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Changes in cell-cycle protein expression during experimental mesangial proliferative glomerulonephritis

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Changes in cell cycle protein expression during experimental mesangial proliferative glomerulonephritis. A characteristic response to mesangial cell injury is proliferation, which is closely linked to mesangial matrix accumulation and the progression of glomerular disease. Cell proliferation in non-renal cells *in vitro* is regulated at the level of the cell-cycle by specific cyclins and their catalytic partners, cyclin dependent kinases (CDK). Cyclin kinase inhibitors (CKI) prevent proliferation by inhibiting cell-cycle progression. However, the expression of cell-cycle regulatory proteins in the kidney and in renal disease is unknown. To determine this we studied the expression of cell-cycle proteins *in vivo* in normal rats and rats with experimental mesangial proliferative glomerulonephritis (Thy1 model). Normal quiescent rat glomeruli have a differential expression for CKI's, where $p27^{Kip1}$ is highly expressed, and the levels for p21 (Cip1, Waf1, Sdi1, Cap20) (p21) are low. The onset of mesangial cell proliferation in Thy1 glomerulonephritis is associated with a reduction in $p27^{Kip1}$ levels when mesangial cell proliferation is maximal. Mesangial cell proliferation *in vivo* is also associated with an increase in glomerular expression of cyclin A, and an increase in expression and activity for CDK2. The resolution of mesangial cell proliferation was associated with a return to baseline levels for $p27^{Kip1}$, while the expression for p21 increased substantially. Furthermore, mesangial cell p21 expression was maintained following the resolution of proliferation. These results provide evidence for a complex interplay of cell-cycle regulatory proteins during the glomerular response to injury *in vivo*. The marked increase in CDK2 expression during mesangial cell proliferation and the sustained increase in p21 expression following the resolution of mesangial cell proliferation suggests that the *in vivo* expression of certain cell-cycle proteins may differ from that described in non-renal cells *in vitro*.

The mesangial cell (MC) is an active participant in many progressive glomerular diseases such as IgA nephropathy, membranoproliferative nephritis, lupus nephritis and diabetes [1]. A characteristic response of the MC to injury in human disease is cell proliferation, which may be associated with extracellular matrix accumulation [reviewed in 2]. This phenomenon also occurs in experimental models of glomerular injury where MC injury occurs. In the remnant kidney model [3] and the Thy1 model of mesangial proliferative glomerulonephritis, MC proliferation has been closely linked to the progression of glomerular disease [4], and maneuvers that reduce MC proliferation in these experimental models, such as low protein diet [5], heparin infusion [6] or complement depletion [7] also reduce subsequent matrix expansion or sclerosis. These studies suggest that the mechanisms responsible for the MC proliferative response to injury *in vivo* may also be important in the development of glomerulosclerosis.

In the past few years major advances have been made in the understanding of how cell proliferation is regulated *in vitro* at the level of the cell-cycle [8]. Cyclins and cyclin-dependent kinases (CDK) constitute the major positive cell-cycle regulatory proteins [reviewed in 9, 10]. Cyclins are periodically expressed during specific phases of the cell-cycle, where the levels are regulated transcriptionally and post-translationally [11]. As cells enter the cell-cycle from quiescence (G0), D-type cyclins are synthesized during the G1 interval. Cyclin E is maximal in late G1, and is essential for G1 to S phase transition [12]. Cyclin A is expressed during the S phase of the cell cycle [13]. Cyclins bind to and activate specific cyclin dependent kinases (CDK). D-cyclins bind to CDK4, whereas cyclins E and A bind to CDK2 [10]. Cyclin dependent kinases are responsible for phosphorylating and functionally inactivating the retinoblastoma protein, an event that is associated with entry into S phase [14].

The cell-cycle is also regulated at various "check points" by negative regulatory cell-cycle proteins known as the cyclin kinase inhibitors (CKI) [reviewed in 15]. There are two families of CKI. The first includes the INK4 family [16, 17], while the second family includes p21 (Cip1, Waf1, Sdi1, Cap20) (p21) [18], $p27^{Kip1}$ and the more recently recognized member, $p57^{Kip2}$ [15]. CKI arrest the cell-cycle by inhibiting specific cyclin-CDK complexes. All CKI inhibit cyclin D-CDK4 complexes, while only p21 and $p27^{Kip1}$ inhibit cyclin-CDK2 complexes [15].

The mitogenic and antiproliferative growth factors that regulate MC proliferation both *in vitro* and *in vivo* are well known [reviewed in 19, 20]. In glomerular disease, multiple growth factors and cytokines are activated and the injured mesangial cells' proliferative response reflects a balance of mitogenic and antiproliferative growth factors and cytokines [2]. Preisig and Franch have shown a role for specific cell-cycle proteins in renal epithelial cells in culture [21]. However, the expression of cell-cycle regulatory proteins in the glomerulus is not known, and while the expression of cell-cycle proteins has been extensively

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studied in various non-renal cell types *in vitro*, the expression of these nuclear proteins in inflammatory states *in vivo* remains to be elucidated. Furthermore, observations that have been made *in vitro* do not always occur *in vivo*. Accordingly, the first aim of the current study was to determine the expression of cell-cycle regulatory proteins in the normal glomerulus *in vivo*, while a second aim was to determine the expression and activity of specific cell-cycle regulatory proteins during mesangial cell proliferation *in vivo* in glomerular disease. Our results show that mesangial cell proliferation *in vivo* in the Thy1 model of experimental mesangial proliferative glomerulonephritis [22] is associated with an increase in expression and activity for cyclin A-CDK2, and a decrease in p27 expression. Resolution of mesangial cell proliferation *in vivo* was associated with a normalization of p27 levels and an increase in p21 expression.

Methods

Animal model

The Thy1 model of experimental mesangial proliferative glomerulonephritis was induced in 180 to 220 gram male Wistar rats (Simonsen Laboratories, Gilroy, CA, USA) by an intravenous injection of goat anti-thymocyte plasma (0.35 ml/100 g body wt) [22–24]. Rats were sacrificed on days 1, 2, 3, 4, 5, 7 and 10 (N = 6 at each time point), where renal biopsies were taken from each animal for immunostaining, and glomeruli were isolated by differential sieving [25] for protein extraction. Age- and weight-matched Wistar rats from the same colony served as normal controls (N = 6).

Immunohistochemistry

Multiple renal biopsies were taken from each rat and fixed in different ways. Biopsies were fixed in formalin or methyl Carnoy's solution, embedded in paraffin, and cut into 4 μm thick sections [6, 7]. For staining on frozen sections, tissue was embedded in O.C.T. Compound (Miles, Elkart, IN, USA) and rapidly snap frozen in isopentane in liquid nitrogen. Sections of 4 μm thick cryostat were fixed at room temperature for 10 minutes in either acetone, methanol, acetone-alcohol or alcohol-ether. Pepsin digestion was used to unmask antigens in acetone fixed sections as previously described [26]. We performed indirect immunoperoxidase double-immunostaining on frozen and paraffin embedded sections to determine the nuclear expression of cell-cycle proteins, and colocalize the cell-type expressing the antigen [27].

