

The cyclin kinase inhibitor p21^{WAF1/CIP1} is required for glomerular hypertrophy in experimental diabetic nephropathy

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The cyclin kinase inhibitor p21^{WAF1/CIP1} is required for glomerular hypertrophy in experimental diabetic nephropathy.

Background. Diabetic nephropathy is characterized by glomerular hypertrophy. We have recently shown that experimental diabetes mellitus is associated with an increase in glomerular expression of the cyclin kinase inhibitor p21^{WAF1/CIP1} (p21). Furthermore, *in vitro* glucose-induced mesangial cell hypertrophy is also associated with an up-regulated expression of p21. In this study, we tested the hypothesis that p21 mediates diabetic glomerular hypertrophy *in vivo*.

Methods. Experimental diabetes mellitus was induced by streptozotocin in mice in which p21 was genetically deleted (p21 ^{-/-}) and in wild-type mice (p21 ^{+/+}). Kidney biopsies were obtained from diabetic and control (citrate injected) p21 ^{+/+} and p21 ^{-/-} mice at day 60. The tissue was used for morphologic studies of glomerular size (measured by computer image-analysis system), glomerular cellularity (cell count), glomerular matrix expansion (silver stain), apoptosis (TUNEL), and expression of transforming growth factor- β_1 (TGF- β_1) by *in situ* hybridization.

Results. The glomerular tuft area increased 11.21% in diabetic p21 ^{+/+} mice at day 60 compared with control ($3329.98 \pm 244.05 \mu\text{m}^2$ vs. $2994.39 \pm 176.22 \mu\text{m}^2$, $P = 0.03$), and the glomerular cell count did not change in diabetic p21 ^{+/+} mice at day 60 compared with the control. These findings are consistent with glomerular hypertrophy. In contrast, the glomerular tuft area did not increase in diabetic p21 ^{-/-} mice at day 60 compared with the control (3544.15 ± 826.49 vs. 3449.15 ± 109.65 , $P = 0.82$), nor was there an increase in glomerular cell count (41.41 ± 13.18 vs. 46.95 ± 3.00 , $P = 0.43$). Diabetic p21 ^{+/+} mice, but not p21 ^{-/-} mice, developed an increase in proteinuria at day 60 compared with the control. Tubular cell proliferation, measured by proliferating cell nuclear antigen immunostaining, was increased in both diabetic p21 ^{+/+} (2.1-fold) and p21 ^{-/-} (7.61-fold) mice compared with controls. Glomerular cell apoptosis did not increase in diabetic mice. Although glomerular TGF- β_1 mRNA levels increased in both strains of diabetic mice at day 60, the glomerular matrix did not expand.

Conclusions. Hyperglycemia was associated with glomerular hypertrophy in p21 ^{+/+} mice. Despite the increase in TGF- β_1 mRNA, diabetic p21 ^{-/-} mice did not develop glomerular hypertrophy, providing evidence that the cyclin kinase inhibitor p21 may be required for diabetic glomerular hypertrophy induced by TGF- β_1 . The loss of p21 increases tubular but not glomerular cell proliferation in diabetic nephropathy. The absence of glomerular hypertrophy appears protective of renal function in diabetic mice.

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) in the United States [1]. Unlike most forms of ESRD, the kidney size is typically increased in diabetes, at least early in the natural history of the disease [2–6]. This is primarily because of two underlying events. First, although diabetic nephropathy may be associated with some low-grade glomerular cell proliferation [7], hypertrophy is the major means of enlargement in the glomerulus [reviewed in 8, 9]. Second, diabetes is associated with increased tubular proliferation and hypertrophy [reviewed in 10]. Normalizing blood glucose levels prevent these growth events and the development of progressive glomerular sclerosis and, in some cases, may even reverse glomerular sclerosis over time [11].

The mechanisms underlying the development of diabetic glomerular hypertrophy have not been fully established, and it is also not known why the glomerulus undergoes little or no proliferation in diabetes, whereas tubular epithelial cell proliferation is readily demonstrated. There is evidence to support a role for growth factors such as TGF- β [12–16], certain intracellular signaling pathways [17], and specific transcription factors [18–20] in mediating diabetic-associated glomerular hypertrophy. However, attempts only recently have been made to determine the role of cell cycle regulatory proteins in diabetic nephropathy.

Cell proliferation requires that certain cyclin dependent kinases (CDKs) be activated by a specific partner

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cyclin during each phase of the cell cycle [21, 22]. Cyclin kinase inhibitors (CKIs) bind to cyclin-CDK complexes, which inactivate the kinase, causing cell cycle arrest and the inhibition of proliferation [23]. There are two families of CKIs, which are classified according to their structural homology and which cyclin-CDK complexes they inhibit. The INK4 family (p15, p16, p18, and p19) inhibits CDKs active in the G1 phase of the cell cycle [24, 25], whereas members of the Cip/Kip family, currently comprising p21^{CIP1/WAF1} (p21), p27^{KIP1}, and p57^{KIP2}, contain a CDK-binding domain and inhibit both G1- and S-phase CDKs [26–29].

