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CLINICAL INVESTIGATION

Expression of transforming growth factor-β1 during diabetic renal hypertrophy

STUART J. SHANKLAND and JAMES W. SCHOLEY, with the technical assistance of HAO LY and KERRI THAI

Department of Medicine, University of Toronto, Toronto, Ontario, Canada

Expression of transforming growth factor-β1 during diabetic renal hypertrophy. Experimental type I diabetes mellitus is characterized by an early increase in kidney weight and glomerular volume, but changes in gene expression accompanying diabetic renal growth have not been fully elucidated. In the current study, total RNA was extracted from renal cortex and isolated glomeruli of streptozotocin-induced diabetic rats 24 hours, 48 hours, 96 hours, one and two weeks after the onset of hyperglycemia (blood glucose >15 mmol/liter), insulin-treated diabetic rats (blood glucose <6.0 mmol/liter), and normal rats. RNA samples were reverse transcribed (RT) and subjected to polymerase chain reaction (PCR) amplication with specific 5′ and 3′ primers for rat transforming growth factor (TGF-β1) and β-actin. RT-PCR analysis revealed that glomerular TGF-β1 mRNA levels increased relative to β-actin as early as 24 hours after the onset of hyperglycemia, reaching a plateau after 96 hours that was sustained at one and two weeks. In cortical samples, TGF-β1 mRNA levels increased less abruptly, reaching a peak one week after the onset of hyperglycemia. Intensive insulin treatment to normalize blood glucose levels attenuated the rise in glomerular and renal cortical TGF-β1 mRNA. Cryostat sections of rat kidneys were immunostained for TGF-β1 utilizing a polyclonal anti-porcine TGF-β1 antibody and semi-quantitative scoring of TGF-β1 immunostaining revealed a twofold increase in diabetic glomeruli after two weeks compared to normal glomeruli. Increased segmental immunostaining for TGF-β1 was also evident in cortical tubules of diabetic rats. These studies establish that TGF-β1 expression in the kidney increases during the phase of rapid renal hypertrophy in diabetic rats. Normalization of blood glucose levels with insulin treatment attenuates the increase in TGF-β1 expression.

Experimental type I diabetes mellitus is characterized by an early increase in kidney size, although the mechanism(s) underlying this response have not been completely elucidated. Studies of streptozotocin-induced diabetic rats have shown that there is a phase of rapid renal growth that follows the onset of hyperglycemia so that both whole kidney weight and glomerular volume increase. The increase in kidney size occurs predominantly by a process of cell hypertrophy [1], although there is a component of tubule cell hyperplasia. The early increase in glomerular volume is accompanied by an increase in filtration surface area, a mechanism that may account in part for the increase in glomerular filtration rate [2]. During the early rapid phase of diabetic glomerular hypertrophy there is a proportional increase in the mesangium, but the later stage of type I diabetes mellitus is characterized by progressive mesangial expansion, eventually leading to a decrease in filtration surface area and a decrease in glomerular filtration rate [3].

In vitro studies have shown that when mesangial [4] and proximal tubule cells [5] are exposed to high glucose concentrations, the cells increase production of the cytokine, transforming growth factor-β1 (TGF-β1). TGF-β1 is an important modulator of cell growth [6]. It inhibits cell division in glomerular mesangial [4, 7–10], epithelial, and endothelial cells [11]. Moreover, studies by Dzau and coworkers have shown that angiotensin II-induced hypertrophy of vascular smooth cells is due to an autocrine effect of TGF-β1 [12]. Recently, Choi and coworkers have shown that mesangial cells hypertrophy when exposed to TGF-β1 *in vitro* [13]. Thus, an early increase in TGF-β1 expression *in vivo* could mediate cellular responses during the early phase of renal and glomerular hypertrophy that follows the onset of hyperglycemia. A sustained increase in TGF-β1 expression after diabetic glomerular hypertrophy is established, as has been demonstrated by Yamamoto et al [14] and Nakamura et al [15], and would then link changes in gene expression during hypertrophy to subsequent glomerular injury.

Accordingly, we sought to determine whether TGF-β1 expression increased in glomeruli and renal cortical tissue of diabetic rats during the early phase of rapid renal hypertrophy.

Methods

Animal model

Male Sprague-Dawley rats weighing 280 to 300 g received streptozotocin, 60 mg/kg body weight, via a single tail vein injection. A first group received no insulin treatment while a second group received insulin twice daily (heat-treated ultralente insulin, Novo Industri A/S, Copenhagen, Denmark) to normalize blood glucose levels. Age-matched normal rats served as the control group. Tail samples of blood were obtained beginning six hours after administration of streptozotocin for determination of blood glucose concentration with an Ames Accutest Glucometer. After the onset of hyperglycemia, defined as blood glucose levels greater than 15 mmol/liter, the blood glucose level was measured daily in the untreated diabetic rats and twice daily in the diabetic rats treated with insulin. Rats were excluded if their blood glucose levels were below 15 mmol/liter. All of the animals had unlimited access to chow (Purina Rat Chow) and water.

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Untreated diabetic rats were studied at 24, 48, and 96 hours, and one and two weeks after the onset of hyperglycemia. Treated diabetic rats were studied one and two weeks after the onset of hyperglycemia. Two groups of age-matched normal rats served as separate control groups. At each time point, the kidneys from five rats were pooled for isolation of total RNA from glomeruli and renal cortical tissue. For immunohistochemical analysis, kidneys of three untreated diabetic rats were obtained two weeks after the onset of hyperglycemia along with three age-matched normal rats.

Glomerular isolation

Rats were anesthetized with Brietal (50 mg/kg) intraperitoneally and the kidneys were rapidly removed. Perirenal fat was trimmed, the capsule stripped off, and the cortex separated from the medulla. The tissue was placed in ice-cold PBS buffer (pH 7.4) and cut into 2 mm^3 pieces. Approximately 1/10 of the tissue was homogenized and used for cortical RNA analysis, and the remainder of the tissue was used for glomerular isolation by the technique of differential sieving. This was performed by passing the tissue through a 250 μm sieve with a glass rod, collected onto a 106 μm sieve, and resuspended in ice-cold PBS before centrifugation at 2000 g for 10 minutes at 4°C. After resuspending the pellet in ice-cold PBS (pH 7.4), the suspension was drawn up into a 10 cc syringe through a 20 gauge needle and discharged several times before centrifugation at 2000 g for 10 minutes at 4°C. The tissue pellet was resuspended, centrifuged and then resuspended in ice-cold PBS (pH 7.4), then pushed through a 75 μm sieve with ice-cold buffer. After the glomeruli were collected on the 75 μm screen and resuspended in ice-cold PBS (pH 7.4), the purity of the preparation was examined with a microscope. On average, there were fewer than five tubular fragments per 100 glomeruli. These glomeruli were then used for RNA isolation.

RNA isolation

Total RNA from both the glomerular and renal cortical tissue was extracted by the single step method of Chomzynsky and Sacchi [16]. Two milliliters of a solution containing guandinium isothiocyanate and 2-mercaptoethanol were added to the glomerular suspension and vortexed; 0.2 ml of 2 M Na acetate, 2.0 ml of phenol, and 0.4 ml of chloroform were then added, vortexed and the solution was placed on ice for 15 minutes. An equal volume of isopropanol was added to the aqueous phase and incubated at −20°C for 30 minutes. Total RNA was pelleted by centrifugation at 8000 rpm for 20 minutes, resuspended in 300 μl of a solution containing guandinium isothiocyanate and 2-mercaptoethanol and 300 μl of isopropanol, and incubated at −20°C for 30 minutes. After centrifugation, the pellet was resuspended in 300 μl of cold 70% ethanol, and the RNA stored in DEPC-treated water at −70°C until used. After thawing, the purity and concentration was determined by measuring the optical densities at 260 and 280 nm. The A260/A280 ranged from 1.75 to 1.95. Twenty micrograms of total RNA were separated by electrophoresis on 1% agarose gels containing ethidium bromide, transferred to a nylon filter (Hybond N), and cross-linked by UV illumination.

