

Direct in vivo Inhibition of the Nuclear Cell Cycle Cascade in Experimental Mesangial Proliferative Glomerulonephritis with Roscovitine, a Novel Cyclin-dependent Kinase Antagonist

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Abstract

Glomerular injury is characterized by mesangial cell (MC) proliferation and matrix formation. We sought to determine if reducing the activity of cyclin-dependent kinase 2 (CDK2) with the purine analogue, Roscovitine, decreased MC proliferation in vitro and in vivo. Roscovitine (25 μ M) inhibited FCS-induced proliferation ($P < 0.0001$) in cultured MC. Rats with experimental mesangial proliferative glomerulonephritis (Thy1 model) were divided into two groups. A prevention group received daily intraperitoneal injections of Roscovitine in DMSO (2.8 mg/kg) starting at day 1. A treatment group received daily Roscovitine starting at day 3, when MC proliferation was established. Control Thy1 rats received DMSO alone. MC proliferation (PCNA+OX7+ double immunostaining) was reduced by $> 50\%$ at days 5 and 10 in the Roscovitine prevention group, and at day 5 in the treatment group ($P < 0.0001$). Early administration of Roscovitine reduced immunostaining for collagen type IV, laminin, and fibronectin at days 5 and 10 ($r = 0.984$; $P < 0.001$), which was associated with improved renal function (urinary protein/creatinine, blood urea nitrogen, $P < 0.05$). We conclude that reducing the activity of CDK2 with Roscovitine in experimental glomerulonephritis decreases cell proliferation and matrix production, resulting in improved renal function, and may be a useful therapeutic intervention in disease characterized by proliferation. (*J. Clin. Invest.* 1997. 100:2512–2520.) Key words: cyclins • Roscovitine • kidney • proliferation • cell cycle

Introduction

Glomerular disease is a major cause of end-stage kidney disease. In glomerulonephritis, injury to the mesangial cell (MC)¹ results in MC proliferation (1). MC proliferation in turn is

tightly linked with mesangial matrix expansion, and mechanisms that inhibit this proliferative response can markedly reduce matrix expansion (1), suggesting that this cascade may be a future therapeutic approach to these diseases.

Cell proliferation is ultimately governed at the level of the cell cycle by cell cycle regulatory proteins (2, 3). Under appropriate stimuli, quiescent cells engage the cell cycle at early G1, and transit through the cell cycle as a consequence of activation of specific cyclin-dependent kinases by cyclins (4, 5). Thus, D-type cyclins bind to CDK4-6 (6), cyclin E binds CDK2 (7), cyclin A binds CDK2 and cdc2 (8), and cyclin B binds to cdc2 (9). CDK2 is required for G1/S transition and DNA synthesis (10). Two families of cyclin-dependent kinase inhibitors (CKI) regulate cyclin-CDK complexes (reviewed 11). Our knowledge of the cell cycle, however, has been largely derived from in vitro studies, and less is understood about the role of specific cell cycle proteins in inflammatory disease in vivo.

Experimental mesangial proliferative glomerulonephritis induced by antibody to Thy1 has been extensively studied as a model of mesangial cell proliferation that occurs as a consequence of immune injury (12, 13). Binding of the Thy1 antibody to the mesangial cell causes mesangiolysis (12, 13) followed by a marked and excessive mesangial cell proliferative response, which is PDGF- and bFGF-dependent (14). Furthermore, mesangial cell proliferation in this and other models of experimental glomerular disease is linked to overproduction of matrix proteins (for review see reference 1). We have recently shown that the onset of mesangial cell proliferation in Thy1 glomerulonephritis is associated with an increase in the expression and activity of cyclin A-CDK2 complex (15). Furthermore, the increase in CDK2 activity was associated with a decrease in levels of the CKI p27^{Kip1} (15). Similar findings were shown during mesangial cell proliferation in culture induced by PDGF and bFGF (16).

Attempts have been made to reduce cell proliferation in vitro by interrupting specific cell cycle proteins, and studies have focused on inhibiting cyclin-CDK complexes because they are required for DNA synthesis (for review see references 17–19). Activity of cyclin-CDK complexes can be regulated at different levels, including changes in cyclin levels (20), altering the phosphorylation status of CDKs, or binding specific cyclin kinase inhibitors (18, 21). Our previous studies support a central role for CDK2 in mesangial cell proliferation in disease, and raise the possibility that selectively inhibiting CDK2 might have a beneficial effect in disorders characterized by cell proliferation. In this study we used Roscovitine, a purine analogue that inhibits the activity of CDK2, CDK5, and cdc2 (17) in certain nonrenal cells in vitro by binding to the ATP-binding pocket of CDKs (22) without changing the levels of these cell cycle proteins (23). Our results provide the first data available on the effects of blocking CDK2 activity in a disease process in vivo, and document a dramatic effect of CDK

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1. Abbreviations used in this paper: CDK2, cyclin-dependent kinase 2; MC, mesangial cell; PCNA, proliferating cell nuclear antigen.

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inhibition on renal function in experimental mesangial proliferative glomerulonephritis.

Methods

Cell culture. To determine if Roscovitine reduced rat mesangial cell proliferation *in vitro*, and to determine a concentration of Roscovitine that could be used for *in vivo* studies, early (4–10) passaged rat mesangial cells were plated in 15% FCS, and were allowed to attach overnight as described elsewhere (16). MC were washed three times with HBSS, and growth arrest was achieved by serum starvation for 48 h. MC were then stimulated for 18 h with either 15% FCS or 15% FCS with Roscovitine at concentrations of 7.5, 12.5, or 25 μ M. DNA synthesis was measured at 18 h by a 4-h pulse of [3 H]thymidine incorporation into DNA as described elsewhere (16). Pilot studies were undertaken to ensure that DMSO, the solvent in which Roscovitine was dissolved, did not interfere with DNA synthesis. To examine CDK2 activity and the levels of S phase cell cycle proteins, nuclear protein was extracted from MC as previously described (16) at 0, 6, 12, and 18 h after stimulation with 15% FCS or 15% FCS and Roscovitine (7.5, 12.5, or 25 μ M). To ensure that Roscovitine did not affect mesangial cell viability, the exclusion of Trypan blue was performed at each concentration of Roscovitine. Serum-starved mesangial cells were used as a positive control.