When a cell engages the cell cycle, the protein content increases in anticipation of preparing the cell for division [21]. However, DNA synthesis occurs only during the S phase of the cell cycle. Hypertrophy is defined as an increase in the ratio of protein to DNA [10]. We and others have hypothesized that one mechanism of hypertrophy is the inhibition of DNA synthesis in cells that have engaged the cell cycle. Accordingly, we have been interested in the potential role of specific CKIs in the pathogenesis of diabetic glomerular hypertrophy. We have recently shown that glucose-induced mesangial cell hypertrophy *in vitro* is associated with increased levels of the CKI p21 [30]. Furthermore, glomerular hypertrophy in the streptozotocin (STZ) model of diabetic nephropathy in mice is also associated with increased p21 expression [30].

In this study, we have extended these studies by inducing experimental diabetes in p21 ^{+/+} and p21 ^{-/-} mice to test whether the presence of p21 is essential for the observed hypertrophy in these experimental systems.

METHODS

Animal model and experimental design

Experimental diabetes mellitus was induced in p21 wild-type (^{+/+}) mice ($N = 7$) and in p21 null (^{-/-}) mice ($N = 12$) with single intraperitoneal injections of streptozotocin (STZ; 160 mg/kg; Sigma, St. Louis, MO, USA) on two consecutive days [13, 30]. Mice were six to eight weeks old. A second group of age-matched p21 ^{+/+} ($N = 5$) and p21 ^{-/-} ($N = 4$) mice were injected with sodium citrate (vehicle for STZ) and served as controls. A third group of normal age-matched unmanipulated p21 ^{+/+} ($N = 11$) and p21 ^{-/-} ($N = 7$) mice served as baseline.

Blood glucose was measured in p21 ^{+/+} and p21 ^{-/-} mice prior to intraperitoneal injections of STZ and citrate, three days postinjection and at sacrifice using the One Touch glucometer (Lifescan INC, Milpitas, CA, USA). Prior to sacrifice, body weight was measured in grams. Control and diabetic p21 ^{+/+} and p21 ^{-/-} mice were sacrificed at day 60, when the kidney weight was also measured in grams. Kidney biopsies were obtained,

and tissues were fixed in methyl Carnoy's solution or 10% buffered formalin as previously described [30].

Urinary protein

To evaluate proteinuria, mice were placed in metabolic cages with free access to water but not chow, and a 24-hour urine collection was obtained one day before sacrifice. Urinary protein was measured using the sulfasalicylic acid (SSA) method [31]. Briefly, urine was diluted in phosphate-buffered saline, and three parts of SSA was added to one part of diluted urine. Precipitated protein was measured by spectrophotometry at an optical density of 650 nm. The protein concentration was measured against a standard curve, and the 24-hour excretion was determined by measuring urine volume and was reported as milligrams per 24 hours.

Morphometric measurements

To determine the role of p21 in diabetic glomerular hypertrophy, the glomerular tuft area, a measure of glomerular size, was assessed [32, 33] on periodic acid-Schiff (PAS)-stained slides. In this study, we used an Optimas computer image analysis system, version 6.1 (Bothell, WA, USA). A Leica DMRB microscope was used and was connected to a computer system via a Nikon UFX camera lens and a microimage video system. Images were viewed using a Sony computer monitor. To measure the glomerular tuft area, a line was manually drawn around the boundary of each glomerular tuft in a blinded manner by one of the authors. The area inside the line (glomerular tuft area) was measured in μm^2 . Measurements were done on 20 consecutive cortical glomeruli at a $\times 630$ magnification. Pilot studies showed that evaluating 20 glomeruli in each kidney biopsy was sufficient to obtain statistically significant morphometric measurements that were not meaningfully altered by evaluating up to 90 additional glomeruli per biopsy (data not shown). Glomeruli were excluded if the tuft area was more than two SD from the mean ($<1\%$ excluded). The tuft area was expressed as mean $\pm$ SD for both mouse strains at each time point. Immunostaining for p21 was performed as previously reported using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Glomerular cell count

In order to determine if glomerular cell proliferation was increased in the absence of p21 in experimental diabetic nephropathy, the glomerular cell number was measured on PAS-stained slides. The total number of cells in each glomerular tuft was measured using the Optimas computer image analysis system, and the glomerular cell count was expressed as mean $\pm$ SD for individual animals. Twenty consecutive cortical glomeruli were evaluated in each control and diabetic p21 ^{+/+} and p21 ^{-/-} animal.

Measuring DNA synthesis

DNA synthesis was evaluated by proliferating cell nuclear antigen (PCNA) staining in methyl Carnoy's fixed tissue. A mouse monoclonal IgM antibody (Coulter clone #6604541; Coulter, Miami, FL, USA) was used as a primary antibody at a concentration of 1:700, followed by a horseradish peroxidase rat antimouse IgM (Zymed, San Francisco, CA, USA), as described elsewhere [30]. Renal tissue from a mouse uninephrectomy model was used as positive control tissue for PCNA staining.