RT-PCR

One microgram of total RNA (10 μl vol) was combined with 10 U RNAsin and 300 pmol of random hexamers. The mixture was heated to 65°C for five minutes and cooled on ice. After the addition of 20 U of Moloney Murine Leukemia Virus reverse transcriptase (M-MLV-RT), 10 U of RNAsin, and 4 μl of 5× RT buffer so that the final volume was 20 μl, the reaction mixture was incubated at 42°C for two hours. The reaction was stopped by heating at 95°C for five minutes. The sample was then diluted to 100 μl with deionized water and stored at −70°C prior to subsequent amplification.

Primers for the polymerase chain reaction (PCR) were designed to flank at least one putative intron site for rat TGF-β1, alpha 2 type IV collagen, and β-actin. For TGF-β1, the sense primer corresponded to base pairs 1142-1168 and the antisense primer to base pair 1520-1546 [17]. The two primers were constructed to cross intron #5 and #6. For alpha 2 type IV collagen, the sense primer corresponded to base pairs 5562-5584 and the antisense primer to base pair 6100-6123, as described by Peten et al [18]. For β-actin, the sense primer corresponded to base pairs 331-354 and the antisense primer to base pair 550-571 [19]. The two primers were constructed to cross intron #2. The primer sequences were as follows:

Rat TGF-β1	5′	CGA GGT GAC CTG GGC ACC ATC CAT GAC
	3′	CTG CTC CAC CTT GGG CTT GCG ACC CAC
Mouse α 2 Type IV collagen	5′	ACT CAT TCC AAC CGT CTG TCA GC
	3′	GCA AAT CAT TGA CAG TGG CGT CTA
Rat β-actin	5′	AAC CCT AAG GCC AAC CGT GAA AAG
	3′	TCA TGA GGT AGT CTG TCA GGT

The expected PCR product size for the TGF-β1 primers was 405 base pairs, for the β-actin primers, 240 base pairs, and for the α 2 type IV collagen, 562 base pairs. For amplication, 5 μl of the RT product were combined with 6.5 μl of PCR mix containing 0.1 μM of each of the primer pairs. Two units of Taq polymerase were then added. Coamplification of β-actin was performed to standardize the amount of RNA subjected to reverse transcription for each time point. The sample was placed onto a Perkin Elmer DNA Thermal Cycler (Model 480) and heated to 94°C for four minutes, followed by 30 temperature cycles. Each cycle consisted of three periods: (1) denature, 94°C for three minutes; (2) cool-anneal, 60°C for three minutes; (3) heat-extend, 72°C for three minutes. When the number of temperature cycles was increased to 34, there was an increase in both the 405 bp and 240 bp PCR product with the above conditions. Serial dilutions (1:2 and 1:4) of the RT products were also subjected to PCR amplication for 30 temperature cycles for the normal and two-week untreated diabetic samples. After amplification, the PCR products were separated by electrophoresis on a 1.0% agarose gel containing ethidium bromide, photographed, and transferred to a nylon membrane (Hybond N).

An antisense oligonucleotide, synthesized to serve as amplification product-specific probe for TGF-β1, spanned base pairs 1394–1424 (5′ GCT GTA CTG TGT GTC CAG GTC CAG GCT CCA AAT GTA 3′), respectively. A second antisense oligonucleotide was synthesized to serve as amplification product-specific probes for β-actin and spanned base pairs 427–456 (5′ CAC AAT GCC AGT GGT ACG ACC AGA GGC ATA 3′), respectively. A third antisense oligonucleotide was synthesized to serve as amplication product-specific probes for the α 2 chain of type IV collagen and spanned base pairs 5781–5804 (5′ CCT GCA GTC TTC CTA AAA TGA GGC 3′), respectively [18]. By design, each oligonucleotide localized to a sequence that was inside the amplification primer sequences. The oligonucleotides was labeled with ^{32}P-ATP (4500 Ci/mmol) utilizing T4 DNA kinase. The nylon

filters were hybridized overnight at 55°C, washed and exposed to X-ray film at room temperature with an intensying screen for 30 to 120 minutes. The autoradiograms were quantitated with a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments) utilizing a MacIntosh Classic II (System 7.0) and Dynamax HPLC Method Manager (V1.2).

Immunohistochemistry

Following brietal anesthesia (50 mg/kg), the kidneys were removed and the capsule excised. Blocks of renal cortical tissue were immersed in OCT, and snap frozen in liquid nitrogen. Cryostat sections (5 μm) were mounted on poly-L-lysine coated slides. Immunoperoxidase staining was performed by the ABC method. Sections were fixed in acetone (4°C), air dried, then quenched in methanol containing 3% H_2O_2 at room temperature for 20 minutes. The sections were washed for five minutes in tap water followed by a 15 minute incubation in pepsin to activate antigenic sites. The sections were incubated at room temperature overnight with specific TGF-β1 antiserum (R&D Systems, Minneapolis, Minnesota, USA) and diluted 1:100 in antibody diluting buffer (Dimension Laboratories Ltd., Missisauga, Ontario, Canada). After a PBS wash, sections were incubated at room temperature for 30 minutes with an antirabbit IgG biotinylated antibody (Vectastain Elite Kit, Vector Lab, Burlingame, California, USA), diluted 1:200 with PBS. Finally, sections were washed and then incubated for 30 minutes with an avidin-biotin-peroxidase complex (diluted 1:50 in PBS). The sites of peroxidase activity were visualized by incubation in DAB solution (Zymed Lab, California, USA) for two minutes. Sections were counterstained with Harris modified hematoxylin with acetic acid (Fisher Scientific) for 45 seconds, washed, cleaned, and covered with a glass cover slip. Negative control experiments were performed by either (1) replacing the primary antibody with antibody diluting buffer, or (2) replacing the primary antibody with nonimmune rabbit antiserum (1:4000).

Semiquantitative assessment of immunohistochemical staining for TGF-β1

The immunostaining was scored by examining each glomerular profile (range 18 to 27 profiles) in cryostat sections from three normal and three untreated diabetic rats. Each profile was assigned a score from 0 to 4, where 0 is no staining, 1 minimal (25% of the glomerular tuft), 2 mild (50% of the glomerular tuft), 3 moderate (75% of the glomerular tuft), and 4 marked (100% of the glomerular tuft). A score was derived for each animal:

$$\text{Score} = \sum_{i=1}^{4} \text{Fi}(i)$$

where Fi is the percentage of glomeruli in the rat with a given score i [20].

Mean glomerular volume

Glomerular tuft volume (VG) was determined by measuring the mean glomerular cross-sectional area (AG) of all glomerular profiles in the cryostat sections. A 100 point grid was superimposed over each glomerular profile. Each point represented 625 $\mu 2$ and the number of points overlying each profile was enumerated. Mean glomerular volume was calculated from AG by the method of Weibel:

$$\text{VG} = \frac{\beta}{k} \times (\text{AG})^{3/2} \times (\mu^3)$$

where $\beta = 1.38$ is the shape coefficient for spheres (the idealized shape of glomeruli) and $k = 1.1$ is a size distribution coefficient [21].