Animal model and experimental design. To determine if glomerular injury was reduced when the activity of CDK2 was inhibited in inflammatory disease by Roscovitine, experimental mesangial proliferative glomerulonephritis (Thy1 model) was induced in Wistar rats (180–200 g; Simonsen Laboratories, Gilroy, CA) by a single injection of goat anti-Thymocyte antibody (400 μ l/100 g body weight) as described elsewhere (15, 24). In this animal model, mesangial cell proliferation begins at day 2, peaks at day 5, and resolves by days 10–14 (25). Pilot studies were performed to determine the optimal concentration of Roscovitine required to reduce mesangial cell proliferation in Thy1 nephritis, to determine the side effect profile, and to ensure that DMSO, the vehicle for Roscovitine, did not alter the course, severity, or renal function of rats with Thy1 glomerulonephritis. Based on these pilot studies, Roscovitine was given as a single daily intraperitoneal injection at a concentration of 2.8 mg/kg body weight in a total volume of 400 μ l DMSO. Control rats with Thy1 glomerulonephritis received daily intraperitoneal injections of DMSO (400 μ l) (Sigma Chemical Co., St. Louis, MO).

Rats with Thy1 glomerulonephritis were divided into two groups: (a) group I, or the prevention group, was studied to determine if reducing CDK2 activity with Roscovitine before the onset of maximal mesangial cell proliferation would reduce mesangial cell proliferation and glomerular injury at days 3, 5, and 10. Roscovitine or DMSO was first given 24 h after the administration of the Thy1 antibody, and daily thereafter for 4 d. Renal biopsies were performed at day 3 ($n = 4$ in each group), day 5 ($n = 8$) and day 10 ($n = 7$) of Thy1 glomerulonephritis; (b) group II, or the treatment group, was studied to determine if the magnitude of mesangial cell proliferation and glomerular injury could be reduced at day 5 and day 10 by inhibiting CDK2 activity with Roscovitine after mesangial cell proliferation had already been established. Rats with Thy1 glomerulonephritis received Roscovitine or DMSO on days 3, 4, and 5 after disease induction, and were killed at day 5 ($n = 8$) and day 10 ($n = 5$).

Before death, a 24-h urine collection for creatinine and protein measurements was obtained from each rat as described elsewhere (26, 27). At death, blood was collected to measure BUN (28), and total complement activity by CH50 (29) and renal biopsies were obtained and fixed in methyl Carnoy's, formalin or OCT and snap frozen for immunostaining (15).

To determine the glomerular kinase activity of CDK2, a separate group of Thy1 rats received either daily injections of Roscovitine ($n = 4$), DMSO ($n = 4$), or nothing ($n = 4$), and were killed at day 4. Glomeruli were isolated from the renal cortex, and glomerular protein was extracted as reported elsewhere (15).

Immunohistochemistry. Renal biopsies were fixed in methyl Carnoy's solution, embedded in paraffin, and cut into 5- μ m sections for indirect immunoperoxidase staining as described elsewhere, using diaminobenzidine with nickel as a chromagen (15). A murine monoclonal IgM antibody against proliferating cell nuclear antigen (PCNA) (19A2; Coulter Immunology, Hialeah, FL) was used to determine DNA synthesis, and double immunostaining with OX-7 (Serotec Ltd., Oxford, United Kingdom), a cell marker specific for mesangial cells, was performed as previously described (15, 30). Immunostaining for matrix proteins was conducted with polyclonal antibodies to collagen type IV (Southern Biotechnology Associates, Inc., Birmingham, AL), laminin (Chemicon International, Inc., Temecula, CA) and fibronectin (Chemicon International, Inc.). To quantitate nonresident cells infiltrating the glomerulus, staining was performed with antibodies to platelets (PL-1; gift of W.W. Bakker, Groningen, The Netherlands) and monocyte/macrophages (ED-1; Serotec Ltd.). Controls included omitting the primary antibody and substituting the primary antibody with an irrelevant antibody of the same IgG class. The glomerular expression of PCNA, matrix proteins, and infiltrating cells was graded semiquantitatively on fifty consecutive glomerular cross-sections from each biopsy in individual rats in a blinded fashion as previously described (15, 31). The number of proliferating mesangial cells was evaluated by counting the number of cells that stained for both PCNA and OX-7 (PCNA+/OX7+ cells), and was expressed as a mean \pm SEM per glomerular cross-section. For the matrix proteins and platelets, scoring reflected the distribution in staining as follows: 0, absent mesangial staining; 1+, up to 25% of glomerular tuft positive; 2+, 25–50% of glomerular tuft with focal strong staining; 3+, 50–75% of glomerular tuft with strong staining; 4+, > 75% of glomerular tuft with increased staining. For each biopsy, a mean score was calculated to generate a mean and SEM for each group.

To examine the glomerular binding of the Thy1 antibody and local complement activation, detection of goat IgG, C3, and C5b-9 in glomeruli was carried out on 4- μ m sections of ether/alcohol fixed frozen kidney tissue using direct (C3, goat IgG) or indirect (C5b-9) immunofluorescence staining as described elsewhere (32). Sections were studied with fluorescein-conjugated IgG fractions of monospecific antisera to goat IgG and rat C3 (Cappel Laboratories, Cochranville, Pennsylvania) and biotinylated 2A1, a murine monoclonal antibody to a neoantigen of rat C5b-9 (25) followed by fluorescein-conjugated streptavidin (Amersham Corp., Arlington Heights, IL), and quantitation was performed as described for matrix proteins above. Total serum complement was measured by CH50 as previously described (29). In brief, serum from Thy1 rats and Thy1 rats given DMSO or Roscovitine was diluted and exposed to sheep red blood cells sensitized with antiserum hemolysin. Hemolysis was measured by spectrometry, and was compared with a pool of rat serum known to have normal complement activity. The results were expressed as a percentage of the positive control. Quantitation of glomerular cellularity was performed on periodic acid-Schiff (PAS)-stained kidney sections by counting the number of cells in 20 consecutive glomerular tufts from individual animals.