The following primary antibodies were incubated overnight at 4°C: rabbit polyclonal antibodies to cyclin A (1:5000 dilution; provided by JMR), cyclin D1 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin E (dilution 1:200; Santa Cruz Biotechnology), cyclin-dependent kinase 2 (1:4000 dilution; Santa Cruz Biotechnology), p27^{Kip1} (1:1000 dilution; provided by JMR) and p21 (1:5000; Santa Cruz Biotechnology). A murine monoclonal IgM antibody against proliferating cell nuclear antigen (PCNA) (1:1000 dilution; 19A2; Coulter Immunology, Hialeah, FL, USA) was also incubated overnight at 4°C. Controls for this step of the double-immunostaining included omitting the primary antibody, substituting the primary antibody with an irrelevant antibody of the same Ig class, and peptide absorption studies for the polyclonal antibodies (peptides were obtained from same source as the primary antibody). The secondary antibody used for the polyclonal antibodies was a biotinylated goat anti-rabbit antibody (1:400 dilution; Vector Laboratories, Inc., Burlingame, CA, USA), and a peroxidase-conjugated rat anti-mouse IgM (Zymed Laboratories, Inc., San Franscisco, CA, USA) was used for the monoclonal antibody. Incubation was for 30 minutes at room temperature, followed by a horseradish peroxidase conjugated avidin D (1:2000 dilution; Vector Laboratories, Inc.) for 20 minutes at room temperature. Black nuclear staining was detected using diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) with nickel as a chromagen.

Double-immunostaining was then performed using a second primary antibody to determine which cell type expressed each nuclear antigen. The following primary antibodies were incubated at room temperature for 60 minutes: OX-7, a marker of mesangial cells (1:4000 dilution; Serotec), ED-1 a marked of macrophages, monocytes and dendritic cells (1:200; Bioproducts for Science, Inc., Indianapolis, IN, USA), and RECA-1 a marker of endothelial cells (gift of A.M. Duijvestijn, Maastricht, Netherlands) [28]. A secondary peroxidase conjugated anti-mouse IgG antibody (1:100 dilution; Zymed Laboratories) was applied for 60 minutes at room temperature. The brown cytoplasmic stain was determined by diaminobenzidine without nickel. The controls used for the second step of the double-immunostaining included omitting the second primary antibody or replacing it with an irrelevant antibody of the same Ig class.

Semiquantitation of immunostaining

The glomerular expression for PCNA and each cyclin (cyclins D1, E, A), cyclin-dependent kinase (CDK2) and cyclin kinase inhibitors (p21, p27) was graded semiquantitatively in a blinded fashion in controls and at each time point in Thy1 glomerulonephritis. Each rat was individually assessed. Thirty to fifty glomerular cross sections from each biopsy were evaluated by counting the number of nuclei that stained positive for each antigen (positive was defined as a black staining nucleus), and the cell type expressing each antigen was also scored (positive was defined as the colocalization of a brown cytoplasmic stain with a black staining nucleus). Mean values per time point were calculated, and are expressed as positive cells per 30 or 50 glomerular cross section $\pm$ SEM.

Western blot analysis

Glomerular protein was extracted from individual animals by isolating glomeruli by differential sieving as previously described [25]. In brief, the kidneys were decapsulated, and the cortex separated from the medulla. The cortex was passed sequentially through mesh sieves of 180, 106 and 75 μm, and the purity of the preparation was checked to ensure that there was less than 5% tubular contamination with each glomerular preparation. Glomeruli were then resuspended in a buffer containing 1% triton, 10% Glycerol, 20 mM HEPES, 100 mM NaCl with 10 μg/ml Leupeptin, 10 μg/ml Antipain, 10 μg/ml Pepstatin, 0.1 mM sodium orthovanadate and 50 mM sodium fluoride (reagents from Sigma). Glomeruli were sonicated for 20 seconds and then placed on ice for 10 minutes, followed by centrifugation at 14000 rpm for five minutes. The protein concentration of the supernatant was measured by the BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

For Western blot analysis, reducing buffer was added to 20 μg glomerular protein extract, the amount loaded in each lane, and boiled for five minutes. Glomerular protein was then separated on

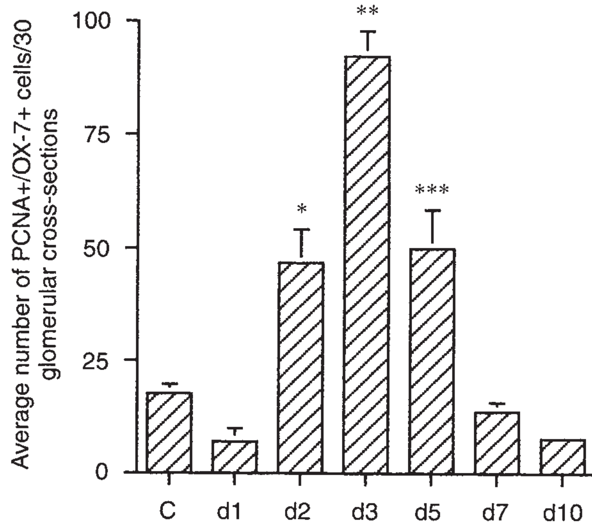


Fig. 1. *Quantitation of mesangial cell (MC) proliferation in the Thy1 model of experimental mesangial proliferative glomerulonephritis.* Cell proliferation was determined by positive nuclear staining for proliferating cell nuclear antigen (PCNA), and the MC was identified by cytoplasmic staining for OX-7 (a marker specific for the MC). MC proliferation begins at day 2, and was maximal at day 3. Resolution of MC proliferation was evident by day 5, with complete resolution occurring by day 7. $^*P = 0.0031$ vs. C; $^{**}P < 0.0001$ vs. C and other time points in Thy1; $^{***}P = 0.0002$ vs. C.

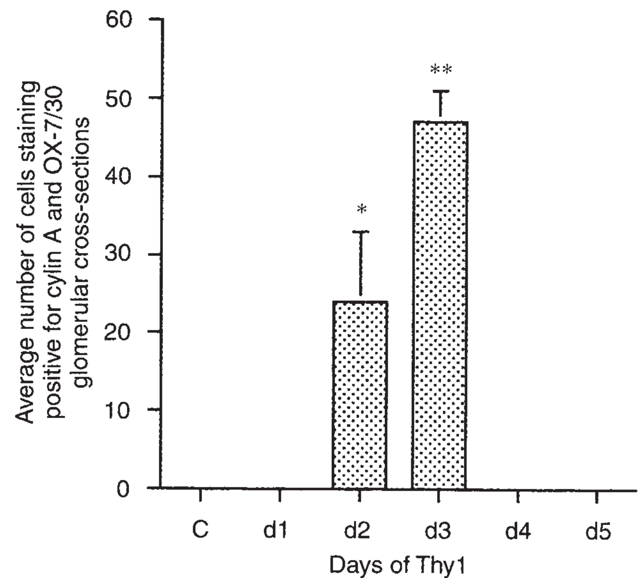


Fig. 2. *Quantitation of cyclin A+/OX-7+ staining.* This figure shows the number of cells that double-stained for cyclin A and OX-7 (a marker of the mesangial cell) in thirty glomerular cross-sections from control rats (labeled as C) and at different days of Thy1 nephritis (days abbreviated as d). Each time point represents the mean ± SEM of six animals. No staining was detected in control rats, or at day 1 of Thy1. There was a significant increase in the number of cells that stained cyclin A+/OX-7+ at day 2 ($P < 0.0001$ vs. control), with maximal staining occurring at day 3 ($P < 0.0001$ vs. control), which coincides with the peak of mesangial cell proliferation. By day 4, cyclin A staining was absent. $^*P < 0.0001$ vs. C, d1, d4, d5; $^{**}P < 0.001$ vs. d2.