Table 1. Values for blood glucose in normal rats (NC), untreated diabetic rats (D-), and insulin-treated diabetic rats (DI-)

Group	Blood glucose *mmol/liter*
NC	4.7 ± 0.1
D- 24 hr	18.4 ± 1.7
D- 48 hr	16.8 ± 1.4
D- 96 hr	17.6 ± 1.9
D- 1 week	> 22.0
D- 2 week	> 22.0
DI- 24 hr	16.0 ± 1.4
DI- 48 hr	10.6 ± 1.1
DI- 72 hr	7.8 ± 0.8
DI- 96 hr	2.9 ± 0.5
DI- 1 week	4.7 ± 0.3
DI- 2 week	5.4 ± 0.8

Values are means ± SE.

Analysis

The statistical significance of differences among values of individual parameters was assessed with an unpaired Student's *t*-test. Significance was defined as $P < 0.05$.

Results

Animal model

Streptozotocin was administered to rats weighing an average of 276 ± 6 g. Over the course of the experiment, diabetic rats lost weight so that by two weeks, the mean body weight was 235 ± 10 g. Average blood glucose levels for normal rats (NC), untreated diabetic rats (D), and the insulin-treated diabetic rats (DI) during the course of the study are shown in Table 1. In accord with the experimental design, blood glucose levels remained above 15 mmol/liter in the diabetic rats, exceeding 22 mmol/liter two weeks after the administration of streptozotocin. Despite the decrease in body weight that accompanied early untreated hyperglycemia, there was an absolute increase in kidney weight compared to age-matched normal rats. As illustrated in Figure 1, whole kidney weight averaged 1.60 ± 0.07 g after two weeks of hyperglycemia compared to 1.31 ± 0.06 g in the normal rats ($P < 0.05$). The ratio of kidney weight to body weight was almost twofold greater in the diabetic rats compared to the normal rats (0.69 ± 0.03% vs. 0.46 ± 0.02%, $P < 0.05$). As expected, there was a significant increase in glomerular volume in the diabetic rats compared to the age-matched normal rats, with mean values averaging 0.97 ± 0.16 10^6 μ^3 in normal rats and 1.97 ± 0.12 10^6 μ^3 in diabetic rats ($P < 0.05$) (Fig. 2). Intensive insulin treatment served to normalize blood glucose levels in streptozotocin-induced diabetic rats (Table 1).

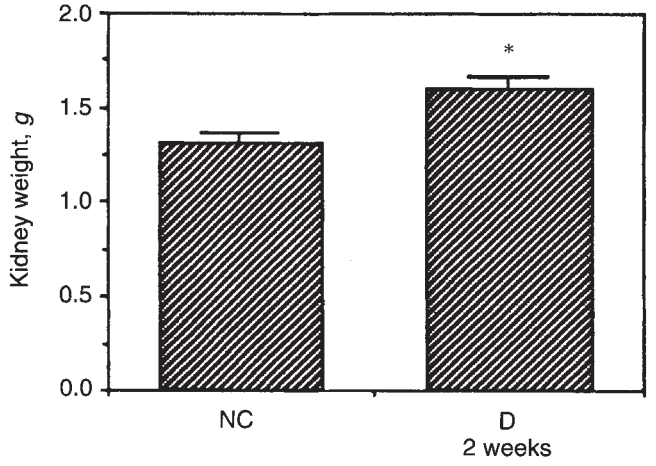

Fig. 1. *Kidney weight in normal rats (NC) and untreated diabetic rats 2 weeks after the onset of hyperglycemia (D- 2 week).* Kidney weight was significantly greater in untreated diabetic rats than in age matched normal rats. Values are mean ± 1 SE. $^*P < 0.05$.

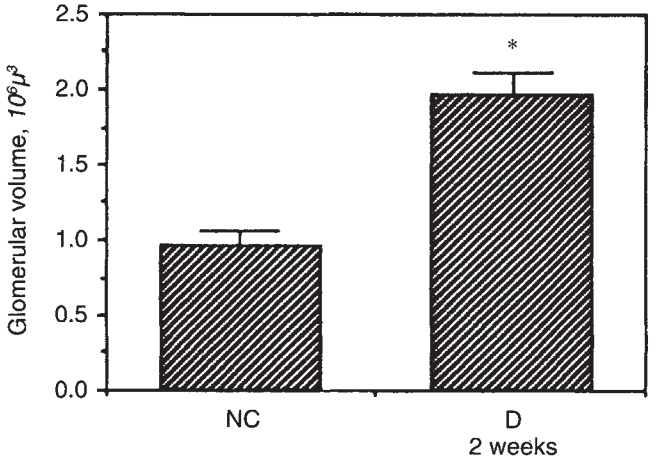

Fig. 2. *Glomerular volume (VG) in normal rats (NC) and untreated diabetic rats 2 weeks after the onset of hyperglycemia (D- 2 week).* VG was significantly greater in untreated diabetic rats than in age matched normal rats. Values are mean ± 1 SE. $^*P < 0.05$.

RT-PCR

The effect of hyperglycemia on steady state levels of TGF-β1 mRNA relative to steady state levels of mRNA for β-actin was determined by coamplication RT-PCR in glomerular and renal cortical samples (Figs. 3 and 4). Figure 3 shows the autoradiographs that were used for densitometry. Individual values for the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin in diabetic rats 24, 48, 96 hours, one week and two weeks after the onset of hyperglycemia, in normal rats, and insulin-treated diabetic rats are listed in Table 2.

In glomerular samples from normal rats, the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin was 0.39 (Fig. 4). A similar ratio was obtained from a separate group of five normal rats. Twenty-four hours after the onset of hyperglycemia there was a 1.75-fold increase in the ratio of the densitometry measures of the PCR products for glomerular TGF-β1 and β-actin so that by 96 hours there was a 2.5-fold increase. The increased ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin was sustained one and two weeks after the onset of hyperglycemia in glomeruli. Intensive insulin treatment reversed the trend observed in untreated diabetic rats, and the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin approached normal values after two weeks. The relative changes in mRNA levels were also observed when serial dilutions of the RT products from normal and two-week untreated diabetic rats were subjected to PCR amplication for 30 temperature cycles.

In renal cortical samples from normal rats, the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin was 0.61 (Fig. 4). A similar ratio, 0.58, was obtained in a separate group of five normal rats. The change in the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin was delayed in renal cortical tissue compared to glomeruli. Twenty-four hours after the onset of hyperglycemia there was only a 1.25-fold increase in the the ratio of the densitometry measures of the PCR products for renal cortical TGF-β1 and β-actin. By 96 hours there was a 2.0-fold increase the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin. The ratio continued to increase so that one week after the onset of hyperglycemia there was a 3.0-fold increase in the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin. Like the measures obtained for glomeruli, the increase in the ratio was sustained two weeks after the onset of hyperglycemia in renal cortical tissue. Intensive insulin treatment initiated after the onset of hyperglycemia reversed the trend observed in the untreated diabetic rats, and the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin approached normal values after two weeks.