Histone H1 kinase assay and Western blot analysis. Protein was extracted from mesangial cells *in vitro* (16) and from isolated glomeruli *in vivo* (15) using a buffer containing 1% triton, 10% glycerol, 20 mM Hepes, 100 mM NaCl with 10 μ g/ml Leupeptin, 10 μ g/ml Antipain, 10 μ g/ml Pepstatin (Sigma Chemical Co.), 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride. The activity of cyclin-dependent kinase 2 was determined by the Histone H1 kinase assay as previously described (15). In brief, 500 μ g of protein extract was immunoprecipitated with an antibody to CDK2 (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C. 50 μ l of Protein A sepharose beads (Repligen, Cambridge, MA) were added to each immunoprecipitation and incubated for 30 min at 4°C. Negative controls included substituting preimmune rabbit serum for the primary antibodies. Recombinant cyclin A-CDK2 (provided by J.M. Roberts, Fred Hutchinson Cancer Research Center, Seattle, WA) were used as positive controls. Histone H1 (Boehringer Mannheim, Indianapolis,

lis, IN), ATP (Pharmacia LKB Biotechnology, Piscataway, NJ) and [32 P]dCTP (DuPont-NEN, Boston, MA) were added for a 60-min incubation at 37°C. Reduced sample buffer was then added, and the mix was separated on a 15% SDS-PAGE. The gel was exposed to autoradiographic film (Amersham Corp., Arlington Heights, IL), and quantitation was performed by densitometry. Histone H1 assays were performed in duplicate.

For Western blot analysis, 40 μ g of protein extract was separated under reducing conditions on a 15% SDS-PAGE gel and transferred to PVDF membranes (Millipore Corp., Bedford, MA) by electroblotting for incubation with the antibodies to cyclin A and CDK2 (Santa Cruz Biotechnology). An alkaline phosphatase-conjugated secondary antibody (Promega Corp., Madison, WI) was used for detection with the chromagen 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Chemical Co.). Controls included omitting the primary antibody and peptide absorption. Western blots were performed in duplicate.

Statistical analysis. Results in this study and scores for immunohistochemical staining were expressed as mean \pm SEM unless stated otherwise. Statistical significance (defined as $P < 0.05$) was evaluated by use of the Student's *t* test or one-way ANOVA with modified *t* test performed with the Bonferroni correction.

Results

Roscovitine inhibits CDK2 activity and mesangial cell proliferation in vitro. Fig. 1 shows the kinase activity of CDK2 in mesangial cells in vitro as measured by a histone H1 kinase assay. CDK2 activity was absent in serum-starved mesangial cells, and increased threefold after 18 h of 15% FCS stimulation. Roscovitine caused a dose-dependent reduction of CDK2 activity. Thus, at concentrations of 7.5 and 12.5 μ M, Roscovitine caused a 25 and 50% decrease in CDK2 activity, respectively. At a concentration of 25 μ M, Roscovitine completely inhibited CDK2 activity, which was similar to the CDK2 activity in serum-starved mesangial cells.

DNA synthesis was measured by [3 H]thymidine incorporation into DNA, and the results are shown in Fig. 2. There was a threefold increase in DNA synthesis when serum-starved mesangial cells were stimulated with FCS ($P < 0.0001$). Fig. 2 shows that Roscovitine caused a concentration-dependent decrease in DNA synthesis so that DNA synthesis was significantly decreased by 33, 71, and 100% at 7.5, 12.5, and 25.5 μ M of Roscovitine, respectively. Roscovitine-treated cells excluded

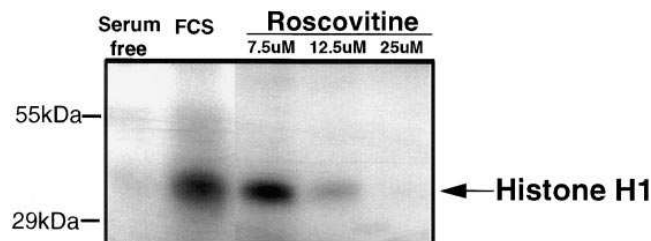


Figure 1. Histone H1 assay for mesangial cells in vitro. CDK2 kinase activity was measured by a Histone H1 assay, where 500 μ g of MC protein at each time point was immunoprecipitated with an antibody specific for CDK2. CDK2 activity was barely detected in serum-starved MC (lane 1). FCS-induced MC proliferation was associated with a threefold increase in CDK2 activity (lane 2). Roscovitine caused a concentration-dependent decrease in CDK2 activity. At 7.5 μ M (lane 3), 12.5 μ M (lane 4), and 25 μ M (lane 5), the activity was reduced by 50, 75, and 100%, respectively.

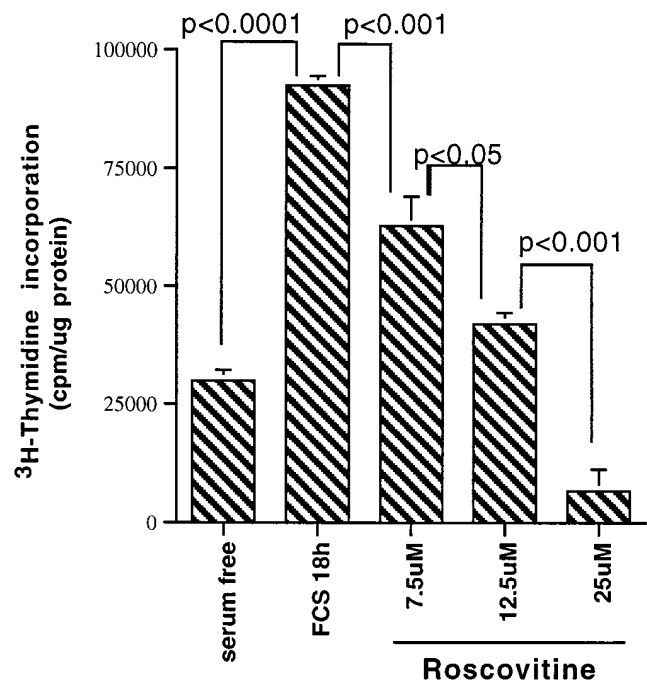


Figure 2. Roscovitine inhibits DNA synthesis in mesangial cells in vitro. The incorporation of [3 H]thymidine into DNA was used to measure DNA synthesis in rat MC, and this incorporation was corrected for cell protein. There was a threefold increase in DNA synthesis when serum-starved MC were stimulated for 18 h with 15% FCS (lane 2). Roscovitine decreased DNA synthesis at a concentration of 7.5 μ M (lane 3), 12.5 μ M (lane 4) and 25 μ M (lane 5).

trypan blue, showing that Roscovitine at the concentrations used in this study did not affect mesangial cell viability.

