Kidney International, Vol. 52 (1997), pp. 973–984

Exposure of human renal proximal tubular cells to glucose leads to accumulation of type IV collagen and fibronectin by decreased degradation

Aled O. Phillips, Robert Steadman, Kimberley Morrisey, John Martin, Lisa Eynstone, and John D. Williams

Institute of Nephrology, University of Wales College of Medicine, Cardiff Royal Infirmary, Newport Road, Cardiff, Wales, United Kingdom

Exposure of human renal proximal tubular cells to glucose leads to accumulation of type IV collagen and fibronectin by decreased degradation. Thickening and reduplication of the tubular basement membrane has been reported as an early event in diabetic nephropathy. In the current study we examined the effects of elevated D-glucose concentrations on human proximal tubular (HPTC) type IV collagen and fibronectin turnover. Incubation of confluent growth arrested HPTC with 25 mM D-glucose led to accumulation of both type IV collagen and fibronectin. This effect was maximal at 48 hours and represented a sevenfold increase for fibronectin ($N = 4$, $P = 0.04$), and a threefold increase for type IV collagen ($N = 3$, $P = 0.03$) over cells exposed to 5 mM D-glucose controls. This increase was not dependent on new gene transcription for either protein. Tissue inhibitor of metalloproteinases (TIMP 1 + TIMP 2) were induced following addition of 25 mM D-glucose, but not when cells were exposed to 5 mM D-glucose. Twenty-four hours after the addition of 25 mM D-glucose there was an eightfold increase in TIMP 1 ($P = 0.009$, $N = 4$), and a tenfold increase in TIMP 2 levels ($P = 0.003$, $N = 4$), over the control values for both inhibitors. The increase in both TIMP 1 and TIMP 2 in response to 25 mM D-glucose was abrogated in a dose dependent manner by the aldose reductase inhibitor sorbinil. Gelatin-substrate gel zymography showed increased activity of gelatinase A, but not of gelatinase B in response to the addition of 25 mM D-glucose to HPTC. The induction of gelatinase A was accompanied by increased gelatinase A mRNA expression, which was inhibited both by protein kinase C (PKC) depletion using PMA pre-treatment, and by the addition of a PKC inhibitor. These data demonstrate that the glucose-induced accumulation of type IV collagen and fibronectin is unrelated to increased gene transcription, but may involve alterations in the degradative pathway of these basement membrane constituents. Furthermore, the data demonstrate that glucose may simultaneously activate two intracellular pathways (the polyol pathway and a PKC dependent activation pathway), which are involved in mediating separate, complementary effects on cell function.

Nephropathy is a major cause of morbidity and mortality in diabetes and is the single most common cause of end-stage renal failure requiring renal replacement therapy [1, 2]. In the past it has been considered to be primarily a glomerular disease. There is now strong evidence, however, that a progressive decline in renal function is also closely correlated with the development of tubulointerstitial lesions [3, 4]. As a result several studies have focused on the mechanisms of induction of these interstitial changes.

There are clear epidemiological data to indicate that both the initiation and the progression of nephropathy are related to poor glycemic control, and that strict metabolic control can delay both the onset and the rate of progression [5, 6]. One of the earliest pathological changes in the renal tubulointerstitium in diabetes, is an increase in tubular basement membrane (TBM) mass that accompanies the development of renal hypertrophy. These changes in the TBM occur before any histological changes within the glomerulus [7].

Previous studies have demonstrated alterations in synthesis of extracellular matrix (ECM) constituents by various cell types in response to exposure to elevated D-glucose concentrations [8–11]. Alterations in the deposition of basement membrane constituents may result from either an increase in their production or a decrease in their degradation. A detailed analysis of ECM components produced by proximal tubular cells has not been carried out. Recent evidence, however, has shown that proximal tubular cells can produce a number of pro-inflammatory molecules enabling them to initiate and influence pathological changes in the renal interstitium [12–14]. In the current study we have examined the effects of glucose on type IV collagen and fibronectin synthesis in cultured human proximal tubular epithelial cells (HPTC). In addition, we have examined associated changes in the synthesis and activity of matrix metalloproteinases and their inhibitors, under the same conditions. By studying the effect of glucose on the synthetic and the degradative pathways of matrix turnover, we have assessed the net effect of glucose on matrix accumulation.

In addition to describing the alterations in the basement membrane constituents seen in response to the exposure of proximal tubular cells to elevated D-glucose concentrations, we have also examined mechanisms by which these changes may occur. Previous studies have suggested that effects of glucose on extracellular matrix may be mediated by the pro-fibrotic cytokine transforming growth factor β1 (TGF-β1) [15–17]. Our previous published observations, however, demonstrated that exposure of HPTC to elevated D-glucose concentrations induced TGF-β1

Key words: type IV collagen, fibronectin, TIMP, gelatinase, glucose.

Received for publication March 21, 1997
and in revised form May 19, 1997
Accepted for publication May 20, 1997

Table 1. Amplification primer

Gene	Primers	Product size	Reference
Type IV (α1) Collagen	5′-CAATGCCCTTCCTGTTCTGC-3′ 5′-GTGGACGGCGTAGGCTTCTT-3′	435bp	[31]
Fibronectin	5′-AGCCGCCACGTGCCAGGATTAC-3′ 5′-CTTATGGGGGTGGCCGTTGTGG-3′	439bp	[32]
Gelatinase A	5′-TTTTCTCGAATCCATGATGG-3′ 5′-CTGGTGCAGCTCTCATATTT-3′	630bp	[33]
TIMP-1	5′-ATCCTGTTGTTGCTGTGGCTG-3′ 5′-GACTGGAAGCCCTTTTCAGA-3′	520bp	[34]
TIMP-2	5′-GTTTTGCAATGCAGATGTAG-3′ 5′-ATGTCGAGAAACTCCTGCTT-3′	540bp	[35]
α-Actin	5′-CCTTCCTGGGCATGGAGTCCT-3′ 5′-GGAGCAATGATCTTGATCTT-3′	204bp	[36]

mRNA synthesis without any associated change in TGF-β1 protein synthesis [18, 19]. In these studies the application of a second stimulus, such as either PDGF or IL-1β, was required to trigger TGF-β1 protein secretion [13, 18]. Further experiments were therefore performed to assess a possible role for TGF-β1 in the glucose mediated alterations in type IV collagen and fibronectin turnover by HPTC.

Numerous biochemical mechanisms have been postulated which could lead to functional changes or tissue damage in diabetes mellitus as a result of hyperglycemia. There is a large body of experimental evidence that activation of the polyol pathway and activation of the protein kinase C system may both be mechanisms forming a link between hyperglycemia and diabetic nephropathy [20–22]. In the current study the relative contributions of these potential mechanisms to the observed changes in type IV collagen and fibronectin accumulation were assessed.

The results of the present study indicate that 25 mM D-glucose increased type IV collagen and fibronectin concentrations, and that these alterations were not dependent on gene transcription. Furthermore the data presented suggest that alterations in the degradative pathway contributed to the accumulation of these matrix components.

METHODS

Materials

D- and L-isoforms of glucose, mannitol, phorbol 12-myristate 13 acetate (PMA), and p-Aminophenylmercuric acetate (PAMA) were purchased from Sigma Chemicals (Poole, UK); Tris, $CaCl_2$, Brij and acrylamide from Imperial Laboratories (Europe) Ltd, and Coomasie Blue from BioRad laboratories Ltd. Blocking experiments were performed in duplicate using pan-specific anti-TGF-β antibody (Genzyme Diagnostics, Cambridge, MA, USA; Ref 80-1835-03). The aldose reductase inhibitor, sorbinil, was a gift from Pfizer Central Research (Pfizer Inc, Groton, CT, USA), and the PKC inhibitor, RO 31-8220/002, was a gift from Roche Pharmaceuticals.

Cell culture

HPTC were isolated and characterised as previously described [18]. Briefly, small fragments of human renal cortical tissue obtained from nephrectomy specimens were passed through a series of mesh sieves, and proximal tubular cells recovered from material retained on a 53-μm pore size sieve. The collected material was digested with collagenase (750 U/ml) for 10 min, and tubular cells isolated by centrifugation. Growth medium for primary and subsequent cultures comprised a 1:1 mixture of DMEM/Ham's F12 with 10% FCS supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), and hydrocortisone (40 ng/ml). Culture media and supplements were obtained from Life Technologies Ltd, (Paisley, Scotland, UK). Cells were grown at 37°C in 5% CO_2 and 95% air. HPTC were characterized to be of proximal tubular origin by enzyme histochemistry and immunofluorescence. Cells stained positively for alkaline phosphatase, acid phosphatase, non-specific esterase, and glucose-6-phosphatase, a pattern which is specific for proximal tubular epithelial cells [21–23]. In addition, cells were positive for cytokeratin and negative for Tamm-Horsfall glycoprotein. Electron microscopy demonstrated the presence of a rudimentary brush border. We have previously demonstrated the viability of HPTC in serum-free media for times up to 10 days [18]. All experiments were performed under serum free conditions following a "growth arrest" period of 72 hours in serum free medium. In all experiments cells were used only up to the third passage. We have previously demonstrated that there were no phenotypic changes up to this passage number [18].