a 15% SDS-PAGE gel, and transferred to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting. Complete protein transfer was ensured by staining the gels with Coommasie Blue. Non-specific background was blocked by incubating the membranes with 5% non-fat dried milk for 30 minutes. Antibodies to cyclin D1, cyclin E, cyclin A, CDK2, p21 and $p27^{Kip1}$ (source for each antibody listed earlier) were incubated for one hour at room temperature. An alkaline phosphatase-conjugated secondary antibody (1:7500 dilution; Promega, Madison, WI, USA) was applied for 60 minutes at room temperature, and protein detection was made with the chromagen, 5-Bromo-4-Chloro-3-Indolyl phosphate/nitro blue tetrazolium (Sigma). Controls included omitting the primary antibody, substituting the primary with pre-immune serum, and peptide absorption of the primary antibody. To ensure that the results obtained by Western blot analysis were not due to uneven protein loading or protein transfer, we routinely stained the filters with Coommasie Blue.

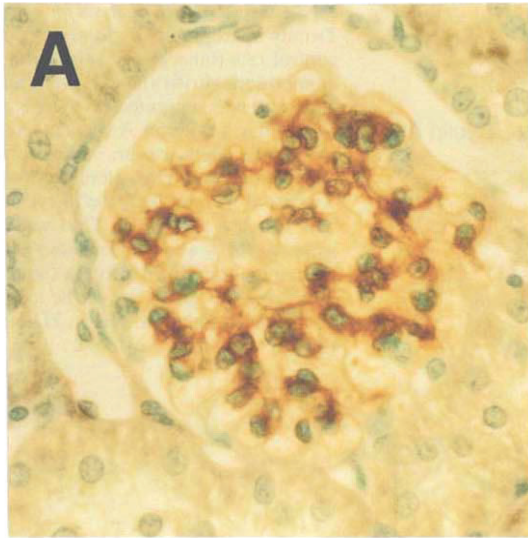
Histone H1 assay

The activity of cyclin-dependent kinase 2 was determined in individual rats by the histone H1 assay as previously described [12]. Briefly, 500 μg of glomerular protein extract from individual control rats, and rats at day 3 and day 5 of Thy1 nephritis was immunoprecipitated with antibodies to cyclin A or cyclin E for 30 minutes at 4°C. 50 μl of Protein A sepharose beads (Repligen, Cambridge, MA, USA) were then added to each immunoprecipitation and incubated for 30 minutes at 4°C. This was followed by the addition of histone H1 (Boehringer Mannheim, Indianapolis, IN, USA), ATP (Pharmacia Biotech, Piscataway, NJ, USA) and [^{32}p] dCTP (Dupont, Boston, MA, USA) for a 60 minute incubation at 37°C. Negative controls included substituting pre-immune

Fig. 3. (Top panels) *Indirect immunoperoxidase double-immunostaining for cyclin A (represented by the black nuclear stain), and OX-7, a marker of mesangial cell (represented by the brown cytoplasmic stain).* In the normal rat glomerulus, no staining was observed for cyclin A (**A**). **B.** Representative staining in a glomerulus at day 3 of Thy1 glomerulonephritis that shows an increase in nuclei staining positive for cyclin A (examples are indicated by the broad arrows). In the glomerulus shown, 2 nuclei stained positive for cyclin A. The majority of cyclin A positive nuclei colocalized with cells that express OX-7, a marker of mesangial cells (represented by the thin arrows). Magnification ×63.

Fig. 6. (Middle panels) *Indirect immunoperoxidase double-immunostaining for cyclin-dependent kinase 2 (CDK2) (represented by the black nuclear stain) and OX-7, a marker of mesangial cell, (represented by the brown cytoplasmic stain).* In the normal rat glomerulus shown, staining was observed for CDK2 in 2 nuclei (**A**). **B.** A representative glomerulus at day 3 of Thy1 glomerulonephritis that shows an increase in nuclear staining positive for CDK2 (examples are indicated by the broad arrows). In this glomerulus 6 cells stained positive for CDK2. The majority of CDK2 positive nuclei colocalized with cells that express OX-7 (represented by the thin arrows). Magnification ×63.