To ensure that the reduction in CDK2 activity and the decrease in DNA synthesis caused by Roscovitine was not due to a decrease in the levels of the G1/S cyclins that we have previously shown to be required for mesangial cell proliferation, Western blot analyses were performed on mesangial cell protein. At each concentration of Roscovitine used in this study, the protein levels of cyclin E, cyclin A, or CDK2 were not altered (results not shown).

Roscovitine does not affect other variables known to modulate severity of mesangial proliferative glomerulonephritis. Pilot studies showed that daily intraperitoneal injections of Roscovitine in excess of 3.0 mg/kg body weight or more caused diarrhea. At 2.8 mg/kg body weight, the concentration of Roscovitine used in this study, all rats were grooming, active, looked healthy, did not lose weight, and did not develop peritonitis, ascites, or diarrhea. The Thy1 antibody binds maximally within 1 h (13). To ensure that Roscovitine or DMSO did not interfere with other variables shown in previous studies to determine the severity of the Thy1 model (antibody deposition, complement activation, platelet and macrophage infiltration), however, immunostaining was performed on frozen tissue sections with antibodies to goat IgG and C5b-9, and total complement levels were measured by CH50. There was no difference in the glomerular distribution or intensity of staining for goat IgG (see Fig. 10) and C5b-9 in rats treated with Roscovitine or DMSO compared to Thy1 rats that did not receive these re-

Table I.

	Thy1	Thy1 + DMSO	Thy1 + Roscovitine
Goat IgG staining	4+	4+	4+
C5b-9 staining	4+	4+	4+
No. ED1+/PCNA-			
cells/glomerular area profile	5.5±1.0	5.7±1.02	5.3±0.75
No. ED1+/PCNA+			
cells/glomerular area profile	9.2±1.4	10.6±1.9	8.2±2.9
Platelets	3+	3+	3+
CH50	83.5±11	93±31	79±15
No. cells/glomerular tuft	105±10.5	101.5±9.1	*54.85±6.7

The results of the quantitation for immunostaining for goat IgG, C5b-9, platelets, and nonproliferating macrophages (ED1+/PCNA-), proliferating macrophages (ED1+/PCNA+), platelets, total serum complement (CH50), and the number of cells per glomerular tuft are shown in unmanipulated Thy1 rats, Thy1 rats given DMSO and Thy1 rats given Roscovitine **P* < 0.05.

agents (Table I). There was also no difference in CH50 levels in Thy1 rats given Roscovitine or DMSO Thy1 compared to unmanipulated Thy1 rats (Table I). Table I also shows that the number of glomerular platelets and macrophages were not different in Thy1 rats given Roscovitine compared to Thy1 rats given DMSO and unmanipulated Thy1 rats.

Fig. 3 shows the results of the CDK2 kinase activity in glomeruli isolated from normal and Thy1 rats. CDK2 kinase activity was absent in normal rats, but CDK2 activity was increased at day 4 in Thy1 rats, and in Thy1 rats given DMSO. In contrast, Roscovitine decreased CDK2 activity in rats with Thy1.

Roscovitine decreases mesangial cell proliferation in experimental glomerulonephritis. Glomerular mesangial cell proliferation

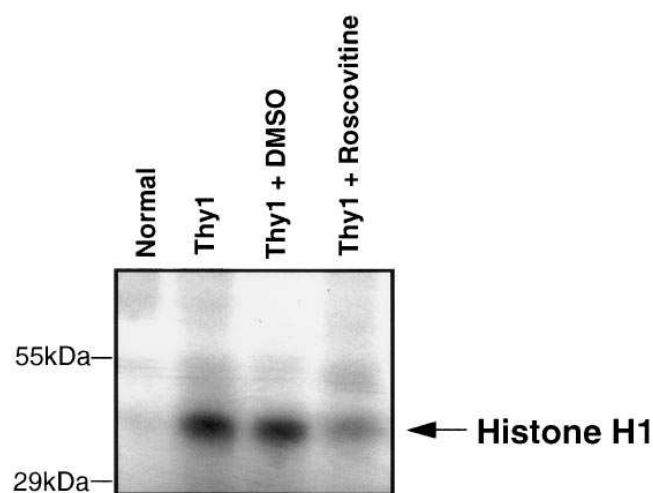


Figure 3. Histone H1 kinase assay in rats with Thy1 glomerulonephritis. CDK2 kinase activity was performed on 500 µg of protein from isolated glomeruli. CDK2 activity was barely detectable in normal rat glomeruli (lane 1), but increased substantially in Thy1 rats (lane 2) and Thy1 rats given DMSO (lane 3). In contrast, CDK2 activity was reduced in Thy1 rats given Roscovitine (lane 4).

was measured by double immunostaining with an antibody to PCNA, a marker of DNA synthesis, and an antibody to OX-7, a marker specific for mesangial cells. Thus, proliferating mesangial cells were identified as PCNA+/OX7+. The effects of Roscovitine on mesangial cell proliferation in the prevention group are shown in Fig. 4, and a representative double immunostain for PCNA and OX7 is shown in Fig. 5. Fig. 4 shows that in Thy1 rats not given Roscovitine or DMSO there was a 12-fold increase in the number of cells staining positive for PCNA+/OX7+ at day 3, and a 20-fold increase at day 5 compared to normal rats. Mesangial cell proliferation had largely resolved by day 10. Similar results were obtained at days 3, 5, and 10 when DMSO was given to Thy1 rats from day 1 (prevention group; Fig. 4). In contrast, Fig. 4 shows that rats with Thy1 glomerulonephritis getting Roscovitine from day 1 of disease, but before the onset of proliferation (prevention group), had a > 50% reduction in mesangial cell proliferation at days 3 and 5 compared to rats given DMSO. The reduction was sustained at day 10, where the number of cells staining positive for PCNA+/OX7+ in the Roscovitine group was similar to normal rats, which was significantly less than Thy1 rats given DMSO. Thus, the magnitude of mesangial cell proliferation was less at each time point studied when Roscovitine was given from day 1 of disease.