To identify renal tubular cells better, an eosin counterstain was added, and PCNA-positive cells were counted in the renal cortical tubules. PCNA-positive cells were counted in all cortical fields (at $\times 400$ magnification) that showed the renal capsule. Overlapping of the fields was avoided, and the average number of cells that stained positive for PCNA in the tubules per high power field (HPF) was calculated.

Terminal deoxynucleotidyl transferase-mediated nick end labeling staining

To determine if glomerular apoptosis (programmed cell death) was increased in diabetic animals, apoptosis was measured by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining on formalin-fixed tissues, as described elsewhere [34, 35].

Transforming growth factor- β_1 *in situ* hybridization

Because transforming growth factor- β_1 (TGF- β_1) has previously been shown to mediate diabetic glomerular hypertrophy [13], TGF- β_1 mRNA was evaluated by *in situ* hybridization. A previously characterized mouse TGF- β_1 cDNA clone was used (gift of Dr. H.L. Moses, Vanderbilt University, Nashville, TN, USA) [36, 37]. The cDNA was transcribed into ^{35}S -labeled antisense and sense cRNA probes corresponding to 974 bp (421 to 1395 bp) of mouse TGF- β_1 . *In situ* hybridization followed a method previously described [38]. Briefly, formalin-fixed, 4 μm tissue sections were digested with 10 $\mu\text{g}/\text{ml}$ proteinase K and prehybridized for two hours in 100 μl of prehybridization buffer [0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM ethylenediaminetetraacetic acid (EDTA) $\times$ 1 Denhardt's solution, 10% dextran sulfate, and 10 mM dithiothreitol (DTT)]. Hybridization was commenced by adding 5×10^5 cpm of ^{35}S -labeled riboprobe in 50 μl of prehybridization buffer. Overnight incubation at 50°C was followed by $\times 2$ standard saline citrate (SSC) washes and was then RNase A digested (20 $\mu\text{g}/\text{ml}$) for 30 minutes at 37°C. Three stringency washes comprising $\times 0.1$ standard saline citrate, 0.5% Tween at 50°C were followed by dehydration and air drying. The slides were dipped in photographic emulsion (Kodak NTB2), exposed at 4°C for three weeks, developed with D-19 developer (Eastman Kodak Co., Rochester, NY, USA), and

counterstained with hematoxylin and eosin. Sense controls were run with every section, and mouse skin containing granulation and scar tissue was used as a concurrent positive hybridization control.

Measuring glomerular matrix expansion

To measure glomerular matrix expansion, the modified Gomori's methenamine silver stain was used, and immunostaining was performed for collagen type IV using a goat antitype IV collagen antibody (#1340-01; Southern Biotech, Birmingham, AL, USA), laminin using a rabbit antirat IgG antibody (Chemicon, Temecula, CA, USA), and fibronectin using a rabbit antirat antibody (Chemicon). Glomerular tuft immunostaining was quantitated for each matrix protein and silver stain using the Optimas computer image analysis system. The color threshold was set by marking three to five separate pixels in areas of positive staining in 20 consecutive glomerular tufts in each animal. Positive staining was expressed as a percentage of the glomerular tuft area.

Statistical analysis

Statistical analysis was performed by the unpaired *t*-test using the StatView computer statistics program (version 4.5; Abacus Concepts, Berkeley, CA, USA). Statistical significance was defined as $P < 0.05$. All values were expressed as mean $\pm$ SD.

RESULTS

Glucose increased in both mouse strains

Blood glucose levels increased significantly in STZ-injected p21 $+/+$ mice compared with their controls (citrate-injected p21 $+/+$ mice) at day 60 (506.29 ± 60.54 mg/dl vs. 184.6 ± 28.5 mg/dl, $P < 0.0001$; Table 1). Similarly, blood glucose levels increased in STZ-injected p21 $-/-$ mice compared with control (citrate-injected p21 $-/-$ mice) at day 60 (439.42 ± 64.79 mg/dl vs. 133.75 ± 9.46 mg/dl, $P < 0.0001$; Table 1).

Proteinuria did not increase in diabetic p21 $-/-$ mice

To evaluate the effect of diabetes on protein excretion, 24-hour urinary protein was measured and is shown in Table 1. Urinary protein was 5.77-fold higher in diabetic p21 $+/+$ mice compared with control (70.72 ± 13.74 vs. 12.26 ± 3.71 mg/24 hr, $P < 0.0001$). In contrast, urinary protein did not increase significantly in diabetic p21 $-/-$ mice compared with controls (23.53 ± 8.66 vs. 30.08 ± 17.33 mg/24 hr, $P = 0.65$).