Fig. 9. (Bottom panels) *Indirect immunoperoxidase double-immunostaining for p21 (represented by the black nuclear stain) and OX-7, a marker of mesangial cell, (represented by the brown cytoplasmic stain).* In the normal rat glomerulus shown, no staining was observed for p21 (**A**). **B.** A representative glomerulus at day 5 of Thy1 glomerulonephritis that shows an increase in nuclear staining for p21 (examples are indicated by the broad arrows), colocalizing with OX-7 staining cells (examples are indicated by the thin arrows). Magnification ×63.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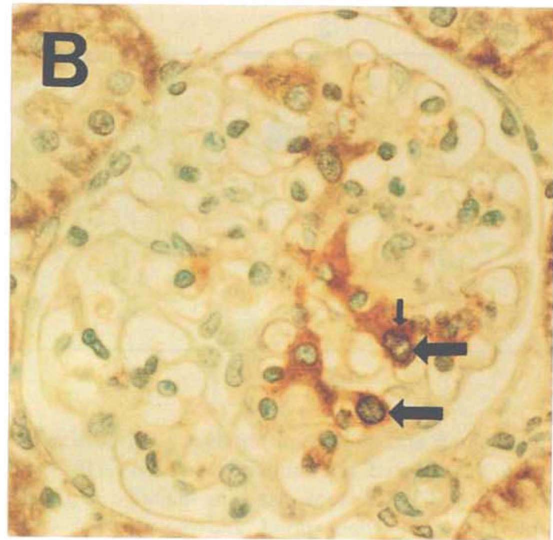
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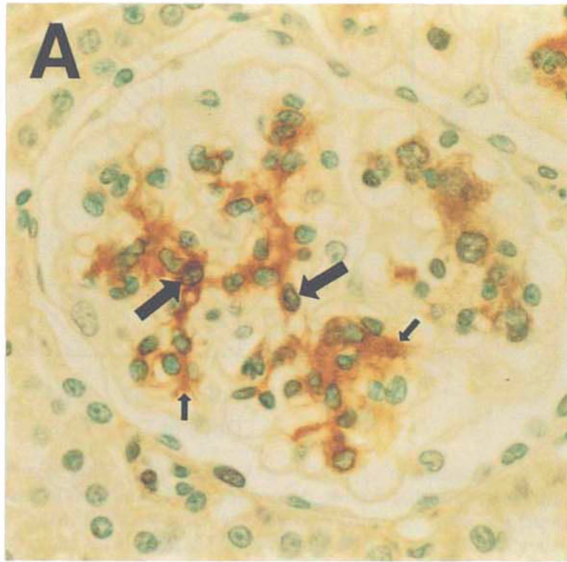
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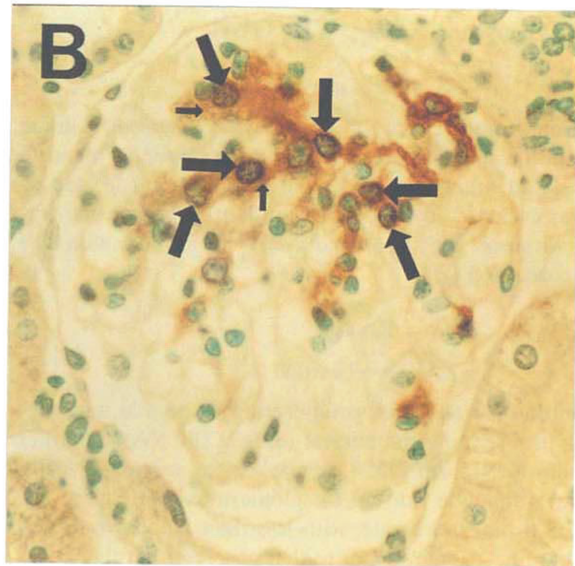
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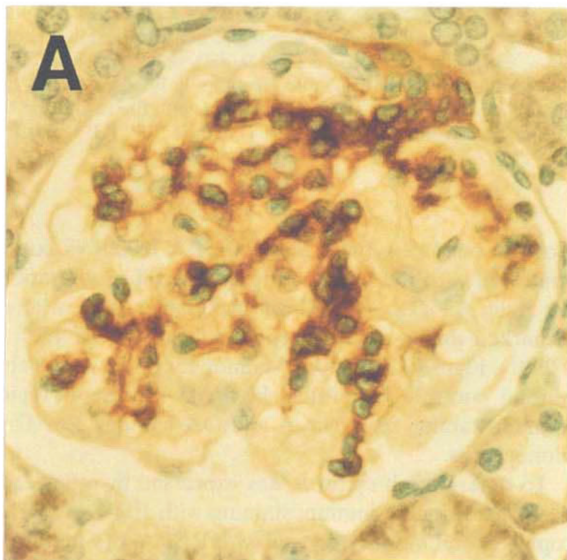
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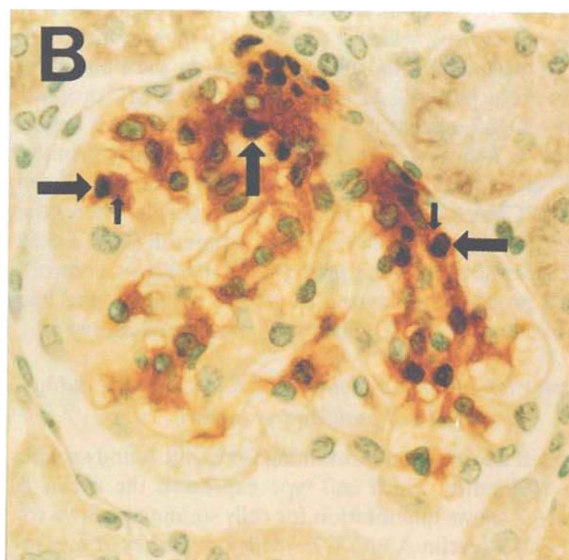
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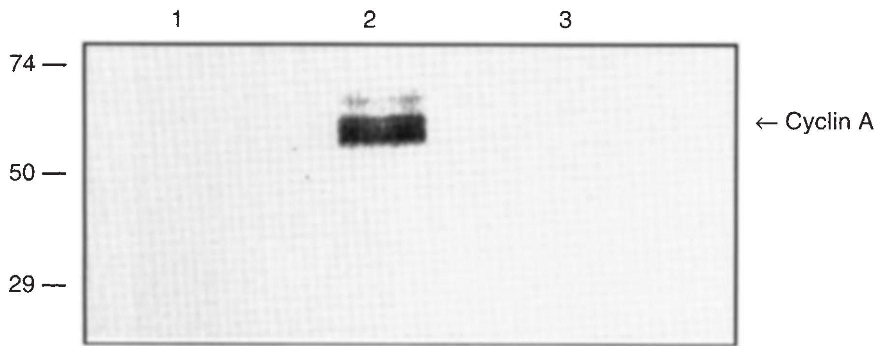

Fig. 4. *Western blot analysis for cyclin A.* Twenty micrograms of glomerular protein from control rats (lane 1) and rats at day 3 of Thy1 glomerulonephritis (lane 2) were loaded in each lane, and was separated on a 15% SDS-PAGE. No protein for cyclin A was detected in control glomeruli. There was an increase in a 55 kDa band at day 3 of Thy1 glomerulonephritis, consistent with an increase in cyclin A protein. The increase at day 3 of Thy1 was not observed when cyclin A was preincubated with recombinant cyclin A protein (lane 3), indicating the specificity of the antibody used. Molecular weight markers in kDa are shown in the left margin.

rabbit serum for cyclin E and cyclin A antibodies, and omitting the primary antibody. Complexed recombinant cyclin E-CDK2 and recombinant cyclin A-CDK2 were used as positive controls. Reduced sample buffer was then added, and the mix was separated on a 15% SDS-PAGE. The gel was exposed to autoradiographic film (Amersham, Arlington, IL, USA).

Statistical analysis

At each time point, scores for immunohistochemical staining was performed on individual animals, and expressed as mean ± SEM unless stated otherwise. Statistical significance (defined as $P < 0.05$) was evaluated by use of the Student's *t*-test or one-way analysis of variance with modified *t*-test performed with the Bonferroni correction [29].

Results

MC proliferation

We measured mesangial cell proliferation by double immunostaining with proliferating cell nuclear antigen (PCNA), a marker for cell proliferation, and OX-7, a marker for mesangial cells. Figure 1 shows that the normal rat glomerulus had a very low mesangial cell proliferative rate, with less than 1 positive PCNA cell per glomerulus, results similar to that observed by others [30]. Administration of the Thy1 antibody causes early mesangiolysis at day 1 (results not shown), which is associated with a reduction in the number of mesangial cells, and a decrease in the number of PCNA positive cells [20–22]. As shown in Figure 1, MC proliferation (PCNA+/OX-7+ cells) in Thy 1 glomerulonephritis begins at day 2 (46.67 ± 7.45 vs. 17.67 ± 2.1 per 30 glomerular profiles; $P = 0.0015$ vs. control). By day 3 of Thy1, mesangial cell proliferation (PCNA+/OX-7+ cells) was maximal, where the increase was 5.21-fold compared to normal glomeruli (92.2 ± 5.62 vs. 17.67 ± 2.1 per 30 glomerular profiles; $P < 0.0001$ vs. control). Mesangial cell proliferation started to resolve by day 5, and had returned to baseline by day 7, which was maintained at day 10.