Determination of fibronectin, type IV collagen and TIMP protein concentrations

Fibronectin concentration in culture supernatant was determined using an antibody competition ELISA [24].

Type IV collagen in supernatant samples was measured by specific antibody competition ELISA [25]. The assay is sensitive to 1 ng/ml with a range up to 200 ng/ml. Inter-assay precision ranged from 2 to 14%, (CV) and intra-assay precision ranged from 2 to 10% (CV). There was no detectable cross reactivity with type I collagen. Rabbit anti-human type IV collagen antibody was obtained from Manosan (Bradsure Biologicals, Market Harborough, UK). It has been previously demonstrated that this antibody reacted with the α1 and α2 procollagen chains as well as the α chains of collagen [25].

Both TIMP I and TIMP II in cell culture supernatants were detected by ELISA as previously described [26]. The assay for TIMP 1 was sensitive to 0.09 nM with a range up to 35 nM, and inter-assay precision of 8.2%, (CV). There was no detectable cross reactivity with TIMP 2. The assay for TIMP 2 was sensitive to 0.15 nM, with a range up to 17.2 nM, and inter-assay precision of 10.6%, (CV). There was no detectable cross reactivity with TIMP 1.

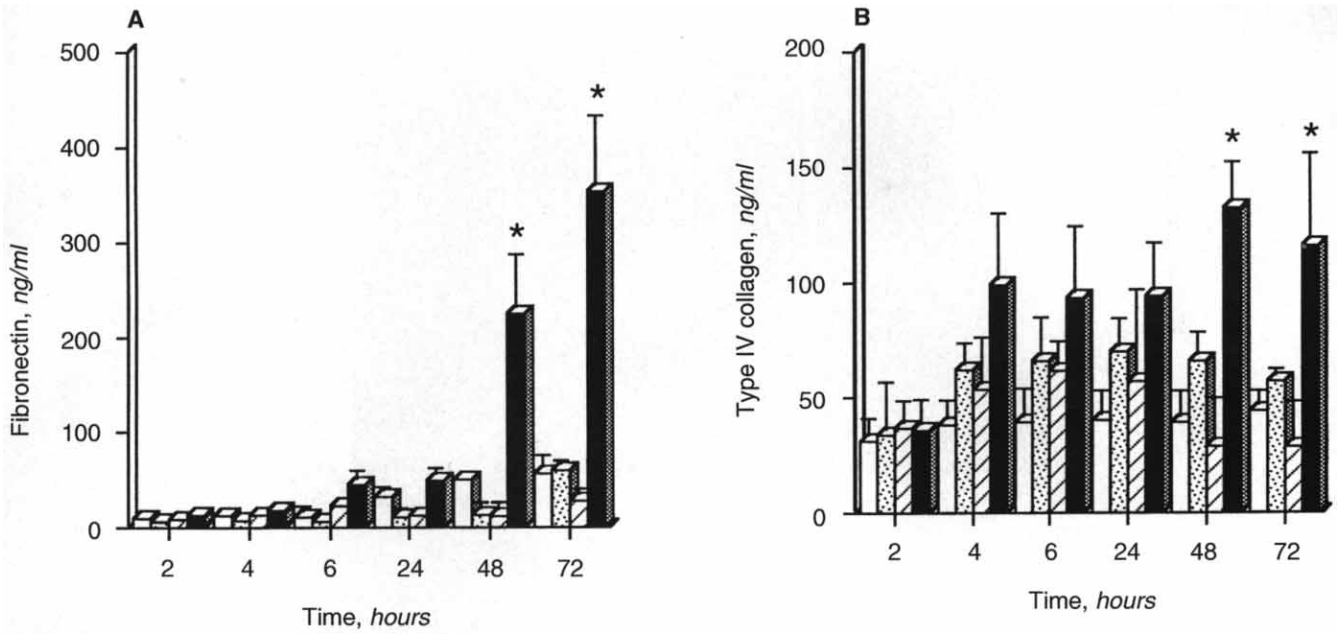

Fig. 1. Time dependent accumulation of fibronectin and type IV collagen following application of 25 mM D-glucose. Cells were grown to confluence and serum deprived for 48 hr prior to the addition of fresh serum free media, containing 25 mM D-glucose (■), L-glucose (⚃) or mannitol (▨), or 5 mM D-glucose (□). Fibronectin and type IV collagen concentration in the supernatant were determined by ELISA. Results are expressed as mean ± SEM of 4 separate experiments, $^*P < 0.05$.

Enzymatic assays

Gelatinase activity was assayed *in vitro* by the degradation of ^{14}C labeled gelatin as previously described [27]. Following collection of medium, samples were centrifuged to remove any debris present and stored at −20°C until assayed for enzymatic activity. One hundred microliters of supernatant was incubated at 37°C with 50 μl of 0.4 M Tris-HCl, pH 7.5 (containing 10 mM $CaCl_2$) and with 10 μl of ^{14}C gelatin (1 mg/ml 0.045 μCi/mg) from denatured interstitial rat skin collagen (95% type I, 5% type III). Fifty microliters of ice-cold 100% (wt/vol) trichloroacetic acid were then added and incubated at 4°C for a further 30 minutes. The insoluble protein was pelleted in a Beckman Microfuge at 11,000 × g for 10 minutes. One hundred and fifty microliters of the supernatant were then dissolved in 4 ml of Ultima Gold (Camberra Packard) and counted in an Camberra Packard Tricarb liquid scintillation analyzer for one minute. *In vitro* activation of gelatinase was performed using p-Aminophenylmercuric acetate (PAMA) which was prepared to a concentration of 10 mM in 0.05 M NaOH and used at 1 mM in the enzyme assay.

Samples were also run at 4°C on a 7% non-reducing SDS polyacrylamide gels containing gelatin at a final concentration of 1 mg/ml at 4°C. The gels were then washed in 50 mM Tris-HCl, pH 7.6, containing 10 mM $CaCl_2$ and 0.05% Brij, and incubated in the same buffer at 37°C overnight. The presence of gelatinase activity was demonstrated by zones of lysis in the Coomasie blue stained gel [28].

RNA isolation, reverse transcription (RT), and polymerase chain reaction (PCR) amplification

The effects of elevated D-glucose concentration on Type IV collagen, fibronectin, gelatinase A, B and TIMP I and II gene expression were examined by RT-PCR. At the defined time periods total cellular RNA was extracted from both control and stimulated cells following lysis with 4 M guanidine isothiocyanate and centrifugation through 5.7 M cesium chloride in 0.1 M EDTA [29]. Total RNA was reverse transcribed to cDNA with M-MLV™ reverse transcriptase (Gibco Life Technologies Ltd, Paisley, UK), using the random hexamers method as previously described [30]. Briefly, the reaction mixture contained 1 μl random hexamers (100 μM; Pharmacia Biosystems Ltd, Milton Keynes, UK), 5 μl NTP's (2.5 mM; Gibco/BRL Life Technologies, Ltd), 2 μl 10× PCR buffer (100 mM Tris/HCl, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% wt/vol gelatin), 2 μl DTT (0.1 M, Gibco/BRL Life Technologies, Ltd), 1 μl RNAase inhibitor (Promega, Southampton, UK), 1 μl M-MLV™ Superscript reverse transcriptase (200 Units, Gibco/BRL Life Technologies, Ltd) and 1 μg of total RNA.

PCR amplification was performed in a total volume of 50 μl (2 μl of reverse transcription product and 48 μl of master mix (36.25 μl H_2O, 1.25 μl 5′-primer (20 μM), 1.25 μl 3′-primer (20 μM), 4 μl NTP's, 5 μl 10× PCR buffer and 0.25 μl Taq polymerase (2.5U, Amplitaq©, ILS Ltd, London, UK) using a Perkin Elmer 480 Thermocycler (Applied Biosystems Ltd, Warrington, UK). The PCR protocol for TGF-β1 mRNA and α-actin was as follows:

The first cycle was at 72°C for 10 minutes, second cycle 94°C for 3 minutes, 55°C for 1 minute, 72°C for 1 minute. The 3 through the 38th cycles 94°C for 40 seconds, 55°C for 1 minute, 72°C for 1 minute. The final cycle was 94°C for 1 minute and 60°C for 10 minutes. PCR was performed for 32 cycles for α-actin, gelatinase A, TIMP 1 and 2, and 25 cycles for fibronectin and type IV (α1) collagen.