Normal glomerular histology in diabetic mice at day 60

The renal pathology in control and diabetic p21 $+/+$ and p21 $-/-$ mice was evaluated by four of the authors (M.A., P.A.J.B., C.E.A., S.J.S.) on PAS- and silver-stained

Table 1. Animal data in control and diabetic p21 +/+ and p21 -/- mice at day 60

Animal group	Blood glucose mg/dl	Urine protein mg/24 hr	Kidney weight	Body weight	Kidney to body weight ratio
			g		
p21 +/+ Control (N=5)	184 ± 28.5	12.26 ± 3.71	0.369 ± 0.03	27.0 ± 0.93	0.014 ± 0.001
p21 +/+ Diabetic (N=7)	506.3 ± 60.5 ^a	70.72 ± 13.74 ^a	0.396 ± 0.04	23.07 ± 2.73	0.017 ± 0.001
p21 -/- Control (N=4)	133.7 ± 9.5	30.08 ± 17.33	0.42 ± 0.08	29.37 ± 1.7	0.014 ± 0.002
p21 -/- Diabetic (N=12)	439.4 ± 64.8 ^b	23.53 ± 8.66	0.406 ± 0.09	22.87 ± 3.75	0.018 ± 0.003

^aP < 0.001 vs. p21 +/+ Control
^bP < 0.001 vs. p21 -/- Control

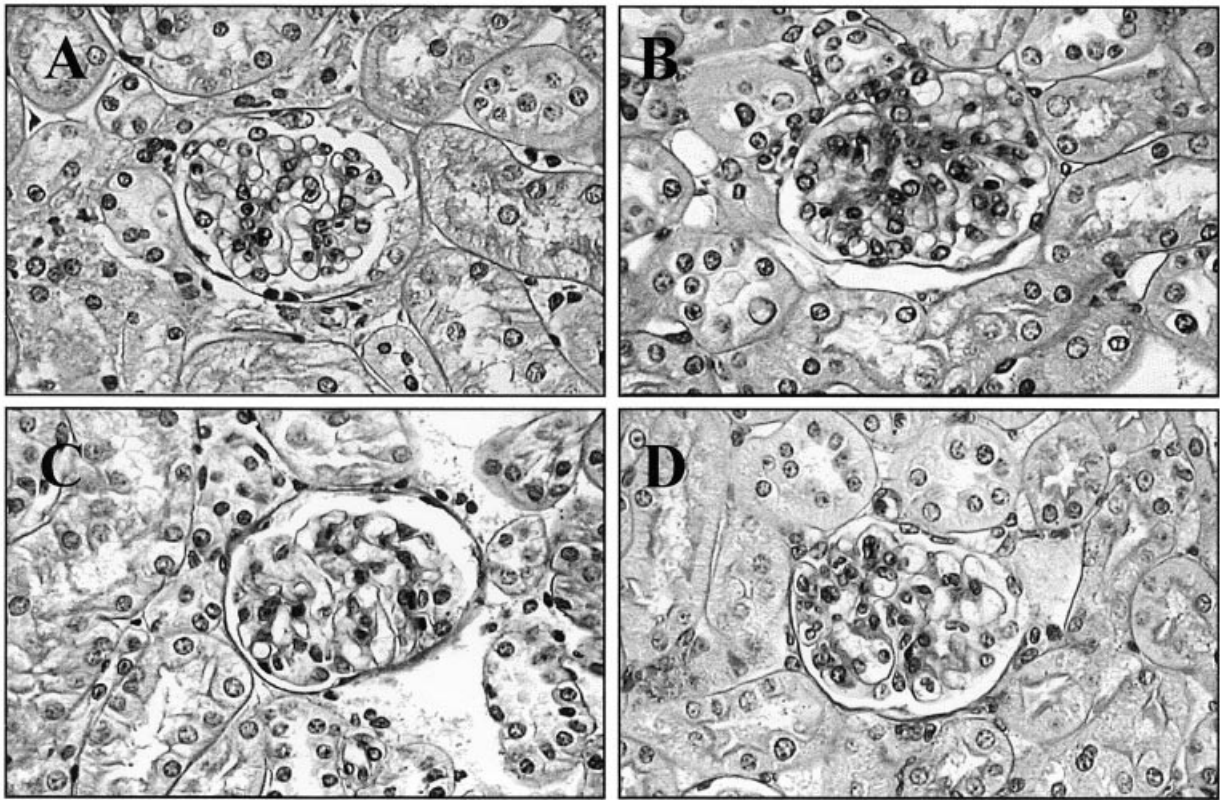


Fig. 1. PAS staining at day 60. The glomerular histology was normal in control (citrate injected) p21 +/+ (A) and p21 -/- (C) mice at day 60. The glomerular tuft area, but not glomerular cellularity, increased in diabetic p21 +/+ mice (B). The glomerular tuft area did not change in diabetic p21 -/- mice (D).

sections (Fig. 1). There was no obvious increase in the mesangial expansion in diabetic mice nor was there any thickening of the glomerular basement membranes. In less than 5% of glomeruli, mild mesangiolysis and microaneurysms were noted, but there was no quantitative difference between p21 -/- and p21 +/+ mice. No abnormalities were noted in the renal blood vessels in diabetic animals, and the tubular epithelial cells were grossly normal.