Cyclin A expression and not cyclins D1 or E, is increased during mesangial cell proliferation in vivo

We performed double-immunostaining for cyclin A and specific cell markers to identify which cell type expressed the cyclin A antigen. Figure 2 shows quantitation for cells staining positive for cyclin A and OX-7 (cyclin A+/OX-7+ cells), a marker of mesangial cells. Cyclin A staining was absent in the normal rat glomerulus. Cyclin A+/OX-7+ cell staining increased at day 2 of Thy1

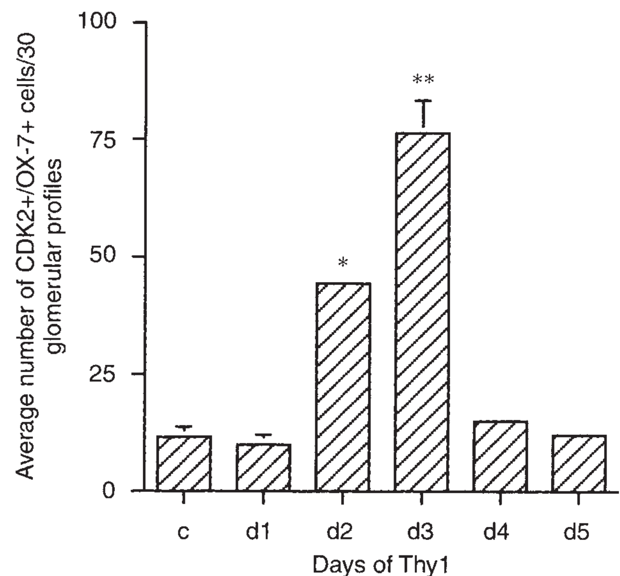

Fig. 5. *Quantitation of CDK2+/OX-7+ staining.* This Figure shows the number of cells that double-stained for CDK2 and OX-7 (a marker of the mesangial cell) in thirty glomerular cross-sections from control rats (labeled as C) and at different days of Thy1 nephritis (days labeled as d). Each time point represents the mean ± SEM of six animals. There was an increase in CDK2+/OX-7+ cells at day 2 of Thy1 ($P < 0.016$ vs. C), coinciding with the onset of mesangial cell proliferation. Peak CDK2+/OX-7+ staining was at day 3 ($P < 0.0001$ vs. C), at which time mesangial cell proliferation was maximal. By day 4, baseline levels for CDK2+/OX-7+ staining was observed, coinciding with the resolution of mesangial cell proliferation. *$P < 0.0001$ vs. C, d1, d4, d5; **$P < 0.016$ vs. d2.

glomerulonephritis (0 vs. 24 ± 9.01; $P < 0.0001$ vs. control), which coincided with the onset of mesangial cell proliferation. Staining for cyclin A+/OX-7+ cells was maximal at day 3, where there was a marked increase compared to controls (0 vs. 47 ± 4.17; $P < 0.0001$). Figure 3 shows that staining for cyclin A was absent in the normal control glomerulus. Figure 3 represents typical double immunostaining for cyclin A and OX-7 at day 3 of Thy1 glomerulonephritis.

To determine if cyclin A was expressed by other cell types, we performed double-immunostaining with ED-1 (a marker of macrophages and monocytes) and with RECA-1 (a marker of glomerular endothelial cells). Eighty percent of the cells expressing cyclin A were mesangial cells (cyclin A+/OX-7+). Of the 20% of

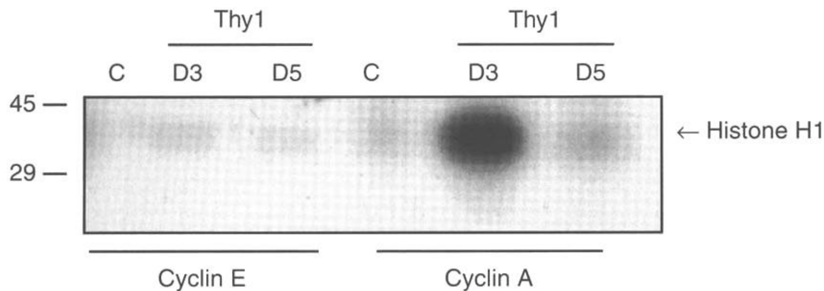

Fig. 7. *Cyclin dependent kinase 2 kinase activity.* Cyclin dependent kinase 2 (CDK2) activity was measured by a histone H1 kinase assay. Each lane represents 500 μg of glomerular protein extracted from either control rats, or day 3 or day 5 of Thy1 glomerulonephritis that was immunoprecipitated with an antibody to either cyclin E or cyclin A. No kinase activity was present in control glomeruli when immunoprecipitated with antibodies to either cyclin E (lane 1) or cyclin A (lane 4). When CDK2 was immunoprecipitated with an antibody to cyclin A at day 3 of Thy1 (lane 5), but not day 5 (lane 6), there was an increase in a protein with a molecular weight of 33 kDA, consistent with histone H1, indicating that CDK2 kinase activity was increased. No kinase activity was detected at days 3 (lane 2) and 5 (lane 3) of Thy1 when an antibody to cyclin E was used for immunoprecipitation. Molecular weight markers (in kDa) are shown in the left margin.

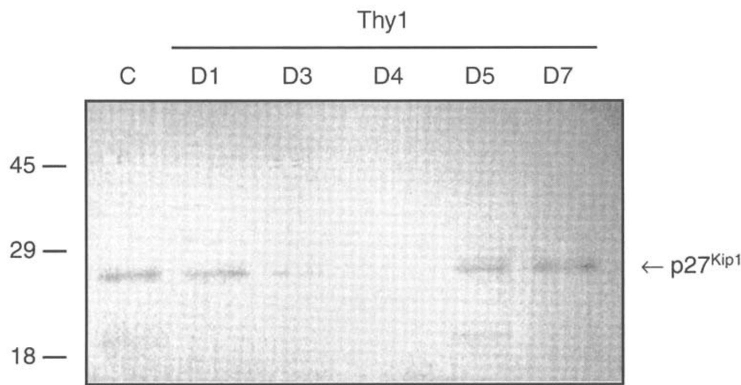

Fig. 8. *Western blot analysis for $p27^{Kip1}$.* Twenty micrograms of glomerular protein was loaded in each lane, and was separated on a 15% a SDS-PAGE, and immunoblotted with an antibody to $p27^{Kip1}$. $p27^{Kip1}$ protein (represented here by a protein with a molecular weight of 27 kDa) is present in control glomeruli (lane 1) and day 1 of Thy1 glomerulonephritis (lanes 2), but was reduced at day 3 (lane 3). $p27^{Kip1}$ levels were barely detectable at day 4 of Thy1 glomerulonephritis (lane 4). $p27^{Kip1}$ expression returned to baseline by day 5 (lane 5). Molecular weight markers in kDa are shown in the left margin.

glomerular cells that stained cyclin A+/OX-7−, 10% were macrophages (cyclin A+/ED-1+), while the remaining 10% were glomerular endothelial cells (cyclin A+/RECA-1+) at day 3. The pattern of staining for cyclin A was similar if OX-7, ED-1 and RECA-1 were omitted.