A comparison was made between the densitometry measures of the PCR products for TGF-β1 and β-actin from euglycemic rats and hyperglycemic rats. Values from normal rats (NC) and euglycemic diabetic rats (DI) were grouped ($N = 4$) and compared to values from the hyperglycemic untreated diabetic rats (D) ($N = 5$). For glomerular samples, there was a significant increase in the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin in hyperglycemic diabetic rats compared to the euglycemic rats (0.87 ± 0.08 vs. 0.57 ± 0.08, $P < 0.05$). For renal cortical samples, there was also a significant increase in the ratio of the densitometry measures of the PCR products for TGF-β1 and β-actin in hyperglycemic diabetic rats compared to the euglycemic rats (0.67 ± 0.05 vs. 1.30 ± 0.18, $P < 0.05$). These results are illustrated in Figure 5.

The effect of hyperglycemia on steady state levels of α 2 type IV collagen mRNA relative to steady state levels of mRNA for β-actin was determined by coamplication RT-PCR in glomeruli of normal rats ($N = 3$) and untreated diabetic rats ($N = 3$) one week after the onset of hyperglycemia (Fig. 6). In samples from normal rats, the ratio of the densitometry measures of the PCR products for α 2 type IV collagen and β-actin was 1:40. One week after the onset of hyperglycemia there was a 1.3-fold increase in the ratio of the densitometry measures of the PCR products for α 2 type IV collagen and β-actin in glomeruli from diabetic rats compared to glomeruli from normal rats.

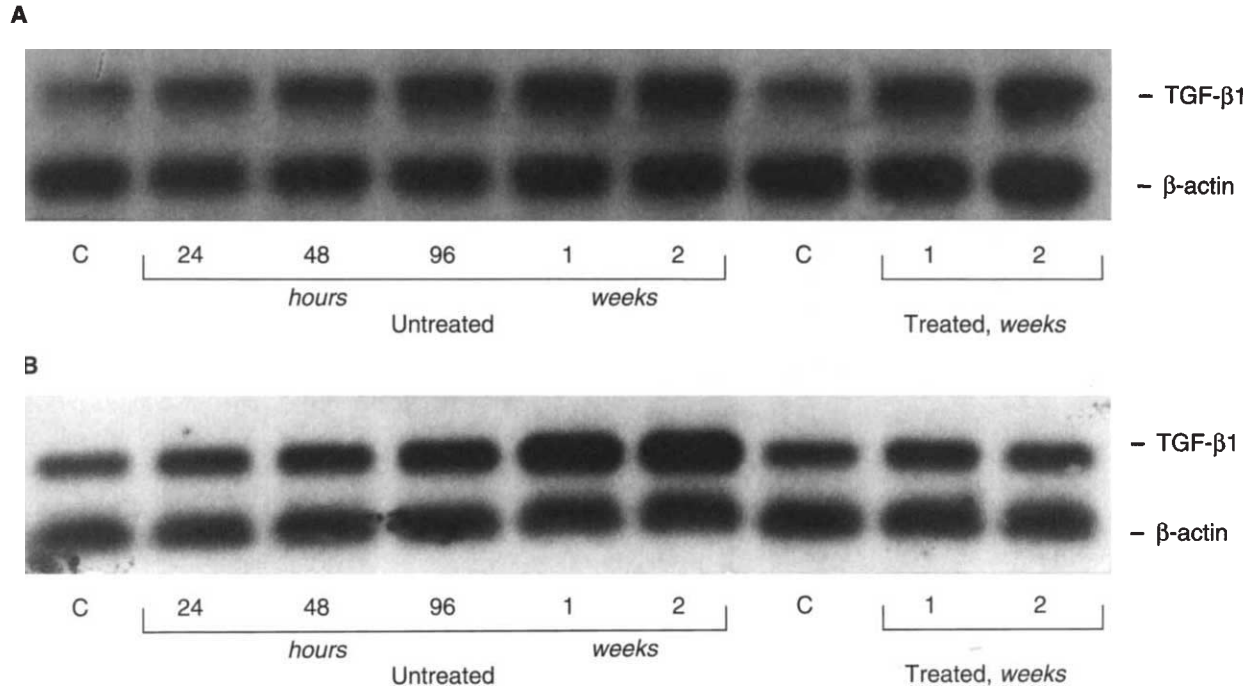

Fig. 3. *Autoradiographs of the coamplification of TGF-β1 and β-actin mRNA in glomeruli (A) and renal cortical tissue (B) from normal rats (C), untreated diabetic rats (D-) and insulin treated diabetic rats (DI-).*

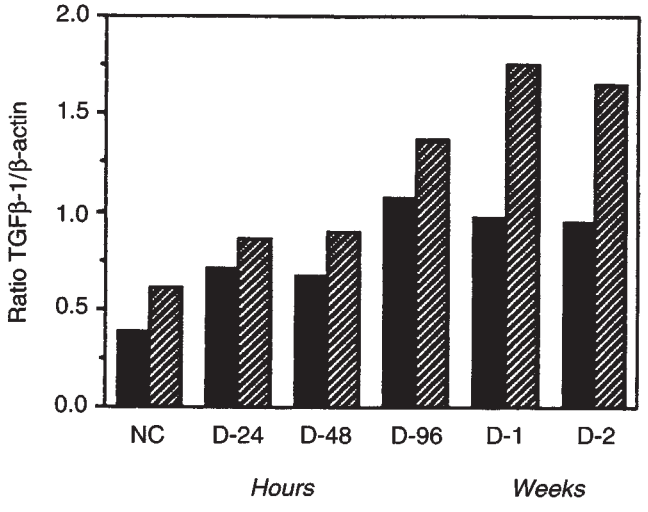

Fig. 4. *Ratios of the densitometry readings for the RT-PCR amplification of TGF-β1 and β-actin mRNA in glomeruli (closed bars) and renal cortical tissue (hatched bars) from normal rats (NC) and untreated diabetic rats (D-).*

Table 2. Ratios of the densitometry readings for the RT-PCR amplification products of TGF-β1 and β-actin in normal rats (NC), untreated diabetic rats (D-), and insulin-treated diabetic rats (DI-)

Time	Renal cortex	Glomeruli
NC	0.61	0.39
D- 24 hr	0.86	0.71
D- 48 hr	0.90	0.67
D- 96 hr	1.37	1.07
D- 1 week	1.75	0.97
D- 2 week	1.65	0.94
NC	0.58	0.39
DI-1 week	0.78	0.68
DI-2 week	0.73	0.66

Immunohistochemistry

The effect of hyperglycemia on immunostaining for TGF-β1 in the renal cortex of the kidney is illustrated in the five panels of Figure 7. Panels A and D show the appearance of kidney tissue from both normal or diabetic rats when the primary antibody is omitted from the protocol or replaced with non-immune serum. In the absence of specific antiserum there is no immunoperoxidase staining of glomeruli (Panel A) or renal tubules (Panel D). Panel B shows typical glomerular immunostaining for TGF-β1 in a normal rat. In this glomerulus, the immunostaining is more prominent at the vascular pole of the glomerulus. Panel C shows typical glomerular immunostaining for TGF-β1 in a diabetic rat two weeks after the onset of hyperglycemia. Marked glomerular immunostaining is present in the glomerular capillary tuft. Panel E shows typical tubular immunostaining for TGF-β1 in cortical tubules of normal rats. The immunostaining pattern was faint and segmental. Panel F shows the increased segmental immunostaining for TGF-β1 that was evident in cortical tubules of diabetic rats two weeks after the onset of hyperglycemia.

Semiquantitative scoring of glomerular immunostaining for TGF-β1 in normal and diabetic rats yielded values of 1.67 ± 0.06 (N = 3) and 3.25 ± 0.30 (N = 3), respectively, so that there was a twofold increase in immunostaining for TGF-β1 during the early phase of diabetic glomerular hypertrophy (Fig. 8).