Glomerular hypertrophy was absent in diabetic p21 -/- mice

The kidney to body weight ratios were calculated in individual mice, and the results are shown in Table 1. Although there was a trend toward an increase in kidney to body weight ratio in diabetic mice, the increase did not reach statistical significance at the time point studied. Hence, the glomerular tuft area was used as a measure of glomerular hypertrophy [32]. In age-matched unmani-

Table 2. Glomerular tuft area (mean $\pm$ SD), a measure of glomerular hypertrophy, increased in diabetic p21 $+/+$ mice, but not in diabetic p21 $-/-$ mice, compared with controls

	Control	Diabetic	
	day 60		<i>P</i> value
p21 $+/+$	2994.39 $\pm$ 176.22	3329.98 $\pm$ 244.05	0.03
p21 $-/-$	3449.15 $\pm$ 109.65	3544.15 $\pm$ 826.49	0.82

Table 3. Glomerular cell number did not increase in diabetic p21 $+/+$ and p21 $-/-$ mice at day 60 compared with controls

	Control	Diabetic	
	day 60		<i>P</i> value
p21 $+/+$	38.83 $\pm$ 1.91	36.26 $\pm$ 4.22	0.24
p21 $-/-$	46.95 $\pm$ 3.00	41.41 $\pm$ 13.18	0.43

pulated mice, there was an increase in glomerular tuft area in p21 $-/-$ mice compared with p21 $+/+$ mice (2973.31 ± 160.64 vs. 2567.48 ± 265.36 , $P = 0.04$). There was an 11.21% increase in glomerular tuft area in diabetic p21 $+/+$ mice at day 60 compared with control (citrate injected; Table 2). In contrast, the glomerular tuft area did not increase in diabetic p21 $-/-$ mice at day 60 compared with the control (Table 2).

To determine whether the increase in glomerular tuft area in diabetic p21 $+/+$ mice was due to cell hypertrophy or increased cell number and whether glomerular cellularity was increased in diabetic p21-deficient animals, glomerular tuft cell count was measured at day 60. There was no difference in glomerular tuft cell count in diabetic p21 $+/+$ mice at day 60 compared with control p21 $+/+$ mice (Table 3). Similarly, there was no difference in glomerular tuft cell count in diabetic p21 $-/-$ mice at day 60 compared with control p21 $-/-$ mice (Table 3). These results and the absence of an increase in extracellular matrix proteins (discussed later in this article) show that the early increase in glomerular tuft area in diabetic p21 $+/+$ mice was consistent with glomerular cell hypertrophy. In contrast, glomerular hypertrophy was not detected in diabetic p21 $-/-$ mice despite a similar degree of hyperglycemia.

There was no difference in glomerular immunostaining for p21 in diabetic and control p21 $+/+$ mice at day 60 (results not shown).

Glomerular matrix is not increased in diabetic p21 $-/-$ mice

Glomerular matrix expansion was quantitated in control and diabetic animals at day 60. There was no increase in silver staining and PAS staining in diabetic p21 $+/+$ mice compared with controls (results not shown). Similarly, silver staining and PAS staining was not increased

in diabetic p21 $-/-$ mice compared with controls at day 60 (results not shown). There was also no increase in glomerular immunostaining for collagen type IV, laminin, and fibronectin in diabetic p21 $-/-$ and p21 $+/+$ mice at day 60 compared with controls (results not shown).

Glomerular TGF- β_1 is increased in diabetic mice

Transforming growth factor- β_1 mRNA was assessed by *in situ* hybridization. All slides were labeled during the same run to minimize technical differences that could occur between procedures. Control p21 $+/+$ mice revealed occasional positive glomerular and interstitial cells using the antisense probe. TGF- β_1 mRNA was increased in glomerular and interstitial cells in diabetic p21 $+/+$ mice (Fig. 2). However, an induction of diabetes resulted in a marked glomerular (and interstitial) hybridization signal in p21 $-/-$ mice, both compared with control p21 $-/-$ and diabetic p21 $+/+$ mice (Fig. 2). Because of the scattering effect of silver grains in this technique, the exact localization to particular glomerular cell types was not possible. Sense control slides revealed only slight background levels of silver grain staining.

Tubular DNA synthesis is increased in diabetic p21 $-/-$ mice

Because previous studies have shown that diabetes is associated with tubular cell proliferation [4, 5], we were particularly interested in determining if DNA synthesis was different in this renal compartment in diabetic p21 $+/+$ and p21 $-/-$ mice. There was a 2.1-fold increase in PCNA staining in tubular cells in diabetic p21 $+/+$ mice at day 60 compared with control (3.08 ± 0.46 vs. 1.47 ± 0.46 cells per HPF, $P = 0.0001$). Moreover, there was a 7.61-fold increase in PCNA staining in tubular cell in diabetic p21 $-/-$ mice at day 60 compared with control (6.66 ± 3.16 vs. 0.87 ± 0.12 cells per HPF, $P = 0.003$).

TUNEL staining, a measure of apoptosis, was not detected in the glomeruli of control or diabetic p21 $+/+$ and p21 $-/-$ mice at day 60.