One-tenth of the PCR reaction from both test and control (α-actin) product were mixed and separated by flat bed electrophoresis in 3% wt/vol NuSieve GTG agarose gels (Flowgen

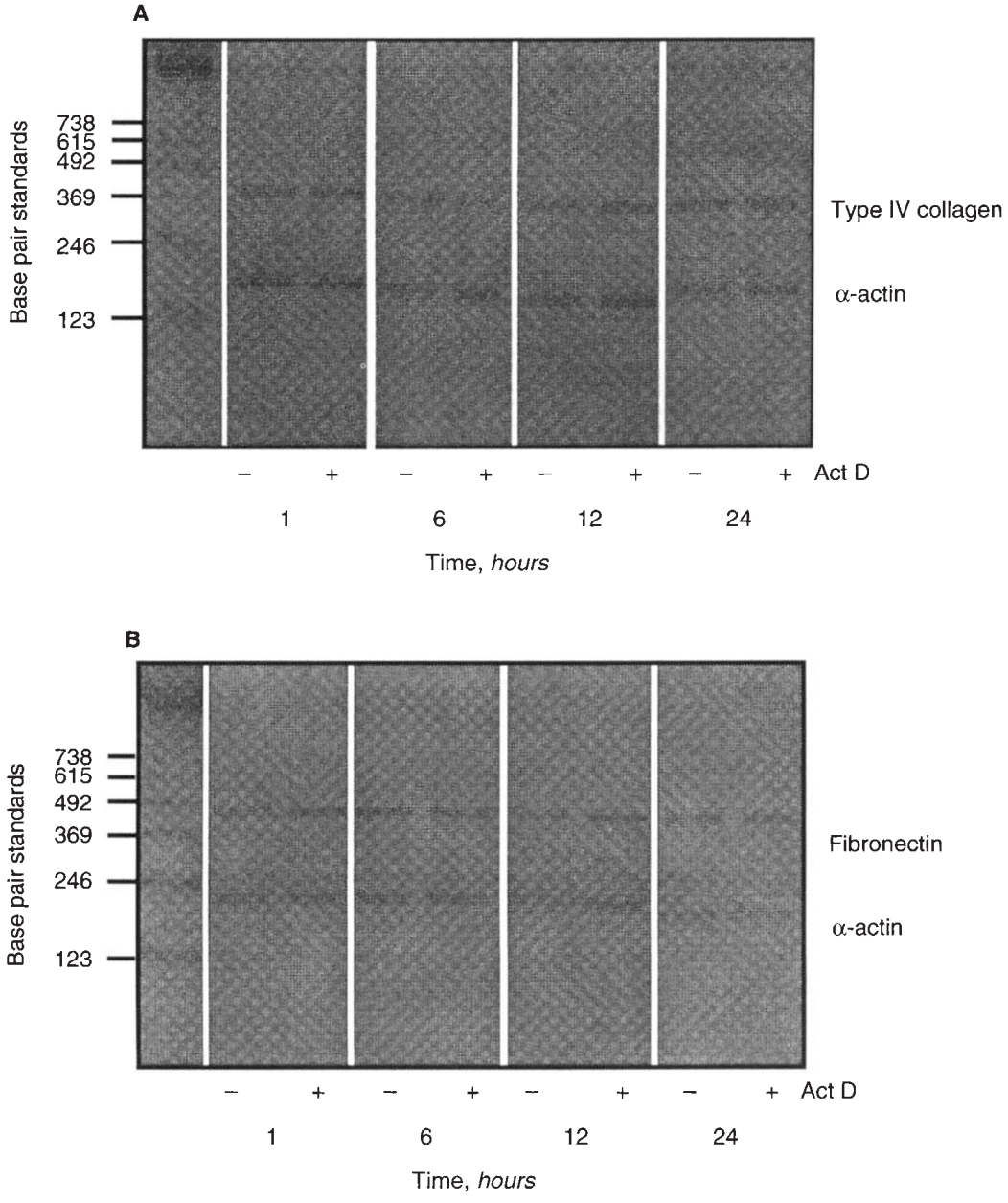

Fig. 2. Effect of actinomycin D on the levels of mRNA for type IV collagen and fibronectin. D-glucose 25 mM was added to confluent serum-deprived HPTC in the presence (+) or absence (−) of 0.5 μg/ml actinomycin D. Total cellular RNA was isolated, and following RT-PCR, type IV collagen (*A*) or fibronectin (*B*) and α-actin mRNA were separated by flat bed electrophoresis in 3%. Scanning densitometry confirmed that there was no difference in the ratios of type IV collagen or fibronectin mRNA to α-actin with or without actinomycin D. One representative experiment ($N = 3$), is shown.

Instruments Ltd, Sittingbourne, UK), stained with ethidium bromide (Sigma) and photographed. The negatives were scanned using a densitometer (Model 620 video densitometer; Bio-Rad Laboratories Ltd) and the density of the bands compared to those of the housekeeping gene. Results were expressed as ratios of the gene of interest to α-actin, normalized to the first time point of each experiment.

The sequences of the amplification primers are presented in Table 1.

Statistics

For each individual experiment the mean of duplicate determinations was calculated. The data are presented as means ± SEM of N experiments (N = experiments performed using cells prepared from separate nephrectomy specimens). Statistical analysis between two groups was performed using the paired Student's t-test, with a value of $P < 0.05$ considered to represent a significant difference.

RESULTS

Collagen and fibronectin production in response to 25 mM D-glucose

Following the addition of 25 mM D-glucose to growth arrested HPTC, there was a time dependent increase in the fibronectin concentration in cell culture supernatants. This increase was

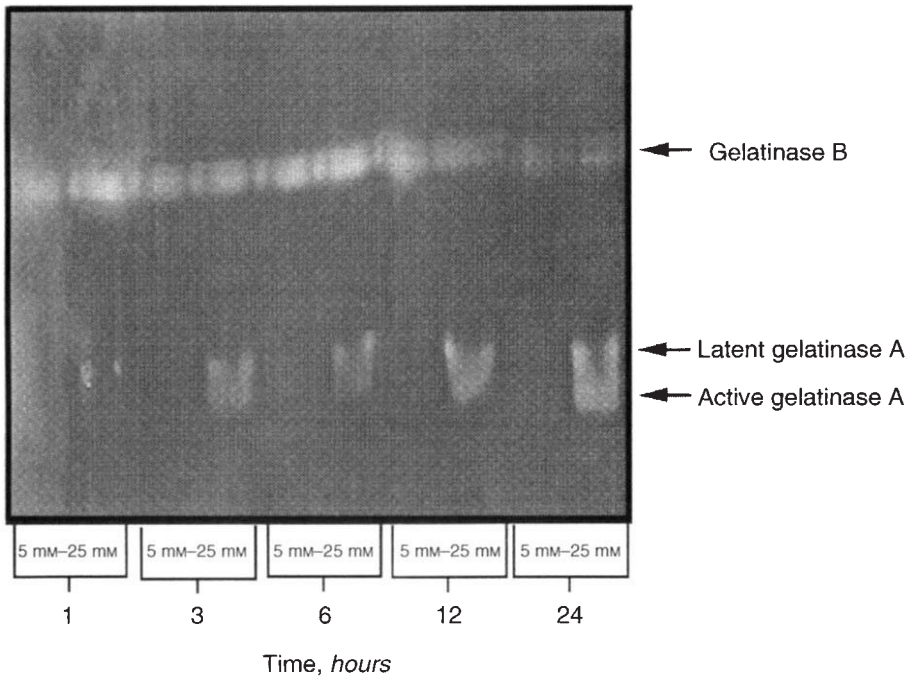

Fig. 3. Time dependent induction of gelatinase A by 25 mM D-glucose. Either 5 mM or 25 mM D-glucose was added to confluent serum-deprived HPTC and supernatant samples collected over the subsequent 24 hours. Gelatinase activity was demonstrated following electrophoresis on SDS-PAGE gelatin substrate gels under non-reducing conditions. One representative experiment is shown.