To further confirm specificity for cyclin A staining in controls and Thy1 glomerulonephritis, we performed Western blot analysis on protein lysates from isolated glomeruli, and the results are shown in Figure 4. No cyclin A protein was present in control glomeruli, but there was a protein of 55 kDa molecular weight at day 3 of anti-Thy1 glomerulonephritis, consistent with an increase in glomerular cyclin A protein expression. When the primary antibody to cyclin A was preincubated with recombinant cyclin A, no protein was detected (Fig. 4), indicating antibody specificity.

We also performed immunostaining and Western blot analysis with antibodies to cyclin D1 and cyclin E in control rats and rats with Thy1 glomerulonephritis. No staining was present in paraffin embedded or frozen fixed tissue, and no protein was detected by Western blot analysis (results not shown). It is important to point out that these antibodies recognize cyclin D1 and cyclin E in rat mesangial cells *in vitro* by immunostaining and Western blot analysis (personal observations).

Expression and activity for CDK2 is increased in disease

We performed staining for CDK2 in control animals, and at each time point in disease, and did double-labeling with cell specific markers to identify which cell types expressed this antigen. Figure 5 shows quantitation for cells staining positive for CDK2 and OX-7 (CDK2+/OX-7+ cells), the latter which stains Thy1, and is specific for mesangial cells. In the normal rat glomerulus there was on average less than one CDK2+/OX-7+ cell per glomerulus, results similar to the PCNA staining. Figure 5 shows there was a substantial increase in CDK2+/OX-7+ cells (11.5 ± 2.17 vs. 44.33 ± 0.67 cells per 30 glomerular profiles) at day 2 of Thy1 ($P < 0.0001$), which coincided with the onset of MC proliferation. The increase in CDK2+/OX-7+ cells was maximal at day 3 (11.5 ± 2.17 vs. 76.33 ± 6.96; $P < 0.0001$), when mesangial cell proliferation also peaked. The double-staining for CDK2 and OX-7 is shown in Figure 6. By day 5 of Thy1, CDK2+/OX-7+ staining was absent, at which time mesangial cell proliferation was resolving.

Of the glomerular cells that stained for CDK2, 75% were mesangial cells (CDK2+/OX-7+ cell). Of the remaining cells that expressed CDK2 (CDK2+/OX-7− cells), 15% were macrophages (CDK2+/ED-1+), while the remainder were glomerular endothelial cells (CDK2+/RECA-1+) (results not shown). The pattern for CDK2 staining was similar if OX-7, ED-1 and RECA-1 were omitted. Nuclear immunostaining was absent when the antibody to cyclin-dependent kinase 2 was peptide absorbed, confirming antibody specificity.

To determine if the activity for CDK2 was also increased during

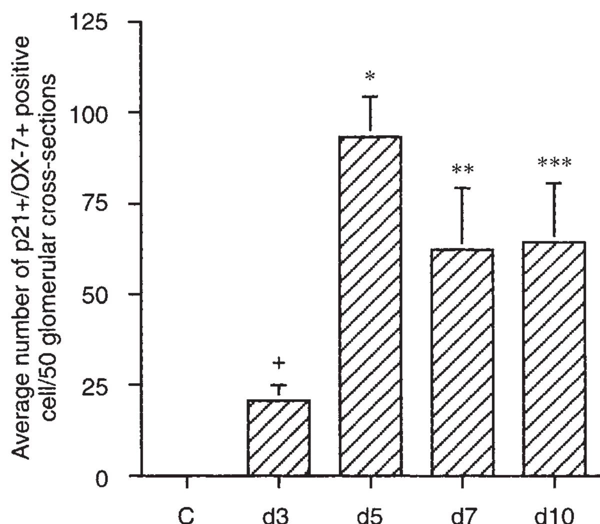

Fig. 10. *Quantitation of p21+/OX-7+ staining.* This figure shows the number of cells that double-stained for p21 and OX-7 (a marker of the mesangial cell) in fifty glomerular cross-sections from control rats (labeled as C) and at different days of Thy1 nephritis (days labeled as d). p21 is absent in the normal rat glomerulus, and is increased at day 3 of Thy1, with a peak at day 5, coinciding with the resolution of mesangial cell proliferation. p21 staining is present at days 7 and 10 of Thy1, when mesangial cell proliferation is absent. $^{+}P = 0.003$ vs. C; $^{*}P < 0.0001$ vs. C, $P < 0.003$ vs. d3; $^{**}P = 0.001$ vs. C, $P = 0.01$ vs. d3; $^{***}P = 0.0008$ vs. C, $P = 0.01$ vs. d3.

mesangial cell proliferation *in vivo*, a histone H1 kinase assay was performed on glomerular lysates. In the normal rat, no kinase activity was observed when CDK2 was immunoprecipitated with antibodies to cyclin E or cyclin A (Fig. 7). In contrast, MC proliferation at day 3 of Thy1 glomerulonephritis was associated with a dramatic increase in CDK2 activity when immunoprecipitated with an antibody to cyclin A (Fig. 7). The increase in kinase activity coincided with the increase in expression for CDK2. No kinase activity was present in Thy1 glomerulonephritis when CDK2 was immunoprecipitated with an antibody to cyclin E (Fig. 7), or when CDK2 was immunoprecipitated with pre-immune serum to cyclin A and cyclin E antibodies (results not shown).

Mesangial cell proliferation is associated with a decrease in the expression for $p27^{Kip1}$

Figure 8 shows a Western blot analysis on glomerular protein lysates with an antibody to $p27^{Kip1}$. $p27^{Kip1}$ is expressed abundantly in the normal quiescent glomerulus, and at day 1 and day 2 of Thy1 glomerulonephritis. However, there was a decrease in $p27^{Kip1}$ levels at day 3, coincident with mesangial cell proliferation, and the levels were difficult to detect at day 4. By day 5 of Thy1, $p27^{Kip1}$ protein levels were at baseline, and this remained at day 7. Specificity for the antibody to $p27^{Kip1}$ was confirmed using a $p27^{Kip1}$ peptide. Coommasie Blue staining of all the filters used in this study showed that protein loading was equal in the Western blots used in the study (results not shown).

