nephron 33:83–90
10. Hara M, Yamamoto T, Yanagihara T, Takada T, Itoh M, Adachi Y, Yoshizumi A, Kawasaki K, Kihara I (1995) Urinary excretion of podocalyxin indicates glomerular epithelial cell injuries in glomerulonephritis. Nephron 69:397–403
11. Harrison DJ, MacDonald MK (1986) The origin of cells in the glomerular crescent investigated by the use of monoclonal antibodies. Histopathology 10:945–952
12. Honda K, Nitta K, Horita S, Yumura W, Nihei H (1996) Morphological changes in the peritoneal vasculature of patients on CAPD with ultrafiltration failure. Nephron 72:171–176
13. Key G, Becker MH, Baron B, Duchrow M, Schluster C, Flad HD, Gerdes J (1993) New Ki-67-equivalent murine monoclonal antibodies (MIB 1–3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encording for the Ki-67 epitope. Lab Invest 68:629–636
14. Lane EB, Alexander CM (1990) Use of keratin antibodies in tumor diagnosis. Semin Cancer Biol 1:165–179
15. Lan HY, Nikolic-Paterson DJ, Mu W, Atkins RC (1997) Local macrophage proliferation in the pathogenesis of glomerular crescent formation in rat anti-GBM glomerulonephritis. Clin Exp Immunol 110:233–240
16. Lebrun DP, Kamel OW, Cleary ML, Dorfman RF, Warnke RA (1992) Follicular lymphomas of the gastrointestinal tract. Pathologic features in 31 cases and bcl-2 oncogenic protein expression. Am J Pathol 140:1327–1335
17. Magil AB (1985) Histogenesis of glomerular crescents. Immunohistochemical demonstration of cytokeratin in crescent cells. Am J Pathol 120:222–229
18. Morita T, Suzuki Y, Churg J (1973) Structure and development of the glomerular glomerular crescent. Am J Pathol 72:349–368
19. Morrin PA, Hinglais, Nabarra B, Kreis H (1978) Rapidly progressive glomerulonephritis. A clinical and pathologic study. Am J Med 65:446–460
20. Nagata M, Yamaguchi Y, Ito K (1993) Loss of mitotic activity and the expression of vimentin in glomerular epithelial cells of developing human kidneys. Anat Embryol 187:275–279
21. Nagata M, Akioka Y, Tsunoda Y, Komatsu Y, Kawaguchi H, Yamaguchi Y, Ito K (1995) Macrophages in childhood IgA nephropathy. Kidney Int 48:527–535
22. Nagata M, Nakayama K, Terada Y, Hoshi S, Watanabe T (1998) Cell cycle regulation and differentiation in the human podocyte lineage. Am J Pathol 153:1511–1520
23. Ophascharoensuk V, Fero ML, Hughes J, Roberts JM, Shankland SF (1998) The cyclin-dependent kinase inhibitor $p27^{Kip1}$ safeguards against inflammatory injury. Nat Med 4:575–580
24. Pabst R, Sterzel RB (1983) Cell renewal of glomerular cell types in normal rats. An autoradiographic analysis. Kidney Int 24:262–631
25. Pardee AB (1989) G1 events and regulation of cell proliferation. Science 246:603–608
26. Polyak K, Kato J, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A (1994) $p27^{Kip\ 1}$, a cyclin-Cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. Genes Dev 8:9–22

27. Shankland S (1997) Cell-cycle control and renal disease. Kidney Int 52:294–308
28. Shankland SJ, Hamel P, Scholey JW (1996) Cyclin and cyclin-dependent kinase expression in the remnant glomerulus. J Am Soc Nephrol 8:368–375
29. Shankland SJ, Hugo C, Coats SR, Nangaku M, Pichler RH, Gordon KL, Pippin J, Roberts JM, Couser WG, Johnson RJ (1996) Changes in cell cycle protein expression during experimental mesangial proliferative glomerulonephritis. Kidney Int 50:1230–1230
30. Shankland SJ, Pippin J, Flanagan M, Coats SR, Nangaku M, Gordon KL, Roberts JM, Couser WG, Johnson RJ (1997) Mesangial cell proliferation mediated by PDGF and bFGF is determined by levels of the cyclin kinase inhibitor $p27^{Kip\ 1}$. Kidney Int 51:1088–1099
31. Shankland SJ, Floege J, Thomas SE, Nangaku M, Hugo C, Pippin J, Henne K, Hockenberry DM, Johnson RJ, Couser WG (1997) Cyclin kinase inhibitors are increased during experimental membranous nephropathy: Potential role in limiting glomerular epithelial cell proliferation in vivo. Kidney Int 52:404–413
32. Sheer CJ, Roberts JM (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9:1149–1163
33. Takemura T, Murakami K, Miyazato H, Yagi K, Yoshioka K (1995) Expression of Fas antigen and Bcl-2 in human glomerulonephritis. Kidney Int 48:1886–1892
34. Thomson NM, Holdworth SR, Glasgow EF, Atkins RC (1979) The macrophage in the development of experimental crescentic glomerulonephritis. Am J Pathol 94:223–240
35. Wang X, Gorospe M, Huang Y, Holbrook NJ (1997) $p27^{Kip1}$ overexpression causes apoptotic death of mammalian cells. Oncogene 15:2991–2997
36. Whitworth JA, Morel-Maroger L, Mignon F, Richet G (1976) The significance of extracapillary proliferation. Clinicopathological review of 60 patients. Nephron 16:1–19
37. Yoshioka K, Takemura T, Akano N, Miyamoto H, Iseki T, Maki S (1987) Cellular and non-cellular compositions of crescents in human glomerulonephritis. Kidney Int 32:284–291