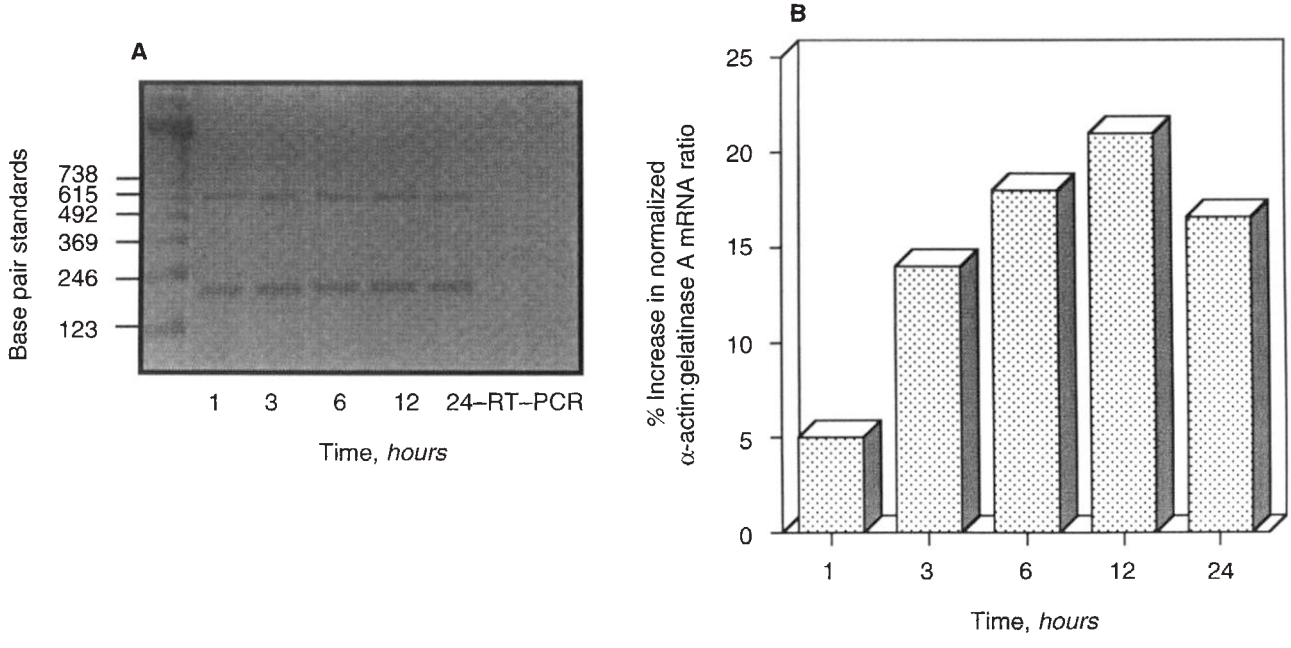

Fig. 4. Time dependent induction of gelatinase A mRNA by glucose. Growth arrested HPTC were stimulated with 25 mM D-glucose for times up to 24 hours under serum-free conditions. Following RT-PCR, gelatinase A and α-actin cDNA were separated by flat bed electrophoresis in 3% agarose gels (*A*) and the results expressed as the densitometric ratios of gelatinase A mRNA to α-actin normalised for the unstimulated ratio (*B*). One representative experiment ($N = 3$) is shown.

significantly greater than that of cells exposed to 5 mM D-glucose ($P = 0.04$, $N = 4$), 25 mM L-glucose ($P = 0.021$, $N = 4$), or 25 mM mannitol ($P = 0.26$, $N = 4$), at 48 hours (Fig. 1). Similarly there was a time dependent increase in type IV collagen following the addition of 25 mM D-glucose. This was also significantly elevated over that seen following the addition of 5 mM D-glucose ($P = 0.03$), 25 mM L-glucose ($P = 0.06$, $N = 4$), or 25 mM mannitol (Fig. 1).

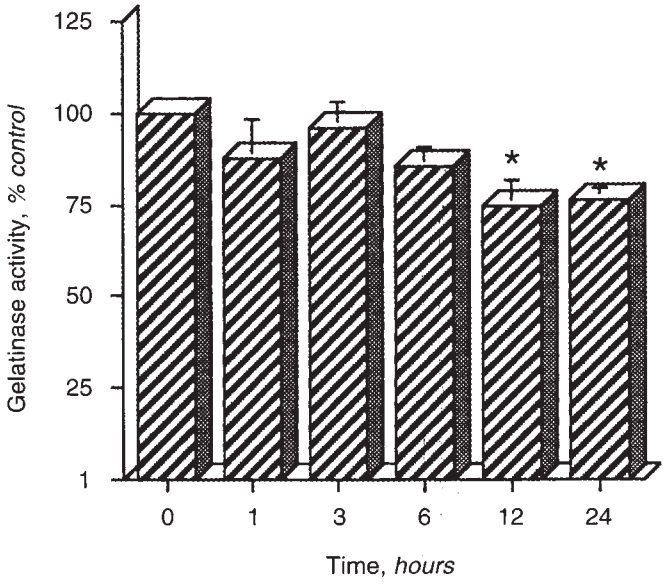

Fig. 5. Effect of 25 mM D-glucose on supernatant gelatinolytic activity. D-glucose 25 mM was added to growth arrested HPTC under serum-free conditions and supernatant samples collected over the subsequent 24 hours. Gelatinase activity was assayed *in vitro* by the degradation of ^{14}C labeled gelatin. Results are expressed as % gelatinase activity of supernatant from 5 mM D-glucose treated HPTC for the same times, and are expressed mean ± SEM, $N = 3$, $^*P > 0.05$.

In contrast to the increase in protein concentration for both fibronectin and type IV collagen, there was no alteration in the amount of corresponding mRNA as assessed by RT-PCR, for either of these matrix constituents. Furthermore, the addition of 25 mM D-glucose in the presence or absence of actinomycin D (0.5 μg/ml) did not alter the amount of mRNA for either type IV collagen or fibronectin, again indicating that the accumulation of protein was not dependent on new gene transcription (Fig. 2).

Metalloproteinase production in response to 25 mM D-glucose

Under serum free conditions 25 mM D-glucose was added to growth arrested HPTC, the supernatants collected over the subsequent 24 hours, and electrophoresed on SDS-PAGE gelatin substrate gels under non-reducing conditions. Constitutive gelatinolytic activity was seen at 95 kDa, corresponding to gelatinase B. This remained unchanged following the addition of 25 mM D-glucose. In contrast, a zone of gelatinolytic activity was induced at 72 kDa, corresponding to gelatinase A, following addition of 25 mM D-glucose, which was not apparent in the cell culture supernatant of growth arrested HPTC incubated with 5 mM D-glucose (Fig. 3).

Examination of gelatinase A mRNA by RT-PCR demonstrated time dependent induction following the addition of 25 mM D-glucose. Maximal induction was seen at 12 hours at which time there was a 21% increase in the gelatinase A:α-actin mRNA ratio (Fig. 4).

Despite the observed increase in the synthesis of gelatinase A, there was a small but significant overall decrease in total gelatinolytic activity in HPTC culture supernatants following incubation in 25 mM D-glucose, as assessed by *in vitro* degradation of ^{14}C labeled gelatin (Fig. 5).

Production of tissue inhibitors of metalloproteinase in response to glucose

To determine the effect of elevated D-glucose concentration on TIMP 1 and TIMP 2 production, 25 mM D-glucose was added to growth arrested HPTC under serum free conditions, and supernatant samples collected over the subsequent 24 hours. Control experiments were performed by incubating in the presence of 5 mM D-glucose. In the presence of 25 mM D-glucose there was a time dependent increase in both TIMP 1 and 2 in culture supernatants. Twenty-four hours after the addition of 25 mM D-glucose there was an eightfold increase in TIMP 1 ($P = 0.009$, $N = 4$), and a tenfold increase in TIMP 2 levels ($P = 0.003$, $N = 4$), over the control values for both inhibitors (Fig. 6). No increase in either TIMP 1 or 2 was detected in control experiments.

Confirmation of TIMP activity in supernatant samples was determined by the *in vitro* inhibition of gelatinase activity. Gelatinase was prepared by neutrophil gelatinase extraction as previously described [27]. Supernatant samples from the 24 hour time point of the experiments in which growth arrested cells had been exposed to 25 mM D-glucose, were serially diluted and added to neutrophil gelatinase. Gelatinolytic activity was determined as described above. The results demonstrated *in vitro* inhibition of gelatinase activity by supernatant samples in which maximal TIMP protein concentration was demonstrated by ELISA (Fig. 7).

Examination of TIMP 1 and 2 mRNA by RT-PCR demonstrated time dependent induction following addition of 25 mM D-glucose. Maximal TIMP 1 induction was seen at six hours, at which time there was a 20% increase in the TIMP 1:α-actin mRNA ratio. Similarly, TIMP 2 mRNA induction was maximal at 12 hours, when there was a 22% increase in the α-TIMP 2:α-actin mRNA ratio (Fig. 8).

Mechanism of D-glucose dependent accumulation of collagen and fibronectin

Effect of TGF-β inhibition on glucose mediated alterations in fibronectin and type IV collagen. To determine if the observed increases in type IV collagen and fibronectin production were mediated by the autocrine action of secreted TGF-β, 25 mM D-glucose was added to growth arrested HPTC in the presence of increasing concentrations of a neutralizing pan-specific TGF-β (1-3) antibody (Genzyme). The addition of this antibody had no affect on the increase in fibronectin or type IV collagen seen following the addition of 25 mM D-glucose (Fig. 9). Furthermore, addition of the antibody did not alter the induction mRNA for gelatinase A, TIMP 1 and TIMP 2, which was seen following the addition of 25 mM D-glucose (data not shown).