The reduction in glomerular cell proliferation in Thy1 rats given Roscovitine from day 1 (prevention group) was associated with a decrease in glomerular cellularity at days 5 (Table

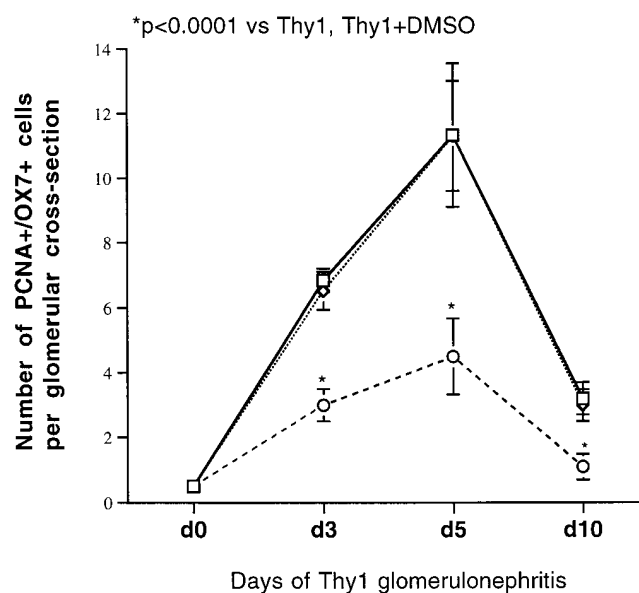


Figure 4. Quantitation of mesangial cell proliferation in rats given Roscovitine at day 1 of Thy1 glomerulonephritis (prevention group). Proliferating MC were identified by cells that stained positive for both PCNA (a marker of DNA synthesis) and OX7 (a marker of MC) (PCNA+/OX7+). At days 3, 5, and 10, there was no difference in the number of PCNA+/OX7+ cells when rats with Thy1 glomerulonephritis were given nothing or given DMSO, the vehicle for Roscovitine, from day 1 of disease. Roscovitine given daily from day 1 caused a > 50% decrease in the number of PCNA+/OX7+ cells per glomerular cross-section at days 3, 5, and 10 of disease. Open squares, Thy1; open diamonds, Thy1 + DMSO; open circles, Thy1 + Roscovitine.

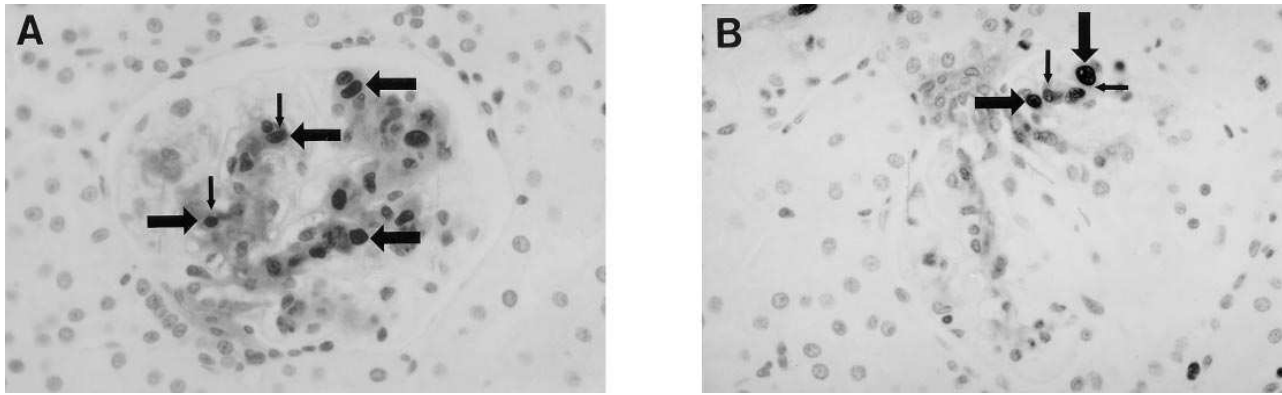


Figure 5. Double immunostain for PCNA and OX7 at day 5 of Thy1 glomerulonephritis. Proliferating cells were identified by nuclear staining for PCNA, and examples are shown by the *broad arrows*. Double immunostaining with OX7 was used to identify mesangial cells, and examples of this cytoplasmic stain are shown by the *thin arrows*. Proliferating mesangial cells double immunostain for PCNA and OX7. Thy1 rats given Roscovitine from day 1 (prevention group) had a marked decrease in the number of proliferating mesangial cells (*B*) compared to Thy1 rats given DMSO (*A*).

I) and 10, and a representative PAS stain is shown in Fig. 6. Furthermore, there was no difference in the number of non-proliferating macrophages (ED1+/PCNA– staining) in Roscovitine-treated Thy1 rats compared to control (Table I), and Roscovitine did not affect the number of proliferating macrophages (ED1+/PCNA+ staining) in Thy1. Thus, the reduced glomerular cellularity in Roscovitine-treated animals was due to a decrease in resident glomerular rather than infiltrating cells.

Mesangial cell proliferation was also measured in Thy1 in rats given Roscovitine from day 3 of disease (treatment group) once proliferation was established; the results are shown in Fig. 7. Roscovitine reduced the number of cells staining positive for PCNA+/OX7+ by 50% at day 5 of Thy1 glomerulonephritis compared to rats given DMSO. The number of cells staining positive for PCNA+/OX7+, however, was lower at day 5 when Roscovitine was given from day 1 (prevention group) compared to when Roscovitine was given from day 3 (treatment group).

Inhibiting CDK2 activity is associated with a decrease in matrix proteins. The matrix proteins collagen type IV, laminin, and fibronectin were measured because mesangial matrix ex-

pansion is linked to cell proliferation, and probably is a consequence of it (13, 14). Fig. 8 shows representative staining for collagen type IV and laminin, and the quantitation for immunostaining is shown in Fig. 9. Similar to previous studies of Thy1 glomerulonephritis, Thy1 rats given DMSO had a substantial increase in immunostaining for these three matrix proteins at day 5, and the increase was sustained at day 10 (Fig. 9). In Thy1 rats given Roscovitine from day 1 (prevention group), there was a marked decrease in glomerular immunostaining for collagen type IV, laminin, and fibronectin at day 5 by 30, 30, and 53% respectively (Fig. 9). The decreased immunostaining for collagen type IV, laminin, and fibronectin was sustained at day 10 of disease in rats given Roscovitine from day 1. Furthermore, the decrease in mesangial cell proliferation at day 3 in rats given Roscovitine correlated with the reduction in immunostaining for collagen type IV, laminin, and fibronectin at days 5 and 10 ($r = 0.94$, $P < 0.001$ for each matrix protein studied).