DISCUSSION

The principle finding in this study is the demonstration that the absence of the CKI p21 prevents the occurrence of glomerular hypertrophy in diabetic mice and that this occurs despite an increase in TGF- β_1 as measured by synthesis of new mRNA. We demonstrated that glomerular hypertrophy in diabetic p21 $+/+$ mice was associated with proteinuria, but proteinuria was not increased in the absence of glomerular hypertrophy in diabetic p21 $-/-$ mice. These results indicate a possible functional defect that can be linked to what is usually identified as the earliest structural alteration in human diabetic nephropathy [3].

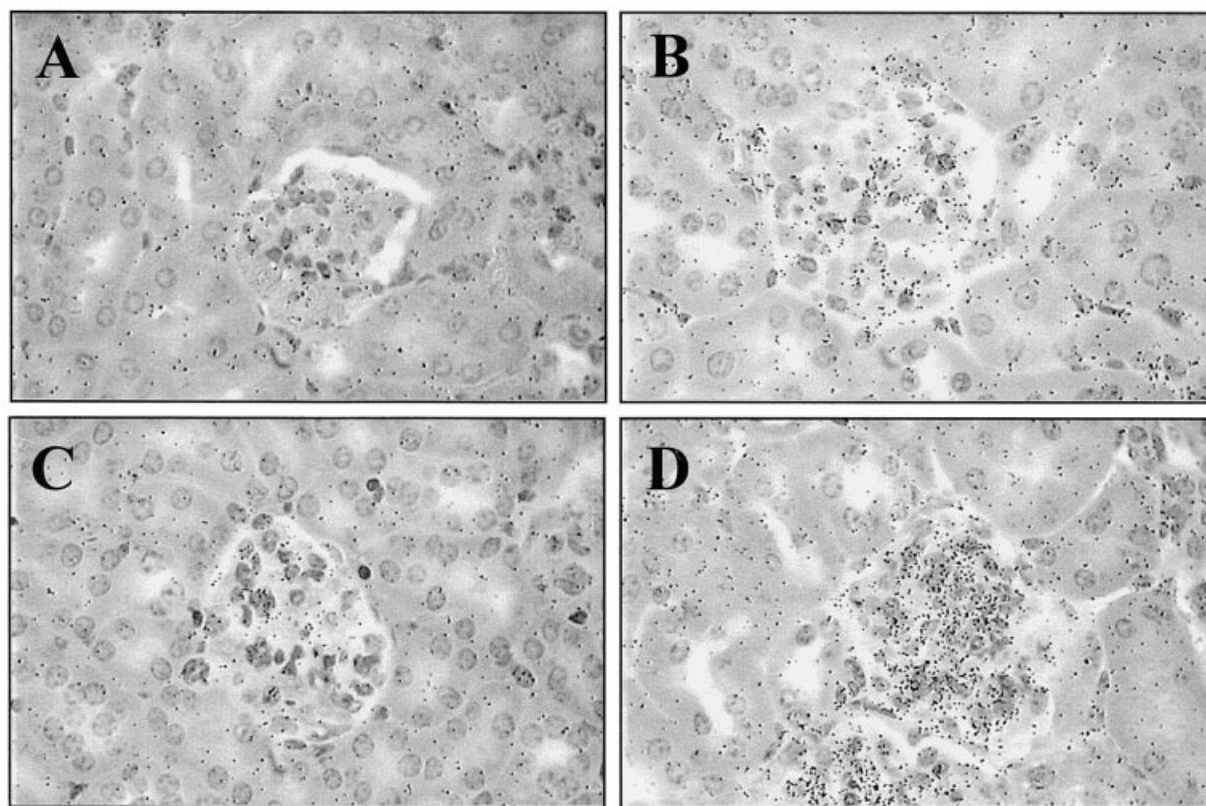


Fig. 2. Transforming growth factor- β_1 (TGF- β_1) *in situ* hybridization at day 60. There was an increase in TGF- β_1 mRNA in glomeruli of diabetic p21 +/+ (B) and p21 -/- (D) mice compared with their respective controls (A and C).

The hallmark of experimental and human diabetic nephropathy is an increase in glomerular size [reviewed in 3, 8, 9, 39]. Early in the course of disease, the increase in glomerular size is predominantly caused by cellular hypertrophy. Later in the course of disease, increased glomerular size is due to hypertrophy and the accumulation of extracellular matrix proteins. Hypertrophy is defined as an increase in the cellular protein to DNA ratio [10], which has led our group and others to ask if hypertrophy is due to an increase in cyclin-kinase inhibitors, which are proteins that inhibit DNA synthesis.

We have recently shown that hyperglycemia-induced mesangial cell hypertrophy *in vitro* was associated with increased levels of the CKI p21. Moreover, glomerular hypertrophy in experimental diabetic nephropathy *in vivo* was associated with a substantial increase in glomerular immunostaining for p21 [30]. Thus, in this study, we asked whether p21 was required for hyperglycemia-induced glomerular hypertrophy. Indeed, hyperglycemia was associated with glomerular hypertrophy in p21 +/+ mice compared with control p21 +/+ mice. However, the first major finding in this study was that despite a marked increase in hyperglycemia induced by STZ, the glomerular tuft area, a measure of glomerular hypertro-

phy, was not increased in diabetic p21 -/- mice compared with control (citrate injected) p21 -/- mice.