Effect of polyol pathway inhibition on glucose mediated alterations in fibronectin and type IV collagen. The contribution of the polyol pathway was assessed by the addition of 25 mM D-glucose together with increasing doses of sorbinil over 24 hours. This compound is an inhibitor of the rate limiting enzyme of the polyol pathway, aldose reductase. Addition of sorbinil resulted in a dose dependent inhibition of glucose induced accumulation of type IV collagen and fibronectin (Fig. 10). Furthermore, inhibition of polyol pathway activation decreased glucose induced TIMP 1 and TIMP 2 mRNA (Fig. 11) and TIMP 1 and 2 protein secretion as assessed by ELISA (Fig. 12). Supernatant samples taken from cells treated with 25 mM D-glucose in the presence of increasing doses of sorbinil demonstrated increasing gelatinase activity as

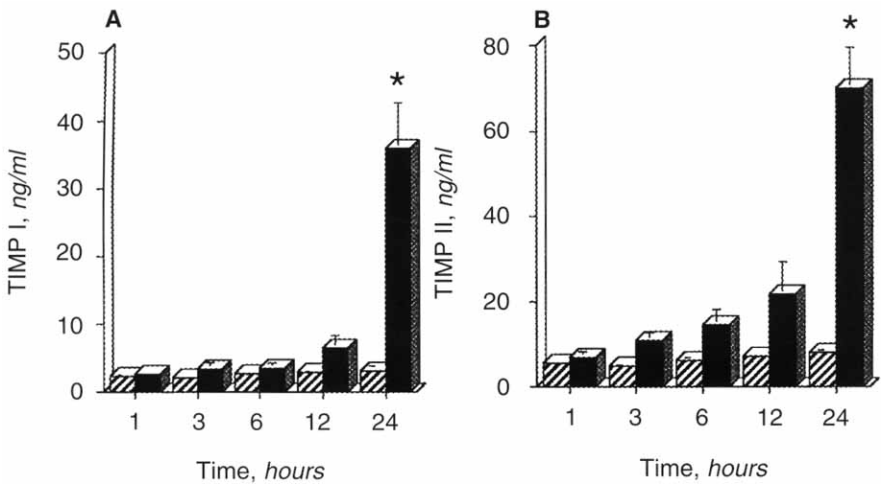

Fig. 6. Time dependent induction of TIMP 1 and TIMP 2 by D-glucose. Symbols are: (■) 25 mM and (▨) 5 mM D-glucose were added to growth arrested HPTC under serum-free conditions. TIMP 1 and TIMP 2 concentrations were determined by ELISA over the subsequent 24 hours. Results are expressed as mean ± SEM, $N = 3$, $^*P > 0.05$.

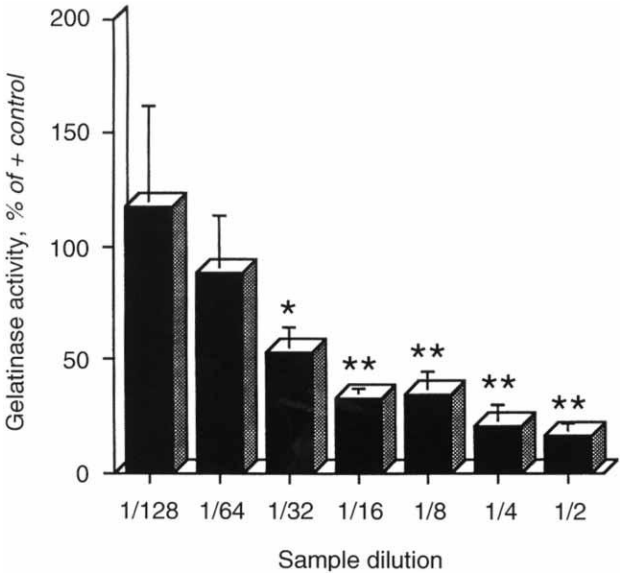

Fig. 7. Gelatinase inhibitory activity of supernatant samples 24 hours after the addition of 25 mM D-glucose to growth arrested HPTC. Supernatant samples were serially diluted and added medium containing neutrophil gelatinase. Gelatinase activity was assayed *in vitro* by the degradation of ^{14}C labeled gelatin. The results are presented as the % gelatinase activity relative to gelatinase samples to which no supernatant was added. Results are expressed as mean ± SEM, $N = 3$, $^*P > 0.05$, $^{**}P > 0.005$.

assessed by *in vitro* degradation of ^{14}C gelatin (Fig. 13). Sorbinil treatment did not affect the induction of gelatinase A mRNA (data not shown). This suggests that the observed increase in gelatinolytic activity following the addition of sorbinil was the result of inhibition of TIMP production.

Effect of PKC inhibition on glucose mediated alterations in fibronectin and type IV collagen. The role of protein kinase C activation was determined by PKC depletion using increasing doses of PMA added prior to the application of 25 mM D-glucose or by the addition of the PKC inhibitor RO-31-8220/002 together with 25 mM D-glucose. Total cellular RNA and supernatant samples were collected 24 hours after the addition of D-glucose. Glucose dependent induction of gelatinase A mRNA was inhibited by both PMA pre-treatment and by the PKC inhibitor (Fig. 14). Depletion of PKC, or inhibition of PKC activity, however, did not influence glucose induced accumulation of type IV collagen or fibronectin, nor did they abrogate glucose induced changes in TIMP 1 and TIMP 2 mRNA (data not shown).

DISCUSSION

Alterations in TBM composition occur early in the course of diabetic nephropathy, and recent reports suggest that these changes may precede any histological alterations within the glomerulus, as assessed by light microscopy [7]. Both *in vitro* and *in vivo* studies have implicated elevation of glucose concentration in the increased production of basement membrane components by endothelial [8, 9, 37] and mesangial cells [10, 11, 38]. The data presented in the current manuscript demonstrate that increased amounts of both fibronectin and type IV collagen were produced by human proximal tubular cells, when cultured in the presence of elevated concentrations of D-glucose. Furthermore, we have demonstrated that this effect was unrelated to alterations in osmolarity, as neither L-glucose or mannitol reproduced the effects seen following the addition of D-glucose. Despite the accumulation of both basement membrane constituents in the cell culture supernatant of 25 mM D-glucose treated cells, RT-PCR suggested that there was no alteration in their mRNA. Experiments performed in the presence of actinomycin-D confirmed that there was no increase in gene transcription of either type IV collagen or fibronectin following the addition of 25 mM D-glucose. These observations are consistent with data derived from streptozotocin treated rats, which have also demonstrated that *in vivo*, there is no alteration in $\alpha 1$ (IV) collagen mRNA in whole kidney [39].

Regulation of matrix synthesis is a dynamic process involving degradation as well as synthesis. Increased synthesis and decreased degradation of basement membrane constituents are not mutually exclusive, and both processes may occur simultaneously at different rates. Altered extracellular matrix interaction with surrounding cells may not result solely from the accumulation of normal components, but may be the result of a distortion of the balance between matrix synthesis and turnover [40]. The present data demonstrate the constitutive expression of gelatinase B by