Nuclear staining for $p27^{Kip1}$ was observed in mesangial cells (OX-7 positive), and also in glomerular endothelial (RECA-1 positive) and epithelial cells (results not shown). There was a progressive reduction in glomerular immunostaining for $p27^{Kip1}$ with the onset of Thy1 glomerulonephritis, with the maximal reduction in staining occurring at days 3 to 4 of disease, similar to that observed by Western blot analysis. All three glomerular cell types exhibited a similar pattern. There was a normalization of $p27^{Kip1}$ staining by day 7, which coincided with the resolution of mesangial cell proliferation (PCNA staining was at baseline levels). Nuclear immunostaining was absent when the antibody to $p27^{Kip1}$ was peptide absorbed, confirming antibody specificity (results not shown).

p21 expression increases, and is sustained in Thy1 glomerulonephritis

The protein expression for p21 was determined by immunostaining and Western blot analysis. We performed double immunostaining to determine which cell type expressed p21. Figure 9 shows that staining for p21 is absent in the normal quiescent rat glomerulus. Quantitation for cells staining positive for p21 and OX-7 (p21+/OX-7+) during Thy1 glomerulonephritis are shown in Figure 10. p21+/OX-7+ staining was first observed at day 3 (0 vs. 20.5 ± 4.3; $P = 0.15$ vs. control), and p21+/OX-7+ staining was maximal at day 5 (93.33 ± 11.05; $P < 0.0001$ vs. control). Of particular interest was that p21+/OX-7+ staining was also present at day 7 and day 10 of Thy1 glomerulonephritis, at which time no proliferation was detected. Double-immunostaining showed that p21 only colocalized with OX-7 (Fig. 9), but not with ED-1 or RECA-1 (results not shown). p21 was therefore only expressed by mesangial cells in this model. A common finding was that p21 staining was localized at the vascular pole of the glomerulus. Nuclear immunostaining was absent when the antibody to p21 was peptide absorbed, confirming antibody specificity. The pattern for p21 staining was similar if OX-7, ED-1 and RECA-1 were omitted.

Figure 11 shows a Western blot analysis for p21 performed on glomerular lysates in control rats, and rats with Thy1 nephritis. In the normal rat glomerulus, there is a very low basal expression for p21. However, there is an increase in p21 protein levels at day 3 of disease, and the increase was progressive at days 5, 7 and was still detected at day 10. Taken together, the increase in p21 expression coincides with the resolution of mesangial cell proliferation *in vivo*.

Discussion

The role of cell-cycle proteins in proliferation has been well documented in certain non-renal cells *in vitro* [8, 12]. However, the expression of cell-cycle regulatory proteins has not been described in the glomerulus, nor in renal disease *in vivo*. In the current study we demonstrate that mesangial cell proliferation *in vivo* in the Thy1 model of experimental mesangial proliferative glomerulonephritis is associated with an up-regulation of cyclin A, and an increase in expression and activity for CDK2. We also show that the normal glomerulus has high endogenous expression of the CKI, $p27^{Kip1}$, and that the levels decrease with the initiation of a MC proliferative response, while the resolution of MC proliferation is associated with the normalization of p27 levels. We also demonstrated that the CKI, p21, has low levels of expression in the normal rat glomerulus, but that resolution of mesangial cell proliferation is associated with an increase in p21 expression.

Quiescent cells *in vitro* do not express cyclins, but once stimulated to enter the cell cycle by extracellular signals such as

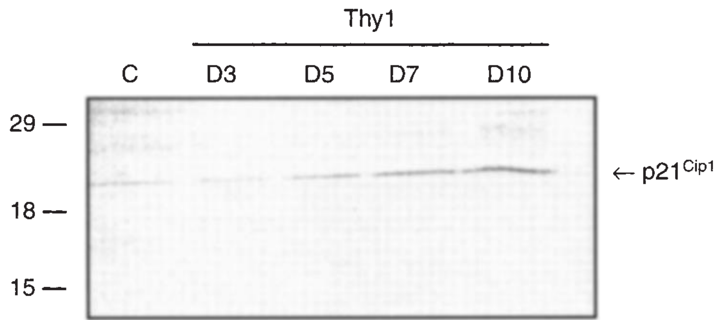

Fig. 11. *Western blot analysis for p21^{Cip1}.* Twenty micrograms of glomerular protein was loaded in each lane, and was separated on a 15% a SDS-PAGE, and immunoblotted with an antibody to p21^{Cip1}, which is represented by a protein of 21 kDa molecular weight (molecular size markers are in the left margin). Protein expression for p21^{Cip1} was barely detectable in control glomeruli (lane 1). There is an increase in p21^{Cip1} levels in Thy1 glomerulonephritis, first observed at day 3 (lane 2), which is sustained at days 5 (lane 3), day 7 (lane 4), and day 10 (lane 5) of Thy1 glomerulonephritis.

cytokines, cyclins are expressed and the levels oscillate throughout the cell-cycle [31]. We initiated our study by examining the expression of cyclins in normal rat glomeruli. Our first observation in the current study was that the normal quiescent glomerulus *in vivo* does not express the proteins for cyclins D1, E or A, results similar to *in vitro* studies in other cell lines [11, 31]. To study mesangial cell proliferation *in vivo* and in disease, we selected the Thy1 model of mesangial proliferative glomerulonephritis, where administration of the anti-Thy1 antibody results in an acute phase of complement-dependent mesangial cell lysis, followed by intense mesangial cell proliferation [22–24] and mesangial matrix accumulation [6]. Because the mesangial cell response to injury is uniform in the Thy1 model, most cells are typically synchronous in their proliferative response to injury and general changes in cell cycle proteins would be evaluated at the glomerular level. In addition, by double immunostaining for the cell-cycle proteins with mesangial cell, glomerular endothelial cell and macrophage markers, it was possible to specifically determine changes in cell cycle proteins in the individual cell populations.

In the current study, mesangial cell proliferation was associated with a substantial up-regulation in expression for cyclin A. The increase in cyclin A coincided with the onset of mesangial cell proliferation in this model. We were unable to determine any increase for cyclin D1 or E despite employing numerous different staining methods. The antibodies to cyclins D1 and E used in this study do recognize the rat antigens in mesangial cells in culture (personal observation). However, we recognize that our inability to detect these proteins may be due to low levels of expression for these cell cycle proteins, their short half lives or because we did not examine them at the time when they may have been increased.

In non-renal cells *in vitro*, the protein levels for cyclin dependent kinases (CDKs) are typically constant throughout the cell cycle, and are regulated post-translationally [32]. CDKs are activated by complexing to cyclins [33]. There is a very low cell turnover in the normal rat glomerulus [30] In the normal rat glomerulus, we observed that there were few cells that stained positive for CDK2. However, our second observation in the current study was that MC proliferation in Thy1 glomerulonephritis was associated with a dramatic increase in the mesangial cell expression for CDK2. The increase was first observed at day 2 which coincides with the onset of mesangial cell proliferation, and was maximal at day 3, coinciding with peak PCNA staining. The increase in CDK2 levels in this *in vivo* model differs therefore from the *in vitro* observations where the levels for CDK2 usually remain constant throughout the cell cycle. We have also observed that the levels for CDK2 increase during rat mesangial cell proliferation *in vitro* (unpublished observations). The increase in cyclin A and CDK2 staining observed in the current study is not due to a general increase in cell number, as there is only a twofold increase in glomerular cell number in the Thy1 model, while the changes for the cell cycle proteins were greater than this. Although PCNA staining peaked at day 3 of Thy1 in the current study, staining persisted at day 5, at which time no cyclin A or CDK2 staining was observed. There are a few possible reasons to explain why there is PCNA but not cyclin A/CDK2 staining at day 5. First, PCNA is a nuclear protein that is also expressed from late G1 phase to the M phase, whereas cyclin A and CDK2 are G1/S proteins [34], and second, PCNA has a half life exceeding that of the G1/S phase cell cycle proteins measured in this study [8, 34].