In contrast, although there was a trend towards a decrease in collagen type IV (3.04 ± 0.32 vs. 2.46 ± 0.21 ; $P > 0.05$), laminin (3.0 ± 0.32 vs. 2.24 ± 0.2 ; $P > 0.05$), and fibronectin (2.9 ± 0.3 vs. 2.39 ± 0.41 ; $P > 0.05$), when Roscovitine was first given to

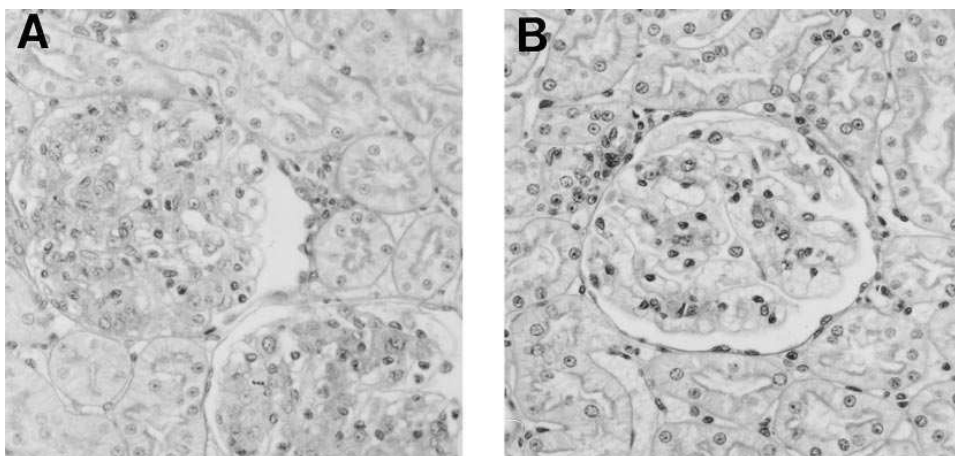


Figure 6. PAS staining in Thy1 glomerulonephritis given Roscovitine from day 1 (prevention group). (*A*) There was a marked increase in glomerular cellularity and matrix accumulation at day 5 of Thy1 glomerulonephritis in rats given DMSO from day 1. (*B*) Roscovitine decreased glomerular cellularity and matrix proteins at day 5 when given to rats from day 1 (prevention group).

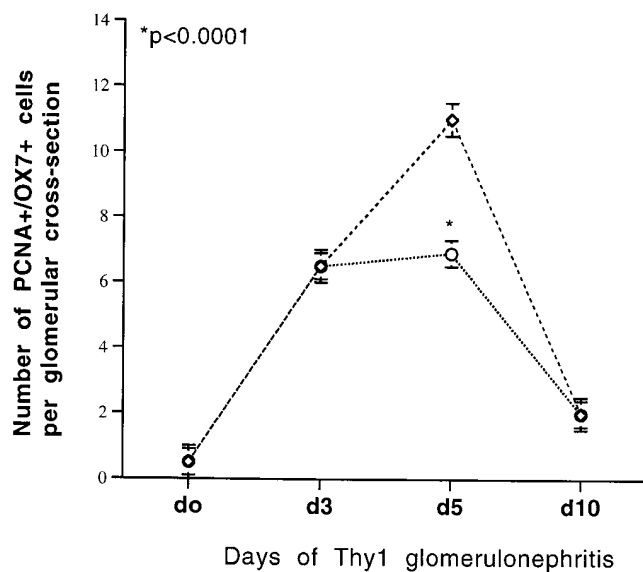


Figure 7. Quantitation of mesangial cell proliferation in Thy1 rats given Roscovitine from day 3 (treatment group). There was a progressive increase in the number of proliferating mesangial cells, identified as cells that stained for PCNA and OX7, a marker of mesangial cells, in Thy1 rats given DMSO from day 3 of disease. In contrast, giving Roscovitine to Thy1 rats from day 3 caused a marked decrease in mesangial cell proliferation at day 5. *Open diamonds*, Thy1 + DMSO; *open circles*, Thy1 + Roscovitine.

Thy1 rats at day 3 (treatment group), the reduction was not statistically significant. Taken together, these results show that the early reduction in CDK2 activity is associated with a decrease in matrix protein production, and that the timing of therapeutic interventions is critical.

Early decrease in CDK2 activity is associated with improved renal function. There was a 30% decrease in the urine protein/creatinine ratio at day 10 in rats given Roscovitine compared to control (4.05 ± 0.9 vs. 1.8 ± 0.8). Reducing CDK2 activity with Roscovitine also decreased the BUN 30% at day 10 compared to control Thy1 rats (28.6 ± 7.5 vs. 42.3 ± 5.8 ; $P < 0.05$).

Discussion

In this study we provide the first evidence that inhibiting the activity of CDK2 can be achieved in vivo, and that this inhibition markedly reduces mesangial cell proliferation and the severity of glomerular injury in an established model of immune-mediated glomerulonephritis.

Roscovitine reduces mesangial cell proliferation in vitro and in experimental glomerulonephritis. The first major finding in this study was that decreasing the activity of CDK2 in vivo with Roscovitine reduces mesangial cell proliferation in vitro and in an animal model of experimental inflammatory kidney disease. Previous studies have shown that an increase in CDK2 activity is essential for DNA synthesis in vitro (10, 21). We have recently reported that the expression and activity of CDK2 is increased in experimental glomerulonephritis, which correlates with mesangial cell proliferation (15). These studies, however, are descriptive; a functional role for CDK2 has not been established in vivo in inflammatory disease. Moreover, there have been no reported in vivo studies showing that pro-

liferation can be reduced by blocking the activity of CDK2, CDK5, cdc2, or another unidentified target. Roscovitine is a purine analogue that inhibits CDK complexes by localizing to the ATP-binding pocket located in the cleft between the small and large lobes of the kinase (23); the crystal structure of the complex between CDK2 and Roscovitine has recently been reported (22, 23).