Discussion

The mechanisms that lead to renal injury in patients with type I diabetes mellitus remain poorly understood. Early investigators

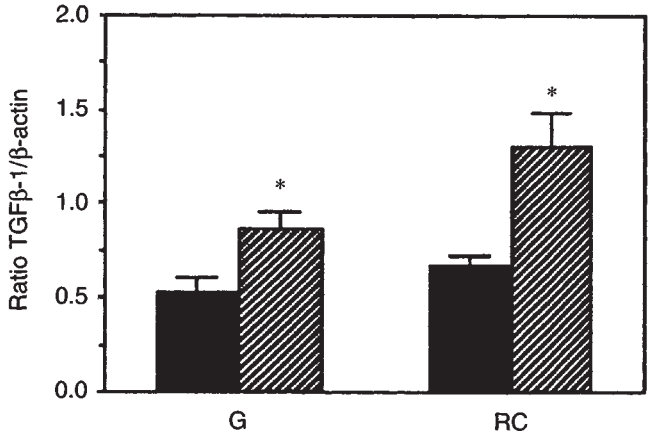

Fig. 5. *Ratios of the densitometry readings for the RT-PCR amplification of TGF-β1 and β-actin mRNA from euglycemic rats (closed bars) (N = 4) and hyperglycemic rats (hatched bars) (N = 5) in glomeruli (G) and renal cortical tissue (RC).* Values are mean ± 1 SE. *$P < 0.05$ vs. euglycemic rats.

showed that the glomerular filtration rate was greater than normal in young adults with diabetes mellitus [22], while further studies established that the increase in glomerular filtration rate was accompanied by an increase in kidney size and glomerular volume [23]. The subsequent observation that enlarged, hyperfiltering kidneys of diabetic patients ultimately fail suggests that mechanisms responsible for maintaining early increases in glomerular filtration rate and kidney size may be important in the pathogenesis of renal injury [24].

More recently, studies by Yoshida, Fogo and Ichikawa [25] of rats with renal ablation have led to the suggestion that an increase in glomerular size is an important determinant of glomerular injury. Glomerular growth in experimental diabetes mellitus is characterized predominantly by structural hypertrophy, as autoradiographic studies have shown that there is no cellular proliferation in the glomerulus [26, 27]. Early glomerular hypertrophy is accompanied by an increase in peripheral capillary wall surface area that parallels the rise in glomerular filtration rate [3]. Later, glomeruli develop localized and/or diffuse expansion of the mesangial matrix, leading to a reduction in glomerular filtration rate [3]. Thus, it is tempting to speculate that changes in gene transcription accompanying diabetes-induced glomerular hypertrophy are important in the development of glomerular injury. Accordingly, the objective of the current study was to examine changes in mRNA levels and protein expression during the phase of rapid glomerular hypertrophy that follows the onset of hyperglycemia.

A number of polypeptide growth factors regulate growth and extracellular matrix production but many recent studies have focused on transforming growth factor (TGF-β1). *In vitro*, TGF-β1 is expressed by proximal tubule cells and glomerular endothelial, epithelial and mesangial cells [7, 9, 11, 28]. TGF-β1 has been implicated in the pathogenesis of mesangial expansion in experimental glomerulonephritis [10, 29], and Wolf et al have reported that exposure to a high glucose concentration doubles levels of mRNA for TGF-β1 in cultured mesangial cells [4]. Therefore, the first major aim of the current study was to test the hypothesis that steady state levels of mRNA for TGF-β1 increase in glomeruli during the phase of rapid glomerular hypertrophy of diabetic rats that follows the onset of hyperglycemia.

Our major finding is that TGF-β1 expression increases during the phase of rapid renal hypertrophy in both the glomeruli and renal tubules of rats with experimental type I diabetes mellitus. Two weeks after the onset of hyperglycemia, when renal hypertrophy is established but before glomerulosclerosis develops, there is a demonstrable increase in immunostaining for TGF-β1 in both the glomeruli and renal cortical tubules. In contrast, MacKay and coworkers found that mRNA levels for TGF-β1 decreased in glomeruli of rats one to two weeks after uninephrectomy [30]. It is tempting to speculate that a sustained increase in TGF-β1 expression may play a role in diabetic glomerular injury. Moreover, taken together, these observations suggest that glomerular hypertrophy *per se* may not be a determinant of injury. The pattern of gene expression accompanying glomerular hypertrophy may be more important.

The RT-PCR analysis further showed that TGF-β1 expression rose more rapidly in glomeruli than in renal cortical tissue. Steady state levels for TGF-β1 mRNA increased approximately twofold 24 hours after the onset of hyperglycemia in glomeruli, while a comparable increase occurred only after 96 hours in renal cortical tissue. This time course corresponds to the phase of rapid glomerular hypertrophy [31]. Rasch and Norgaard have studied ^{3}H-thymidine uptake in glomeruli and renal cortical tissue of diabetic rats during this period [26]. No uptake was demonstrated in glomeruli, but there was an early increase in ^{3}H-thymidine uptake in cortical tubules, indicating some cellular hyperplasia. ^{3}H-thymidine uptake returned to baseline four days after the onset of hyperglycemia.

TGF-β1 has a wide spectrum of cellular effects, and one of the most prominent is inhibition of cell proliferation [32, 33]. Our observations suggest that TGF-β1 may modulate the cellular growth response to hyperglycemia *in vivo* because an early increase in glomerular TGF-β1 expression could function in an autocrine and/or paracrine fashion to prevent cell division. Recent studies by Choi et al support this hypothesis [13]. TGF-β1 directly inhibited mesangial cell proliferation and increased cell size *in vitro* [13]. Moreover, when mesangial cells are exposed to high glucose concentrations, the cells proliferate, but a subsequent rise in TGF-β1 production inhibits proliferation [4, 28]. Neutralizing antibody to TGF-β1 sustains the proliferative response, indicating that TGF-β1 functions in an autocrine fashion to inhibit cell division. These studies suggest that the early increase in TGF-β1 in diabetic glomeruli may play a similar role *in vivo*. Similar effects have been shown *in vitro* with proximal tubule cells [5], so that the early tubular cell hyperplasia in the renal cortex, observed by Rasch and Norgaard may be attenuated by the subsequent rise in TGF-β1 expression.

The mechanism(s) by which TGF-β1 controls the cell cycle has been actively studied. The TGF-β1 receptor is a serine/threonine kinase [34–38], and growth inhibition by TGF-β1 have been linked to the phosphorylation status of the retinoblastoma gene product (RB protein) [39]. TGF-β1 arrests cells in G1 by reducing the phosphorylation of RB protein [40]. This post-translational modification of RB protein prevents the cell from entering the S phase of the cell cycle [26, 39]. In addition, TGF-β1 may also change the phosphorylation state of P53 [41], as well as reduce the level of c-*myc* protein [42], effects that prevent cell proliferation.