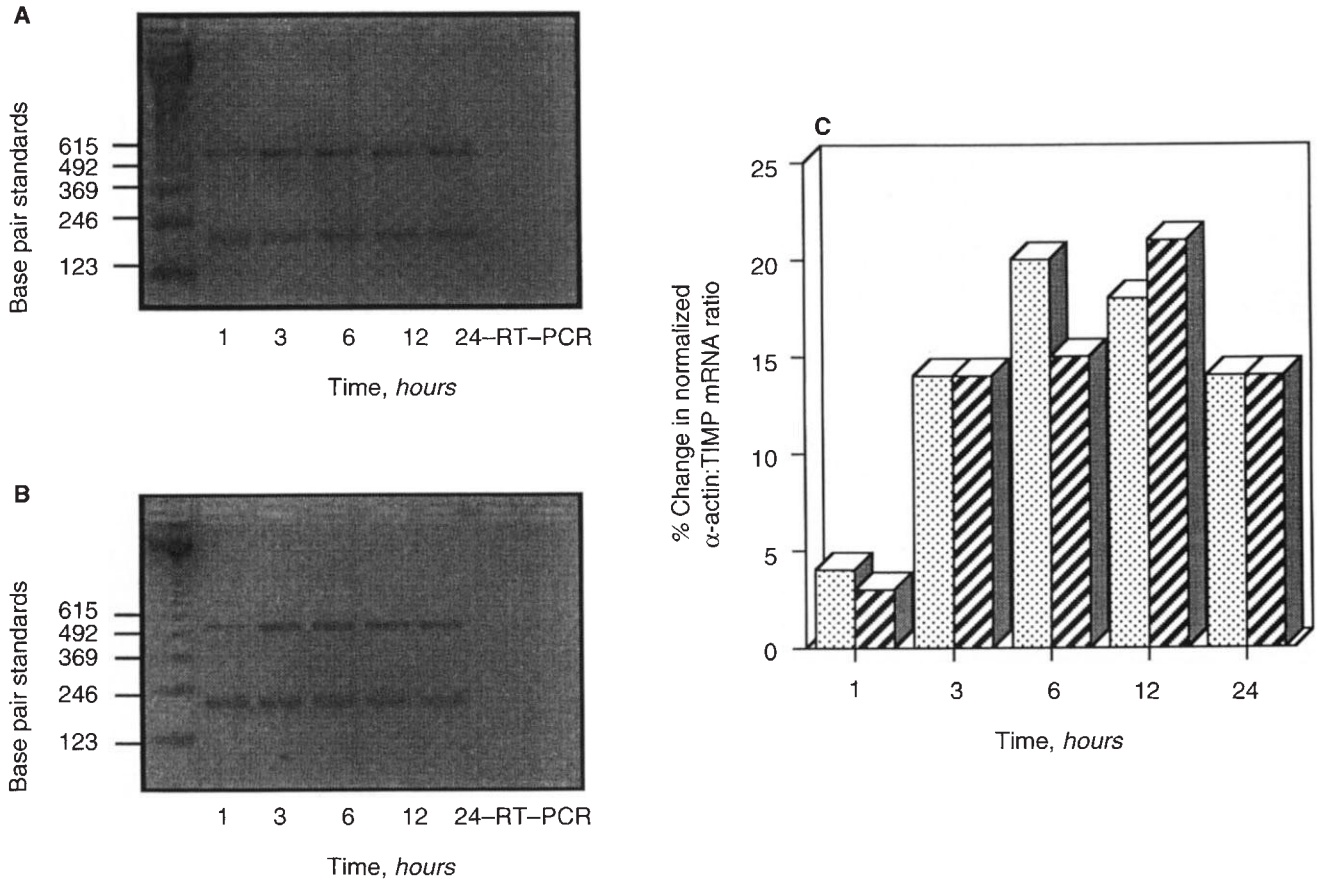

Fig. 8. Time dependent induction of TIMP 1 and TIMP 2 mRNA by D-glucose. Growth arrested HPTC were stimulated with 25 mM D-glucose for times up to 24 hours under serum free conditions. Following RT-PCR, TIMP 1 (*A*) or TIMP 2 (*B*) and α-actin cDNA were separated by flat bed electrophoresis in 3% agarose gels, and the results expressed as the densitometric ratios of TIMP 1 (▨) or TIMP 2 (▨) mRNA to α-actin normalized for the unstimulated ratio (**C**). One representative experiment ($N = 3$) is shown.

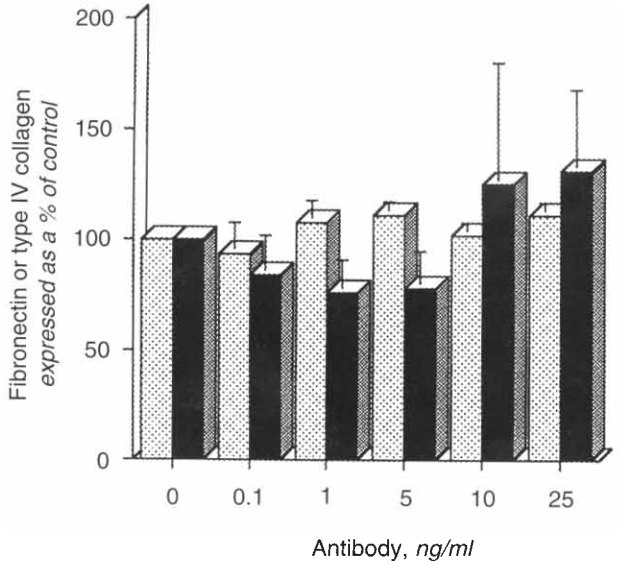

Fig. 9. Effect of anti-TGF-β-antibody on glucose-induced fibronectin and type IV collagen production. Cells were grown to confluence and serum deprived for 48 hours prior to the addition of fresh serum free-media, containing 25 mM D-glucose and increasing concentrations of a neutralizing antibody to TGF-β1, -2 and -3 (Genzyme). Fibronectin (▨) and type IV collagen (■) concentration in the supernatant were determined by ELISA. Results expressed as mean ± SEM of 3 separate experiments. $^*P < 0.05$.

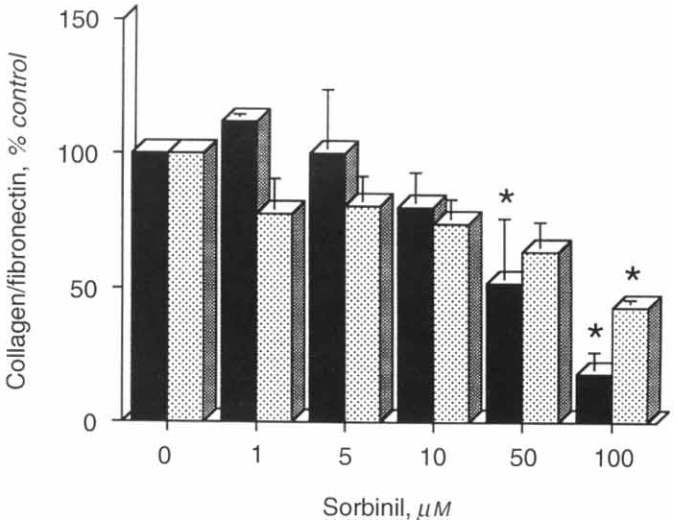

Fig. 10. Effect of aldose reductase inhibitor on type IV collagen and fibronectin accumulation. HPTC were grown to confluence and serum-deprived prior to addition of 25 mM D-glucose in the presence of increasing concentrations of sorbinil (0 to 100 μM) for 24 hours. Type IV collagen (■) and fibronectin (▨) concentration in the supernatant were then determined by ELISA. Results are presented as % inhibition relative to the uninhibited control (0 μM sorbinil) and represent mean ± SEM, $N = 3$, $^*P > 0.05$.