We began our study by determining if Roscovitine inhibits proliferation in rat mesangial cells in vitro. Roscovitine caused a concentration-dependent inhibition of CDK2 activity in rat mesangial cells in vitro, which was associated with a marked reduction in FCS-induced proliferation. Thus, at a concentration of 25 μ M, Roscovitine completely inhibited mesangial cell proliferation in vitro. This effect was specific for CDK2 activity because the protein levels for G1/S cyclins and CDK2 were not altered by Roscovitine. Furthermore, Roscovitine did not effect mesangial cell viability in vitro at the concentrations

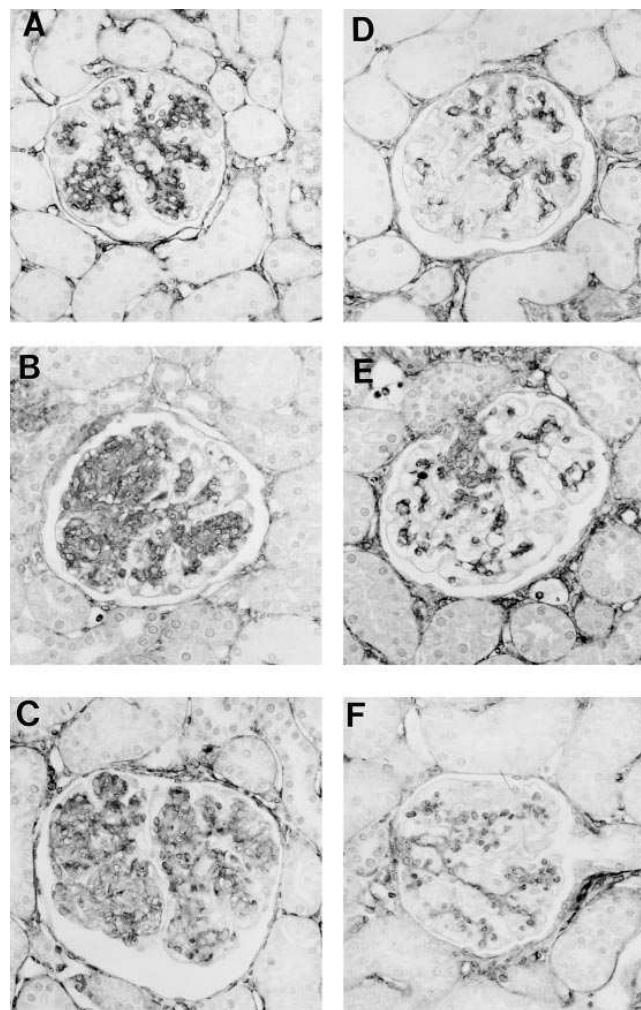


Figure 8. Immunostaining for matrix proteins. Indirect immunoperoxidase immunostaining for matrix proteins is shown at day 5 of Thy1 glomerulonephritis in rats given either DMSO or Roscovitine from day 1. In Thy1 rats given DMSO, there was an increase in immunostaining for collagen type IV (A), laminin (B), and fibronectin (C). In contrast, there was a marked decrease in immunostaining for collagen type IV (D), laminin (E), and fibronectin (F) in Thy1 rats given Roscovitine from day 1.

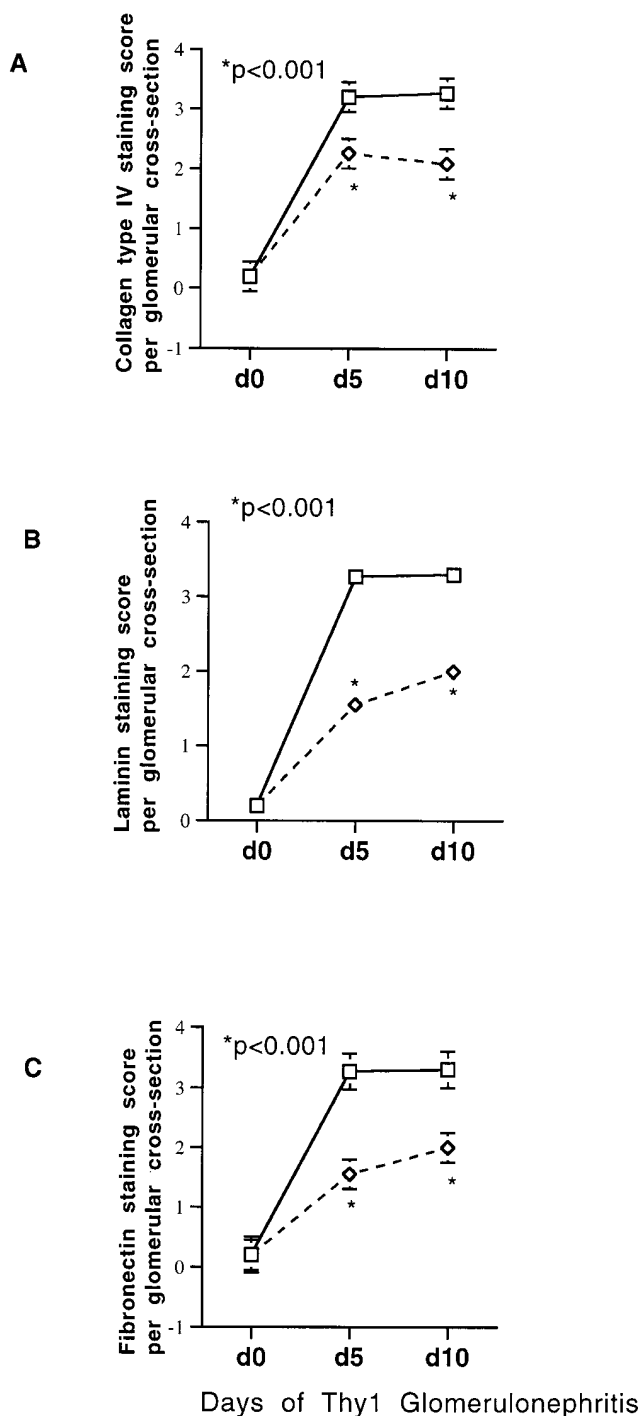


Figure 9. Quantitation of matrix proteins. Immunostaining for matrix proteins was quantitated, and given a score of 1–4 based on glomerular distribution (see Methods). In Thy1 rats given DMSO from day 1, there was an increase in immunostaining for collagen type IV (A), laminin (B), and fibronectin (C) at days 5 and 10. In contrast, giving Roscovitine to Thy1 rats from day 1 significantly reduced immunostaining for all three matrix proteins at day 5 and day 10 of disease. Open squares, Thy1 + DMSO; open diamonds, Thy1 + Roscovitine.

used in this study. Based on these in vitro findings, we gave Roscovitine to rats at a concentration of 2.8 mg/kg body weight.