An increase in expression for CDK2 does not necessarily correlate with kinase activity because activation of kinase activity for each CDK is dependent on complexing with specific cyclins, events that correlate with progression through the cell cycle [35]. Cyclin E and cyclin A associate with CDK2 [36], and cyclin A-CDK2 complex is necessary for continued DNA replication [13]. We next determined if the increased glomerular expression of CDK2 was associated with an increase in kinase activity *in vivo*. Kinase activity of CDK2 was absent in the normal glomerulus, similar to that shown in quiescent cells *in vitro*. However, there was a marked increase in glomerular kinase activity for CDK2 in Thy1 glomerulonephritis. Immunoprecipitation studies showed that the increase was due to CDK2 complexing with cyclin A, and not cyclin E. Because 25% of the cells that stained for CDK2 in this study were not mesangial, it is possible that these cells may have contributed to the kinase activity observed in Thy1 nephritis.

A characteristic of many types of glomerular disease, including Thy1 glomerulonephritis, is the presence of proliferating macrophages in the glomerulus [37]. It was therefore not surprising that cyclin A and CDK2 staining were observed in this cell type in the current study. We have recently shown that there is early capillary repair and angiogenesis in Thy1 [38]. An increase in glomerular endothelial cell expression for cyclin A and CDK2 in the current study was also not surprising.

The cyclin kinase inhibitors (CKI) p21 (also known as Cip1, Waf1, Sdi) [18] and p27^{Kip1} [17] are negative regulators of the cell-cycle, and play a critical role in regulating cell proliferation. p21 and p27^{Kip1} prevent cell cycle progression by inhibiting cyclin D-CDK4, cyclin E-CDK2 and cyclin A-CDK2 complexes [15]. However, the expression of these CKI may be cell type specific, and their levels are also determined by their proliferative state [40–42]. For example, resting macrophages [39] and fibroblasts [40] contain high levels of p27^{Kip1} that decline when these cells are stimulated to proliferate. In contrast, Mv1Lu lung epithelial and human keratinocytes retain a high level of p27^{Kip1} in their

proliferative state [41]. The expression for p21 is absent in quiescent fibroblasts and levels increase during proliferation [40], while p21 levels are high in resting HCA2 cells [42]. Furthermore, a specific cytokine can alter the expression of cell cycle proteins differently in different cell types. For example, TGF-β1 increases the expression of p15 in keratinocytes [16], reduces the levels of CDK4 in others [43], inhibits CDK2 complexes [44, 45] by mobilizing p27 in Mv1Lu mink lung epithelial cells [17], and increases the expression of p21 in the OVCA420 cell line [46].

Our third observation was that $p27^{Kip1}$ is endogenously expressed at high levels in the normal quiescent rat glomerulus, and was present in mesangial cells, and in glomerular endothelial and epithelial cells. Mesangial cell proliferation in Thy1 glomerulonephritis was associated with a decrease in p27 expression, which was marked at day 3 and day 4 of Thy1, when MC proliferation was maximal. Resolution of mesangial cell proliferation was associated with the return to baseline expression for $p27^{Kip1}$ (p27). One may speculate that the $p27^{Kip1}$ expression may help explain in part the low cell turnover that has been observed in the normal rat glomerulus [30]. Although most of the glomerular cell proliferation in this model represents the mesangial cell, there is also proliferation of glomerular endothelial cells that occurs [38]. The decrease in glomerular p27 levels would be consistent with a role for stimulating mesangial and endothelial cell proliferation in this model.

Our fourth finding was that there is a differential expression for the CKIs, p21 and p27, in control and disease. In the normal quiescent rat glomerulus we could not detect staining for p21, which contrasted with the high endogenous expression for p27. While the levels for p27 were decreased during mesangial cell proliferation *in vivo*, there was a marked increase in p21 protein expression by the mesangial cells. Unlike other cell cycle proteins in this study, p21 was only expressed by the mesangial cell during Thy1 glomerulonephritis. The peak in p21 staining coincided with the resolution of mesangial cell proliferation *in vivo*. Furthermore, at day 7 and day 10 of Thy1 where MC proliferation was absent, p21 was still expressed by mesangial cells. The significance of p21 commonly localizing to the vascular pole of the glomerulus is not known. This differs from that described *in vitro* studies in non-renal cells, where p21 levels increase during proliferation, but are absent during quiescence [40]. The reason for the sustained increase is unknown. p21 prevents cell proliferation in two ways. First the N terminus of p21 binds to and inhibits cyclin-CDK complexes [47], and second the C-terminus inhibits proliferating cell nuclear antigen (PCNA) [48], an auxiliary protein to DNA polymerase. A role for p21 *in vivo* was recently shown where the overexpression of p21 prevented vascular smooth muscle cell proliferation in a carotid model of balloon angioplasty [49]. Our results suggest that p21 may be important for the resolution of MC proliferation in the Thy1 model.

The differential expression for the CKI in the Thy1 model emphasizes that not all CKI behave similarly, and that specific CKI may have function in individual cell types or initiating or resolving cell proliferation. This has been suggested in previous studies in which mutations in certain CKI (for example, p16) predispose to cancers in certain cell lineages (for example melanoma), as opposed to a general increase in cancers in all cell types [50]. Furthermore, the studies reported herein again emphasize that *in vivo* observations do not always reproduce what is observed *in vitro*. Thus, the demonstration that *in vivo* mesangial cell proliferation is associated with increased expression of CDK2 has not been observed in non-renal cells *in vitro* [10]. Second, the sustained increase in p21 in mesangial cells *in vivo* even after proliferation has subsided differs from the transient expression of p21 that occurs during non-renal cell proliferation *in vitro* [40].

In the current study we did not determine the mechanisms responsible for the changes in cell-cycle regulatory protein expression that accompanies mesangial cell proliferation *in vivo*. *In vitro* studies have shown that positive and negative regulatory cell-cycle proteins are regulated by various cytokines. Mesangial cell proliferation in the Thy1 model is induced by PDGF and bFGF [51]. TGF-β1 is also increased in this model [52], but the role of this cytokine in regulating MC proliferation during Thy1 nephritis is unknown. The changes observed in cell-cycle protein expression in the current study may be due to these and other cytokines. In conclusion, we have shown that certain cell-cycle regulatory proteins are expressed during mesangial cell proliferation *in vivo*. Further studies are needed to determine their functional role.

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