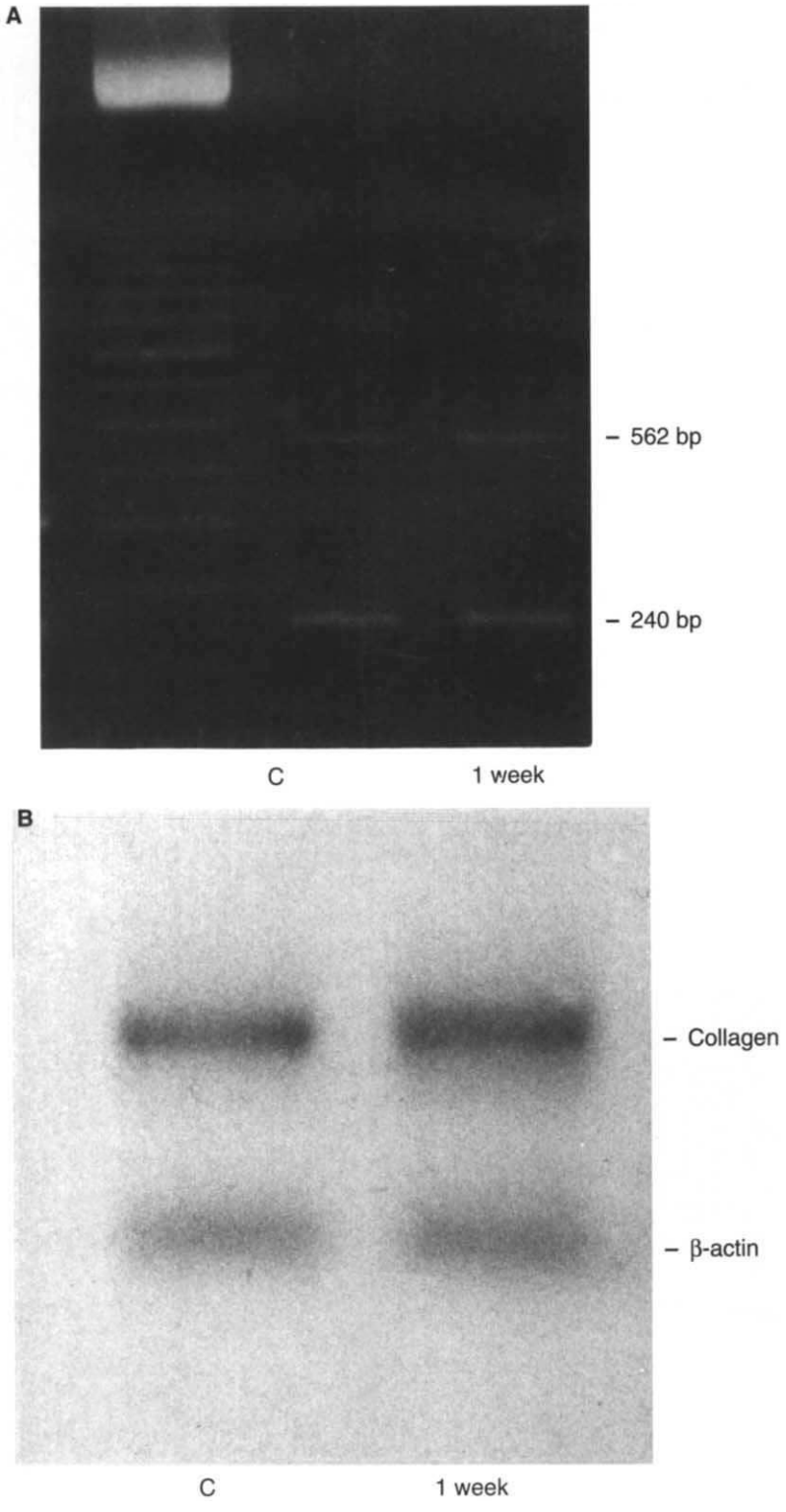

Fig. 6. A. *UV transilluminated photograph of the PCR products for α 2 type IV collagen (562 bp) and β-actin (240 bp) in normal rats* (**C**), *and untreated diabetic rats, one week (D- 1 week) after the onset of hyperglycemia.* **B.** Autoradiographs of the coamplification of α 2 type IV collagen and β-actin mRNA in normal (C) and untreated diabetic glomeruli (D- 1 week).

Previous studies have shown that mRNA levels for TGF-β1 do not always correlate with protein levels [30]. Therefore, the second major aim of the current study was to test the hypothesis that immunostaining for TGF-β1 would increase in glomeruli and renal cortical tissue along with the changes in mRNA levels during the phase of rapid glomerular hypertrophy that follows the onset