The experimental (Thy1) model of inflammatory mesan-

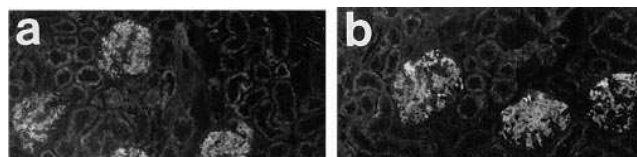


Figure 10. Immunostaining for goat IgG. The binding of the anti-Thy1 antibody was assessed by immunostaining for goat IgG. Immunostaining for goat IgG as identical in Thy1 rats given DMSO (a) or Roscovitine (b).

glial proliferative glomerulonephritis is characterized by mesangial cell proliferation and matrix accumulation (31). Floege et al. have previously shown that mesangial cell proliferation starts at day 2, peaks at day 5, and resolves by day 10–14 in Thy1 glomerulonephritis (31). In this study we asked if mesangial cell proliferation could be reduced by decreasing CDK2 activity with Roscovitine, without altering the levels of CDK2. We showed two novel in vivo results. First, reducing CDK2 activity with Roscovitine before the onset of mesangial cell proliferation decreased the magnitude of later mesangial cell proliferation by > 50% at days 3 and 5 of disease (prevention group). Second, administering Roscovitine at day 3 of disease after mesangial cell proliferation had already been established, also significantly reduced the magnitude of mesangial cell proliferation at day 5 (treatment group), the time when proliferation is maximal in this model. One possible explanation for the latter result is that reducing CDK2 activity inhibits new mesangial cell proliferation. In this study we cannot exclude an effect of Roscovitine on the kinase activity of CDK5 or cdc2, or that proliferation was influenced by an alternative signaling cascade such as extracellular regulated kinases (Erks) (23).

Previous studies have shown that reducing the levels of CDK2 in vivo with antisense can reduce proliferation in an angioplasty model (33); more recently this method has been successfully used experimentally in preventing coronary graft arteriosclerosis (34). Gene therapy, however, is difficult to perform, has problems targeting specific sites, and the transfection efficiency is dependent on the vector used (33, 35, 36). Thus, the current study is the first report where the activity of CDK2 is inhibited in inflammatory disease in vivo. Furthermore, giving Roscovitine is easy, and has a low side effect profile in rats. Although mesangial cell proliferation was not completely inhibited in this study, we were unable to give higher concentrations of Roscovitine by intraperitoneal injection because the rats developed diarrhea. It is likely that giving Roscovitine by continuous infusion or by alternative routes may be even more effective.

Inhibiting CDK2 activity reduces matrix production and improves renal function. The initial decline in renal function in many forms of glomerular disease is in part due to the production of matrix proteins (1). Several previous studies have shown that mesangial cell proliferation is closely linked with matrix production in both experimental and human glomerulonephritis. Indeed, reducing glomerular cell proliferation by blocking specific growth factors (14) or intracellular signaling pathways (37) has been shown to decrease matrix protein production. This study confirms these findings using an agent that acts directly on the cell cycle, which is a second major finding in this study. Reducing mesangial cell proliferation in vivo with Roscovitine caused a decrease in immunostaining for all three

different glomerular matrix proteins studied. The link between the decrease in proliferation and matrix production, however, was not noted at all stages of disease. Thus, early reduction of CDK2 activity and mesangial cell proliferation (prevention group) caused a significant decrease in matrix protein deposition, whereas decreasing mesangial cell proliferation later in disease (treatment group) caused a decrease in matrix proteins that was not statistically significant. This interesting finding suggests that there are specific phases in glomerular disease where decreasing mesangial cell proliferation with therapeutic strategies causes a decrease in matrix proteins, and reemphasizes the importance of early interventions. Furthermore, the decrease in glomerular injury in Roscovitine-treated rats was associated with better renal function compared to controls.

Roscovitine does not affect other variables that alter glomerulonephritis. Because previous studies showed that the Thy1 antibody binds to the mesangial cell within 1 h (13), in this study we elected to first administer Roscovitine or DMSO 24 h after antibody administration to avoid any effects of antibody deposition and disease induction. Our results show that there was no difference in the intensity or distribution of immunostaining for goat IgG or C5b-9 in rats given Roscovitine or DMSO, and that total serum complement levels, measured by CH50, were not altered. Together these results show that the reduction in proliferation and matrix proteins was not due to interference with binding of the Thy1 antibody or complement activation. Because platelets (38) and macrophages (24) are also critical in the development of mesangiolysis and glomerular injury in this model, we quantitated these infiltrating cell types in rats given Roscovitine and DMSO. Our results show that Roscovitine and DMSO did not alter platelet or macrophage influx into the glomeruli of rats with Thy1 glomerulonephritis. This finding was not surprising as these cells maximally infiltrate the glomerulus within the first 24 h of disease (24, 38). Thus, these results demonstrate that the reduction in mesangial cell proliferation observed was a specific effect of inhibiting the cell cycle with Roscovitine.

In summary, this is the first report where cell proliferation has been reduced in vivo in an inflammatory disease by reducing the activity of CDK, without altering the levels of this cell cycle protein. Moreover, the reduction in mesangial cell proliferation achieved was accompanied by a significant reduction in other measures of disease activity (proteinuria, renal function). Uncontrolled cell proliferation characterizes neoplastic and other forms of inflammatory diseases. Our study shows that reducing CDK activity should be considered as a potential future therapeutic strategy to decrease cell proliferation in vivo.

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