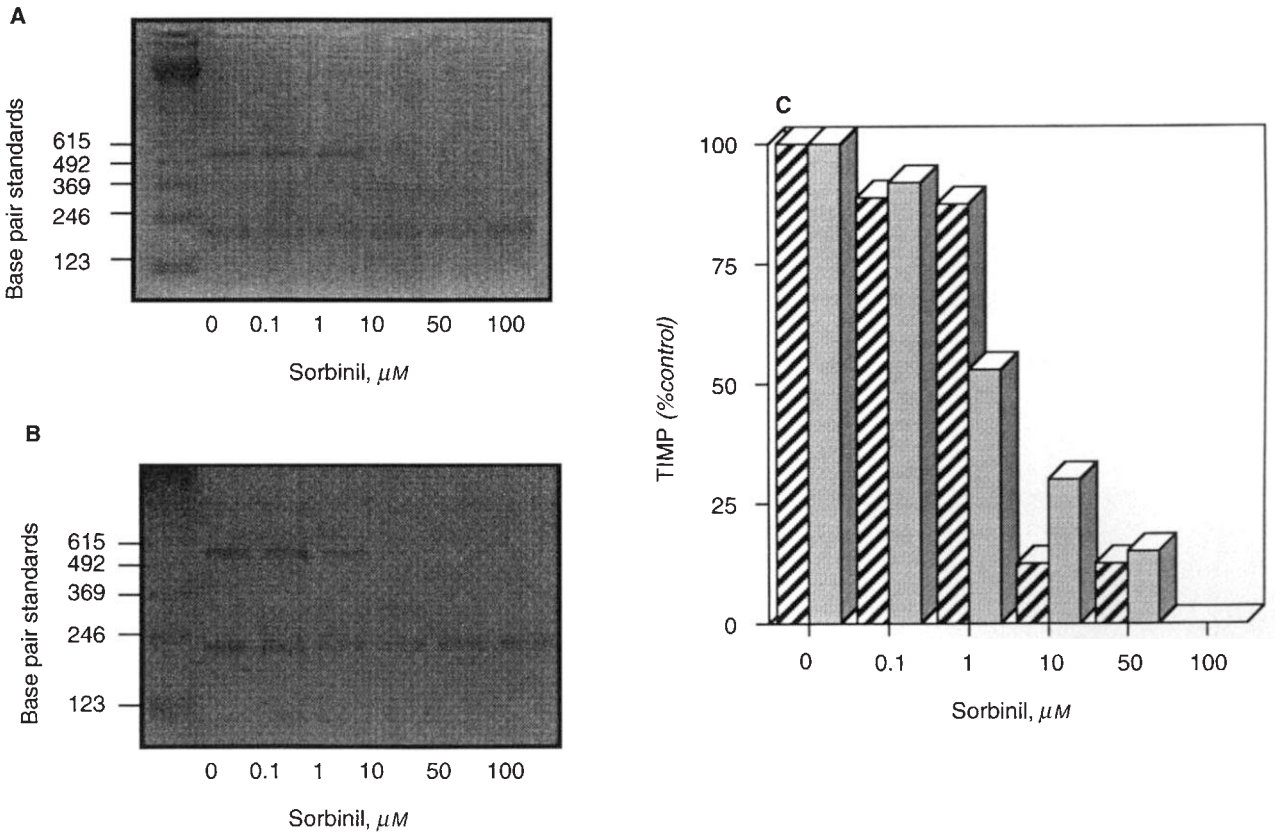

Fig. 11. Effect of aldose reductase inhibitor on TIMP 1 and TIMP 2 mRNA induction. HPTC were grown to confluence and serum-deprived prior to addition of 25 mM D-glucose in the presence of increasing concentrations of sorbinil (0 to 100 μM) for 24 hours. Following RT-PCR, TIMP 1 (*A*) or TIMP 2 (*B*) and α-actin cDNA were separated by flat bed electrophoresis in 3% agarose gels, and the results expressed as the densitometric ratios of TIMP 1 (▨) or TIMP 2 (▩) mRNA to α-actin normalized for the mRNA ratio in the absence of sorbinil (**C**). One representative experiment ($N = 3$) is shown.

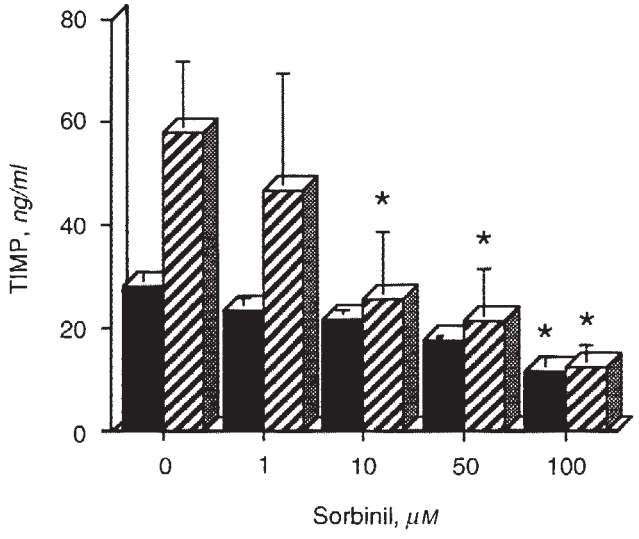

Fig. 12. Effect of aldose reductase inhibitor on TIMP 1 and TIMP 2 production. HPTC were grown to confluence and serum-deprived prior to addition of 25 mM D-glucose in the presence of increasing concentrations of sorbinil (0 to 100 μM) for 24 h. TIMP 1 (■) and TIMP 2 (▨) concentration in the supernatant were then determined by ELISA. Results represent mean ± SEM, $N = 3$, $^*P > 0.05$.

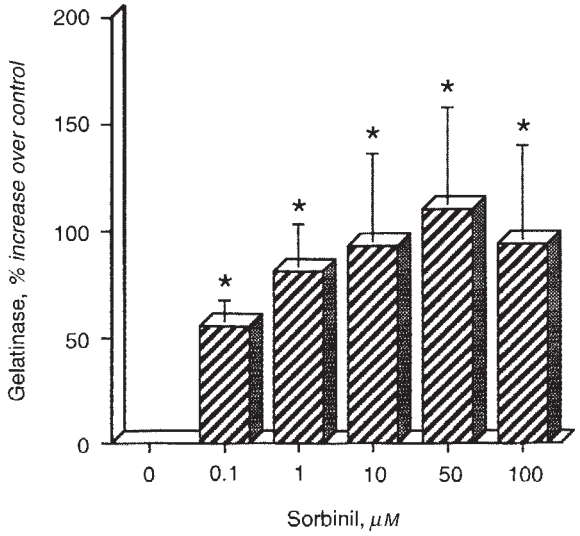

Fig. 13. Effect of aldose reductase inhibitor on supernatant gelatinolytic activity. HPTC were grown to confluence and serum-deprived prior to addition of 25 mM D-glucose in the presence of increasing concentrations of sorbinil (0 to 100 μM) for 24 hours. Gelatinase activity was assayed *in vitro* by the degradation of ^{14}C labeled gelatin. Results are expressed as % increase in gelatinase activity above that of supernatant samples taken from HPTC to which 25 mM D-glucose was added in the absence of sorbinil, and are expressed as mean ± SEM, $N = 3$, $^*P > 0.05$.

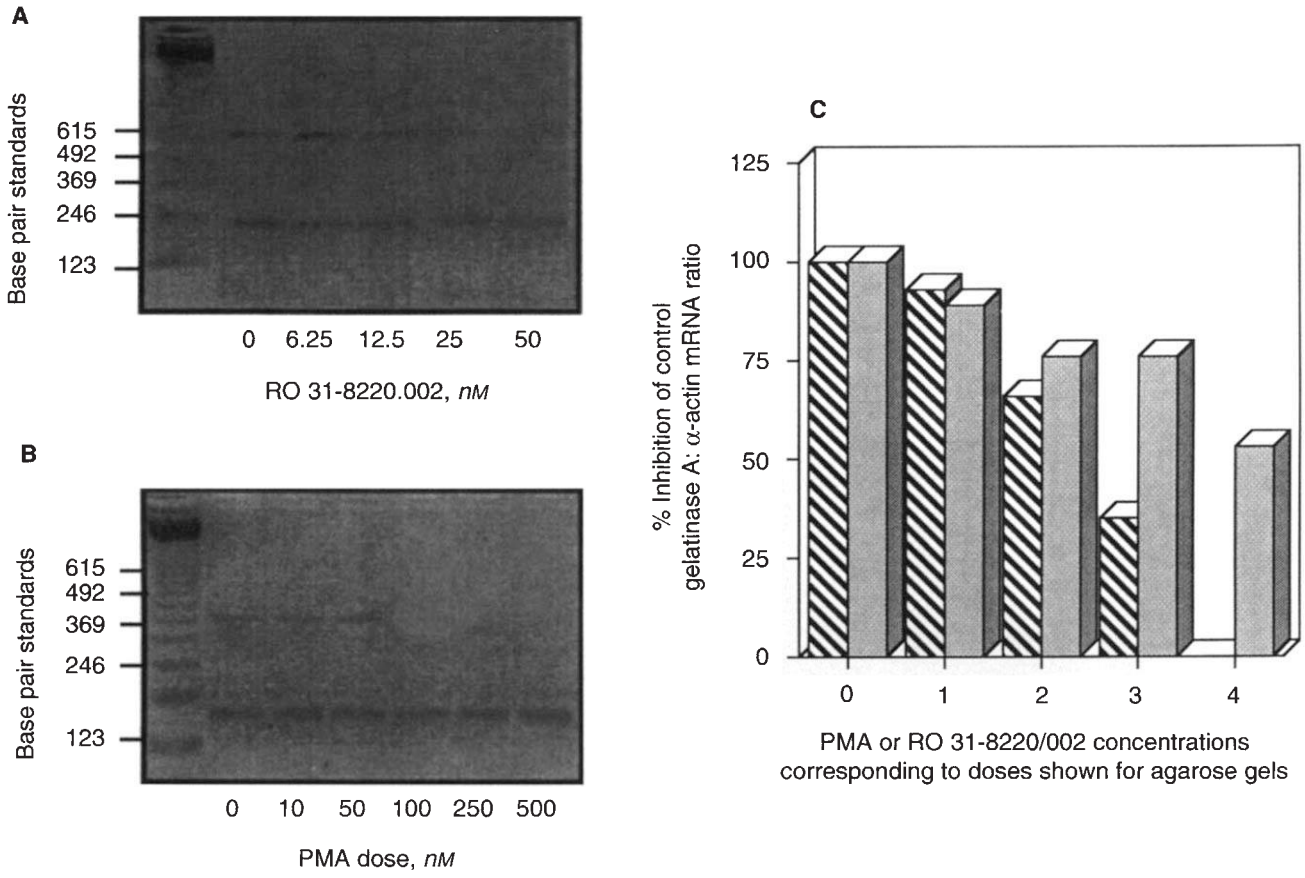

Fig. 14. Role of PKC in the induction of gelatinase A mRNA by glucose, assessed by addition of 25 mM D-glucose to growth arrested HPTC together with increasing doses of the PKC inhibitor RO-31-8220/002 (*A*), or by PMA pre-treatment to deplete PKC prior to the addition of 25 mM D-glucose (*B*). Following RT-PCR, gelatinase A and α-actin cDNA were separated by flat bed electrophoresis in 3% agarose gels and the results expressed as the densitometric ratios of gelatinase A cDNA to α-actin normalized for the cDNA ratio in the absence of either the PKC inhibitor (▨), or PMA pre-treatment (▩) (C). One representative experiment ($N = 3$) is shown.