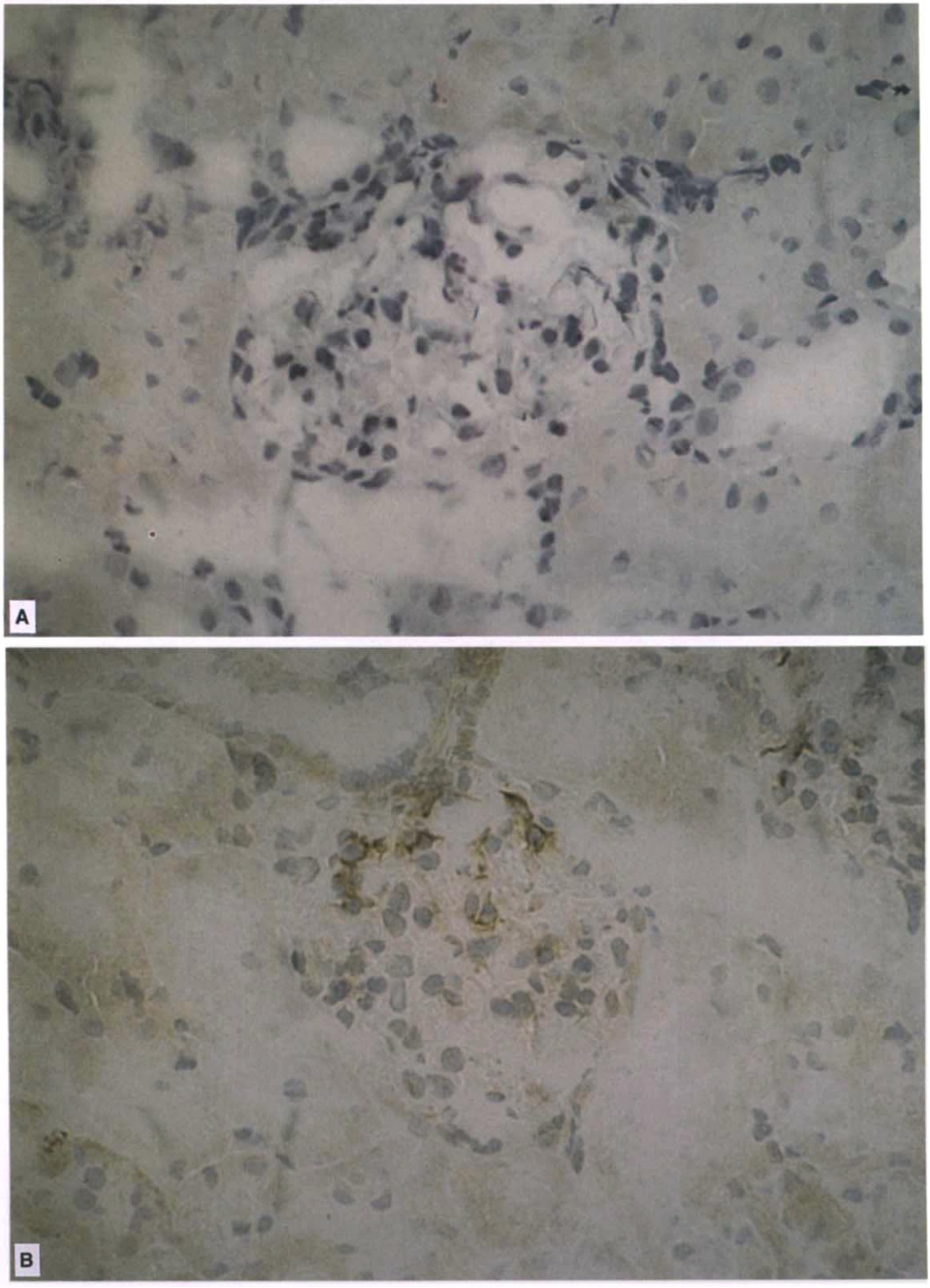

Fig. 7. *Photomicrographs of TGF-β1 immunoreactivity in normal and diabetic rat kidney tissue.* **A.** There is no immunostaining in glomeruli when the primary antibody is ommitted from the protocol or replaced by non-immune rabbit serum. **B.** Typical glomerular immunostaining for TGF-β1 in a normal rat. **C.** Typical glomerular immunostaining for TGF-β1 in an untreated diabetic rat two weeks after the onset of hyperglycemia. **D.** There is no immunostaining in tubules when the primary antibody is ommitted from the protocol or replaced by non-immune rabbit serum. **E.** Typical segmental tubular immunostaining for TGF-β1 in a normal rat. **F.** Typical segmental tubular immunostaining for TGF-β1 in an untreated diabetic rat two weeks after the onset of hyperglycemia (mag ~ 530×).

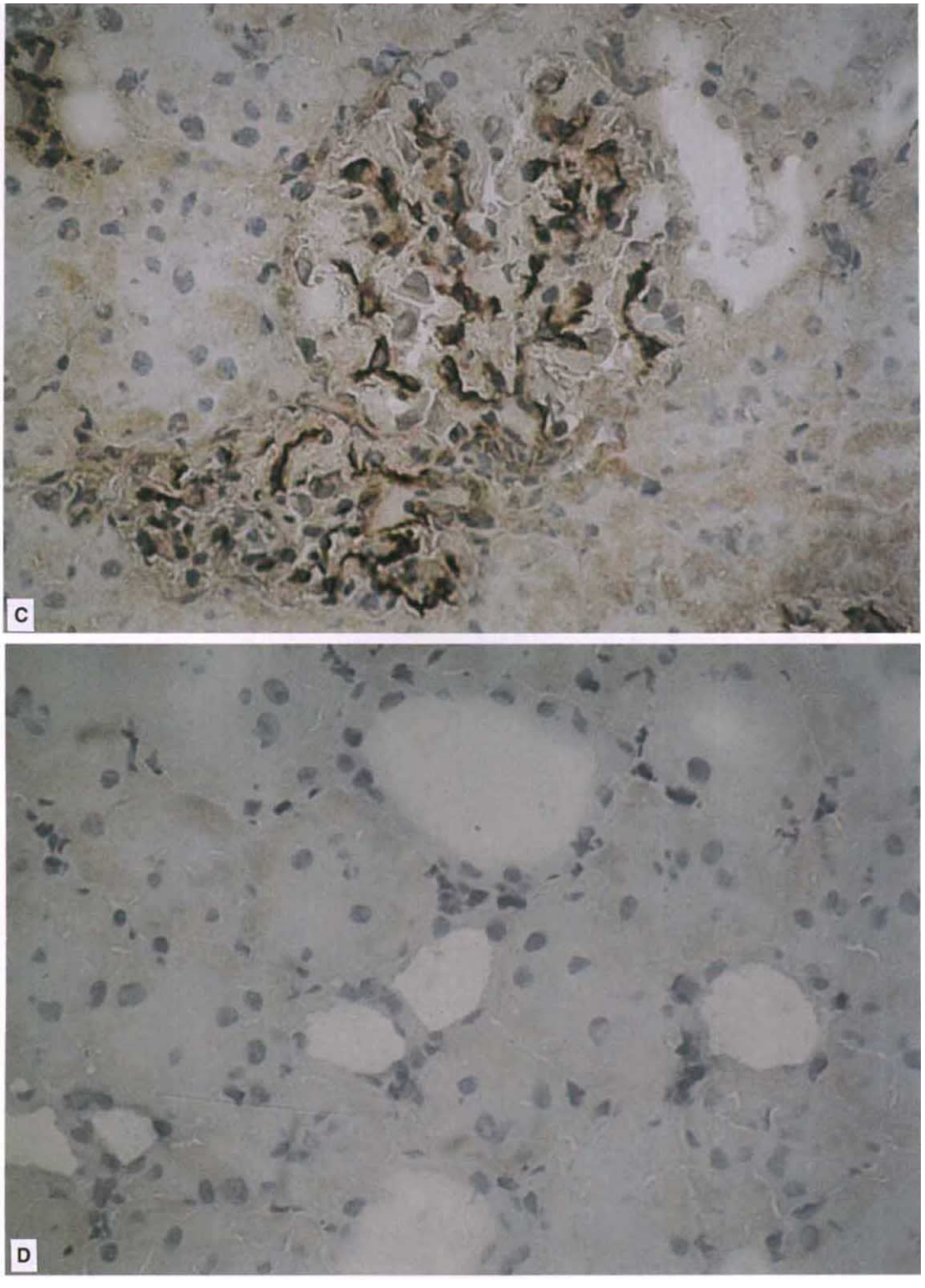

Fig. 7. *Continued.*

of hyperglycemia. Two weeks after the onset of hyperglycemia, when there was an approximately twofold increase in TGF-β1 mRNA levels, we found that there was a marked increase in TGF-β1 immunostaining in both glomeruli and renal cortical tissue, demonstrating that changes in TGF-β1 mRNA levels were associated with a change in protein expression.