In this study, we used the glomerular tuft area as a measure of glomerular hypertrophy. Although the protein to DNA ratio may be more specific, we did not perform these measures in this study because of the difficulty in obtaining a tubule-free glomerular preparation by isolation in mice. Second, the kidney to body weight ratio has been used as a gauge of kidney growth in diabetes. However, because 70% of the kidney weight is the tubular fraction (which proliferates in diabetes), this is not a sensitive measure of glomerular size. Furthermore, the body weight decreases in diabetes, which increases the ratio. Finally, as discussed later here, the increase in glomerular tuft area in this study occurred in the absence of an increase in cellularity or extracellular matrix proteins, results that are consistent with hypertrophy as a cause of increased glomerular tuft area.

A role for CKIs in mediating diabetic and nondiabetic renal hypertrophy is emerging. Wolf et al showed that hyperglycemia increased levels for the CKI p27^{Kip1} (p27) in mesangial cells *in vitro* [40]. Furthermore, reducing p27 levels with antisense prevented glucose-induced mesangial cell hypertrophy *in vitro* [40]. Wolf et al also

showed increased expression for p27 in both the STZ and genetically determined (db/db) models of diabetic nephropathy [41]. A role for CKIs has also been shown in renal tubular cell hypertrophy. It has been shown that p21, but not the CKI p15, mediates angiotensin II-induced proximal tubular cell hypertrophy *in vitro*, and Wolf and Stahl demonstrated a role for p27 in angiotensin II-induced hypertrophy *in vitro* [42]. In this study, using mice genetically deficient for p21, our results show that the CKI p21 may be required for glomerular hypertrophy *in vivo*. Although the kidneys from unmanipulated p21 $-/-$ mice are histologically normal by light and electron microscope, and the renal function is normal, the glomerular tuft area was larger than in unmanipulated p21 $+/+$ mice. The reason for this is not clear.

Many forms of immune- and nonimmune-mediated glomerular injury are associated with glomerular cell proliferation. In contrast, glomerular cell proliferation is not marked in experimental and human diabetic nephropathy [7]. Moreover, recent studies have shown that glomerular cell counts decrease in diabetic nephropathy [43]. CKIs prevent and inhibit renal and nonrenal cell proliferation *in vitro* [23]. Accordingly, we hypothesized *a priori* that the absence of p21 (in p21 $-/-$ mice) would be associated with increased glomerular cell proliferation following the onset of hyperglycemia. However, a second finding in this study was that the glomerular cell count did not increase in diabetic p21 $-/-$ mice and diabetic p21 $+/+$ mice. In contrast, tubular epithelial cell DNA synthesis increased in diabetic p21 $+/+$ and p21 $-/-$ mice, results that have been previously shown in diabetic animals. Moreover, tubular epithelial cell DNA synthesis was more pronounced in diabetic p21 $-/-$ mice compared with diabetic p21 $+/+$ mice. These results suggest a differential effect of p21 on tubular epithelial cells and glomerular cells during hyperglycemia.

There are a number of potential explanations about why glomerular hypercellularity did not increase in diabetic p21 $-/-$ mice. First, the cell number may have decreased by apoptosis [43]. Indeed, Megyesi, Safirstein, and Price showed that tubular cell apoptosis increased in p21 $-/-$ mice with cisplatin-induced renal cell injury [44]. In contrast, in this study, glomerular TUNEL staining did not increase in diabetic p21 $+/+$ mice and p21 $-/-$ mice. However, apoptosis may have occurred at different time points not studied here. Furthermore, if apoptosis increased in podocytes, cell loss into the urinary space may make it difficult to detect. Second, the lack of (expected) glomerular hypercellularity in diabetic p21 $-/-$ mice may be that hyperglycemia is not sufficient to increase the expression and activity of cyclins and cyclin-dependent kinases required for DNA synthesis [30]. Third, the increase in expression for the antiproliferative growth factor TGF- β (discussed later in this arti-

cle) may limit glomerular cell proliferation through a p21-independent mechanism.

The cytokine TGF- β has been of major interest in renal and nonrenal disease because it increases the production of extracellular matrix proteins and also has potent effects on cell growth, where it is antiproliferative to glomerular cells *in vitro* [45], converts tubular epithelial cell proliferation to hypertrophy [46], and induces mesangial cell hypertrophy [47]. TGF- β expression increases in mesangial cells exposed to high glucose *in vitro* [48], and the glomerular expression for TGF- β 1 increases during glomerular hypertrophy in experimental and human diabetic nephropathy [12, 14, 16, 49]. Furthermore, neutralizing TGF- β prevents diabetic glomerular hypertrophy [13]. Taken together, these studies show a role for TGF- β in hypertrophy of diabetic nephropathy.