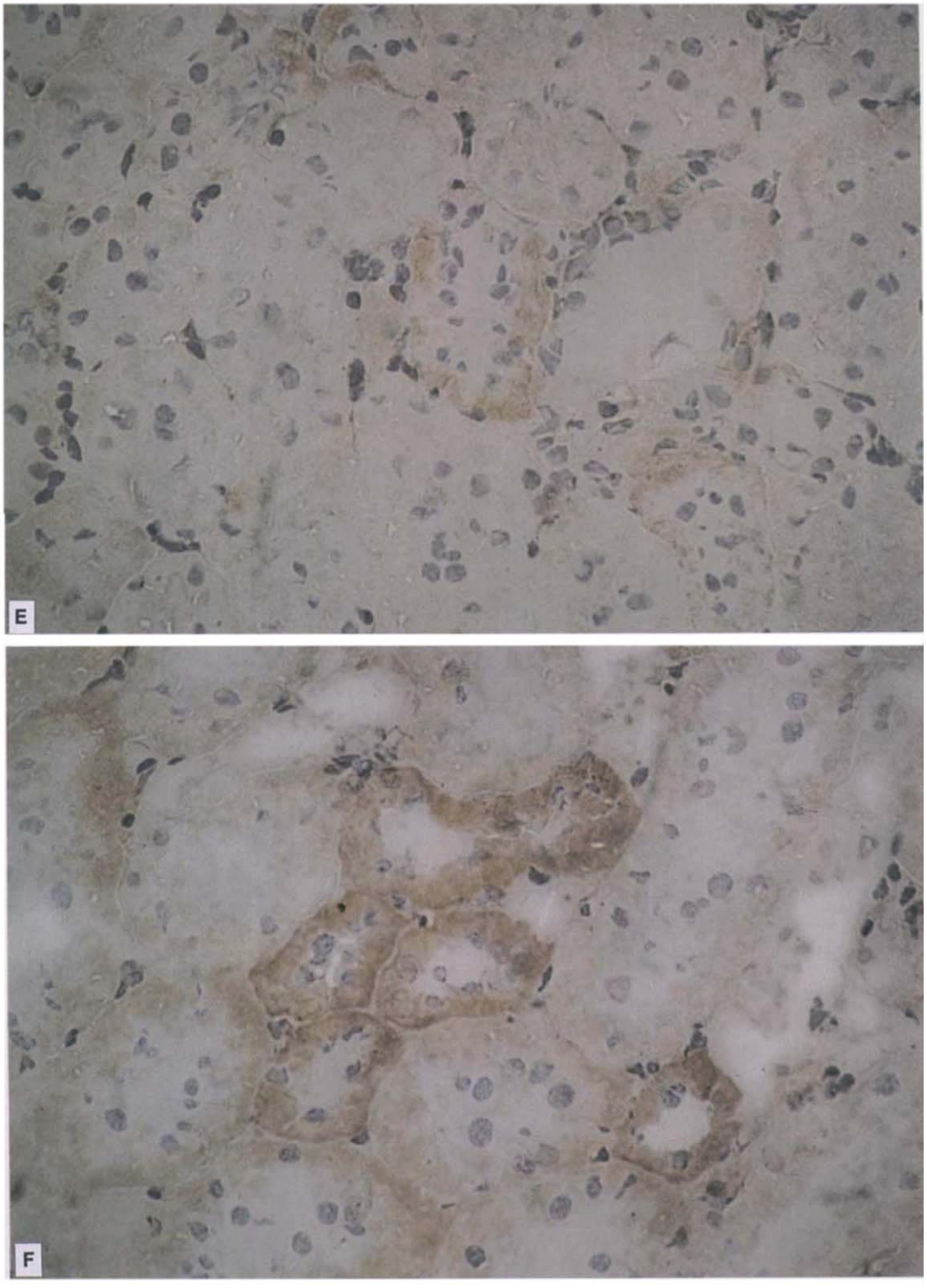

Fig. 7. *Continued.*

Steffes, Brown and Basgen have shown that islet cell transplantation normalizes blood glucose levels and reduces mesangial expansion in diabetic rats [43]. Taken together, these studies suggest that renal growth and factors that lead to mesangial expansion in diabetic glomeruli are at least partially reversible. Accordingly, the third aim of the current study was to test the

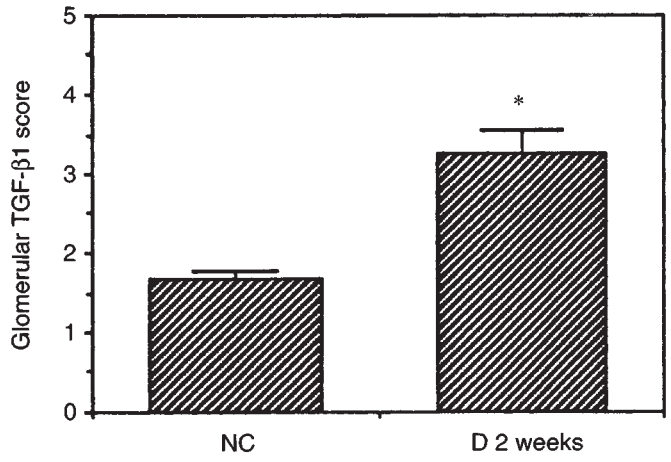

Fig. 8. *Glomerular TGF-β1 immunostaining score in normal rats (NC) and untreated diabetic rats 2 weeks after the onset of hyperglycemia (D- 2 week).* There was a significant increase in TGF-β1 immunostaining in the glomeruli of the diabetic rats. Values are mean ± 1 SE. $^*P < 0.05$.

hypothesis that normalization of blood glucose levels by intensive insulin treatment would prevent the rise in mRNA for TGF-β1 in glomeruli. Importantly, intensive insulin treatment to normalize blood glucose levels attenuated the changes in TGF-β1 mRNA. Moreover, the results of these studies showed that changes in TGF-β1 expression were not due to a non-specific effect of streptozotocin.

Our results are consistent with the hypothesis that there may also be a link between early changes in gene expression during glomerular hypertrophy and subsequent glomerular injury. One week after the onset of hyperglycemia co-amplification RT-PCR showed a modest 1.3-fold increase in glomerular mRNA levels for α 2 type IV collagen compared to β-actin. Fukui et al have shown that mRNA levels for α 1 type IV collagen also increase in the glomeruli of diabetic rats, although Northern blot analysis was performed four weeks after the onset of hyperglycemia [44]. Ihm et al examined α 1 type IV collagen mRNA levels in the glomeruli of diabetic rats one week after the onset of hyperglycemia and were unable to detect any increase in glomerular mRNA levels for α 1 type IV collagen by either slot-blot hybridization or *in situ* hybridization [45]. There was no difference between the glomerular volumes of control and diabetic rats seven or 28 days after administration of streptozotocin in this study, which may account, in part, for the modest increase in α 2 type IV collagen mRNA that we observed in the current study.

It is tempting to speculate that an early rise in TGF-β1 expression in the diabetic glomeruli may be responsible for an increase in α 2 type IV collagen mRNA levels. TGF-β1 has been shown to modulate extracellular matrix production by cells *in vitro* [6, 32, 33, 46–48], and extracellular matrix proteins accumulated in the mesangium and basement membrane of diabetic glomeruli [49–52]. *In vitro*, glomerular mesangial and epithelial cells [53, 54] both increase synthesis of extracellular matrix proteins including proteoglycans, decorin, fibronectin, type IV collagen and laminin in response to TGF-β1 [46, 54–56]. Moreover, TGF-β1 inhibits production of collagenases [57, 58], and increases production of tissue inhibitors of metalloproteinases (TIMP) [59]. Both of these effects could lead to a decrease in extracellular matrix protein degradation, thus contributing to the accumulation of matrix proteins in the diabetic glomerulus [44, 45, 49–52, 60–63].

Studies by Border and coworkers have directly implicated TGF-β1 in the pathogenesis of extracellular matrix protein accumulation in experimental glomerulonephritis because neutralizing antibodies to TGF-β1 attenuate glomerular injury [10, 29, 64], and a recent report by Nakamura and coworkers [54] indicates that mRNA levels for TGF-β1 remain elevated in glomeruli up to 24 weeks after the onset of hyperglycemia [10]. Yamamoto et al have also reported that TGF-β1 immunostaining is increased in the glomeruli of diabetic rats and the glomeruli of patients with clinical diabetes mellitus [14]. In the rats with experimental diabetes mellitus the increase in TGF-β1 correlated with increases in expression of the extracellular matrix proteins fibronectin and biglycan. Taken together, these studies suggest that an early and sustained increase in TGF-β1 expression in diabetic glomeruli may be responsible for the development of diabetic glomerulopathy.

In summary, the current study establishes that there is an increase in TGF-β1 mRNA levels and protein expression in glomeruli and renal cortical tissue during the phase of rapid renal hypertrophy that follows the onset of hyperglycemia in streptozotocin-induced diabetic rats. Normalization of blood glucose levels with insulin treatment attenuates the rise in TGF-β1 mRNA levels. These studies suggest that changes in gene expression during glomerular hypertrophy may link injury and structural hypertrophy.

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Reprint requests to Stuart J. Shankland, M.D., 13 EN-243, The Toronto Hospital (Toronto General Site), 200 Elizabeth Street, Toronto, Ontario M5G 2C4, Canada.

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