A link between TGF- β and the cell cycle has been shown. TGF- β 1 increases the levels for p21 in certain nonrenal cells *in vitro* that may be responsible in part for the antiproliferative effects of TGF- β [50–52]. However, little is known about the role of p21 in mediating hypertrophy induced by TGF- β , and we were therefore interested in studying the relationship of TGF- β 1 and glomerular hypertrophy in diabetic p21 $-/-$ mice. In this study, we showed a marked increase in glomerular TGF- β 1 mRNA in diabetic p21 $+/+$ and p21 $-/-$ mice. However, a third major finding in this study was that despite the increase in TGF- β 1 mRNA synthesis by intrinsic cells comprising the renal parenchyma, glomerular hypertrophy was not detected in diabetic p21 $-/-$ mice at day 60. Taken together, these results show that p21 is required for TGF- β 1-induced diabetic glomerular hypertrophy *in vivo*.

Although hypertrophy is the predominant glomerular lesion in the earlier phase of diabetic nephropathy, the later stages are characterized by increased extracellular matrix protein accumulation, leading to glomerulosclerosis. In this study, glomerular matrix accumulation did not increase in both strains of diabetic mice at day 60, despite the increase in TGF- β 1. However, the lack of increased matrix proteins in diabetic mice at the early time points studied here is consistent with the findings of others, who show that matrix proteins accumulate late in the course of human and experimental diabetes, including the STZ mouse model (Fuad Ziyadeh, personal communication). Striker et al showed that collagen IV and laminin expression were increased in STZ-induced diabetes in mice after 12 weeks of hyperglycemia, which was significantly later in the course of hyperglycemia compared with this study. Moreover, the increase in matrix proteins was mouse-strain specific [53, 54]. Thus, further long-term studies are needed to determine the role of p21 on the accumulation of glomerular extracellular matrix proteins. However, the absence of a detectable

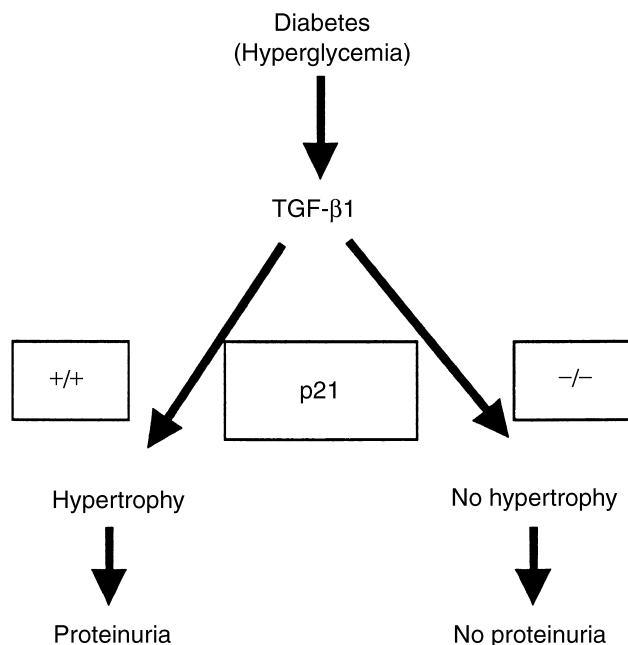


Fig. 3. Schema of proposed hypothesis. Hyperglycemia causes an up-regulation in TGF- β_1 , which increases the levels of the cyclin kinase inhibitor p21 and is associated with glomerular hypertrophy and proteinuria. In the absence of p21 expression, glomerular hypertrophy does not occur despite the increase in TGF- β_1 levels, and proteinuria is absent.

increase in glomerular matrix proteins suggests that any changes in glomerular tuft area in this study were likely not a consequence of matrix proteins, but rather caused by an increase in cell size.

Proteinuria, the hallmark of diabetic nephropathy, is due to a number of different mechanisms [55]. One mechanism is the hyperfiltration that accompanies increased glomerular size. In this study, we showed that proteinuria was increased in diabetic p21 $+/+$ mice. In contrast, proteinuria was not increased in the absence of glomerular hypertrophy in diabetic p21 $-/-$ mice. Long-term studies are needed to study the effect on the glomerular filtration rate.

In summary (Fig. 3), we show that diabetic p21 $-/-$ mice do not develop glomerular hypertrophy, despite a marked increase in TGF- β_1 . These data support the hypothesis that p21 are required for diabetic-related glomerular hypertrophy. The loss of p21, an inhibitor of cell proliferation, did not convert the glomerular lesion from a hypoproliferative state to a proliferative form. These data show that p21 is a critical determinant of diabetic nephropathy.

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APPENDIX

Abbreviations used in this article are: CDK, cyclin dependent kinase; CKI, cyclin kinase inhibitor; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESRD, end-stage renal disease; p21, p21^{CIP1, WAF1}; p21 $+/+$, p21 wild-type mouse; p21 $-/-$, p21 null mouse; PAS, Periodic Acid Schiff; PCNA, proliferating cell nuclear antigen; SSA, sulfasalicylic acid; STZ, streptozotocin; TGF- β , transforming growth factor-beta; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

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