HPTC, which is not affected by 25 mM D-glucose. In contrast, gelatinase A mRNA and protein were both increased following the addition of 25 mM D-glucose. Despite this induction, there was a net decrease in gelatinolytic activity. This is explained by the simultaneous induction of TIMP 1 and 2 by D-glucose. In addition to demonstrating the increased synthesis of these two inhibitors by ELISA, we have demonstrated their "bio-activity" in supernatants of 25 mM D-glucose treated HPTC. Taken together, the data suggest that a decrease in the degradation of both type IV collagen and fibronectin contributes to the accumulation of these basement membrane components, under conditions of elevated D-glucose. The data support the hypothesis that decreased degradation of tubular basement membrane components may contribute to TBM thickening in diabetic nephropathy, and is consistent with *in vivo* observations on the turnover of glomerular basement membrane in streptozotocin-induced diabetic rats [41]. These changes in turnover of the basement membrane may subsequently affect its functional properties and its interactions with neighbouring proximal tubular epithelial cells.

Matrix production is controlled by a complex cytokine network. Of all cytokines studied to date, TGF-β1 has now emerged as a key mediator of matrix remodeling in health and disease. A role for TGF-β1 cannot be inferred simply on the basis of alterations in mRNA, as it is now clear that TGF-β1 synthesis may occur independently at the level of transcription, translation and secretion of pre-formed protein [18, 42–44]. Furthermore, it is difficult to draw conclusions from studies in which TGF-β1 protein is measured unless latent and active TGF-β1 can be distinguished by the assay employed. Numerous studies have implicated TGF-β1 in the extracellular matrix accumulation associated with diabetic nephropathy [15, 16]. These *in vitro* studies, however, utilized SV40 virally transformed cells, and it is known that transformation by SV40 may be accompanied by alterations in response to cytokines [45]. Studies utilizing non-transformed human endothelial cells demonstrated inhibition of cell proliferation and increased gene expression of basement components and TGF-β1 upon the addition of high D-glucose concentrations [8, 37, 46]. Significantly, there was no increase in active TGF-β1 as assessed by bioassay. Furthermore, the effects of elevated D-glucose on cell proliferation and basement membrane component gene expressions were not antagonized by the addition of neutralizing antibodies to TGF-β [47]. Our previous observations have demonstrated that the exposure of HPTC to elevated D-glucose concentrations induced TGF-β1 mRNA without any increase in TGF-β1 protein synthesis [18]. The data presented in the current manuscript demonstrate that glucose mediated accumulation of

basement membrane constituents was not mediated by autocrine TGF-β1 production. These findings are therefore consistent with our previous studies and also the work of other groups studying the effects of glucose on extracellular matrix components in non-transformed cells.

Mechanisms of glucose induced injury in other systems are protein kinase C (PKC) activation by diacylglycerol (generated as a result of increased glycolytic activity) [48], or the increased activity of the polyol pathway [49–51]. Postulated harmful effects of polyol pathway activation involved in the pathogenesis of diabetes include accumulation of sorbitol, and alteration of the redox state of the cell due to the change in NAD^+/NADH ratio which accompanies the conversion of sorbitol to fructose. The data in the current manuscript also demonstrate that the mechanism of 25 mM D-glucose induction of TIMP 1 and TIMP 2 mRNA and protein synthesis were dependent upon polyol pathway activation. Furthermore, inhibition of glucose induced TIMP by the aldose reductase inhibitor, sorbinil, abrogated glucose-induced accumulation of type IV collagen and fibronectin. Under these conditions, gelatinase A was induced by a PKC dependent mechanism, and its unopposed action likely accounts for the observed decrease in collagen and fibronectin.

In conclusion, we have demonstrated that exposure of cultured non-transformed human proximal epithelial cells to 25 mM D-glucose increased type IV collagen and fibronectin in the culture supernatant. Furthermore, we have demonstrated that the accumulation of these basement membrane constituents involved an alteration in the degradative pathway, which was the result of a net increase in TIMP 1 and TIMP 2 activity over gelatinase activity. Finally, we have also demonstrated that glucose may simultaneously activate two intracellular pathways, that is, the polyol pathway and PKC activation, which are both independently involved in mediating its effects on cell function.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the generous supply of TGF-β1 standards and antibody by Dr. A. Mire-Sluis (National Institute for Biological Standards and Control, Potters Bar, UK). In addition, the authors wish to thank Dr. M. Davies for his support and advice. RS is supported by the Kidney Research Unit for Wales Foundation, and KM is supported by the British Diabetic Association.

Reprint requests to Dr. A. O. Phillips, Institute of Nephrology, Cardiff Royal Infirmary, Newport Road, Cardiff. Wales CF2 1SZ United Kingdom

REFERENCES

1. Joint Working Party on Diabetic Renal Failure of the British Diabetic Association, the Renal Association and the Research Unit of the Royal College of Physicians: Renal failure in diabetics in the UK: Deficient provision of care in 1985. *Diab Med* 5:79–84, 1988
2. United States Renal Data Systems: Annual Report 1990. *Am J Kid Dis* 15:22–27, 1990
3. Bohle A, Wehrmann M, Bogenschutz O, Batz C, Muller GA: The Pathogenesis of chronic renal failure in diabetic nephropathy. *Pathol Res Pract* 187:251–259, 1991
4. Lane PH, Steffes MW, Fioretto P, Mauer SM: Renal interstitial expansion in insulin-dependant diabetes mellitus. *Kidney Int* 43:661–667, 1993
5. The Diabetes Control and Complications Research Group: The effect of intensive treatment of diabetes on the development and progression of long term complications in insulin dependant diabetes mellitus. *N Engl J Med* 329:977–986, 1993
6. The Diabetes Control and Complications Research Group: Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. *Kidney Int* 47:1703–1720, 1995
7. Ihm CG, Lee GSL, Nast CC, Artishevsky A, Guillermo R, Levin PS, Glassock RJ, Adler SG: Early increased renal procollagen α1 (IV) mRNA levels in streptozotocin induced diabetes. *Kidney Int* 41:768–777, 1992
8. Cagliero E, Maiello M, Boeri D, Roy S, Lorenzi M: Increased expression of basement components in human endothelial cells cultured in high glucose. *J Clin Invest* 82:735–738, 1988
9. Cagiero E, Roth T, Roy S, Lorenzi M: Characteristics and mechanisms of high-glucose induced over expression of basement membrane components in cultured human endothelial cells. *Diabetes* 40:102–110, 1991
10. Danne T, Spiro MJ, Spiro RG: Effect of high glucose on type IV collagen production by cultured glomerular epithelial, endothelial, and mesangial cells. *Diabetes* 42:170–177, 1993
11. Nahman NS, Leonharat KL, Cosio FG, Hebert CL: Effects of high glucose on cellular proliferation and fibronectin production by cultured human mesangial cells. *Kidney Int* 41:396–402, 1992
12. Frank J, Engler-Blum G, Roderman HP, Muller GA: Human renal tubular cells as a cytokine source: PDGF-B, GM-CSF and IL-6 mRNA expression in vitro. *Exp Nephrol* 1:26–35, 1993
13. Phillips AO, Topley N, Steadman R, Morrisey K, Williams J: Induction of TGF-β1 synthesis in D-glucose primed human proximal tubular cells: Differential stimulation by the macrophage derived pro-inflammatory cytokines IL-1β and TNFα. *Kidney Int* 50:1546–1554, 1996
14. Ong ACM, Fine LG: Tubular-derived growth factors and cytokines in the pathogenesis of tubulointerstitial fibrosis: Implications for human renal disease progression. *Am J Kid Dis* 23:205–209, 1994
15. Ziyadeh FN, Sharma K, Ericksen M, Wolf G: Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor beta. *J Clin Invest* 93:536–542, 1994
16. Wolf G, Sharma K, Chen Y, Ericksen M, Ziyadeh FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-β. *Kidney Int* 42:647–656, 1992
17. Rocco MV, Chen Y, Goldfarb S, Ziyadeh FN: Elevated glucose stimulates TGF-β gene expression and bioactivity in proximal tubule. *Kidney Int* 41:107–114, 1992
18. Phillips AO, Steadman R, Topley N, Williams JD: Elevated D-glucose concentrations modulate TGF-β1 synthesis by human cultured renal proximal tubular cells: The permissive role of platelet derived growth factor. *Am J Pathol* 147:362–374, 1995
19. Phillips AO, Morrisey R, Steadman R, Williams JD: Polarity of stimulation and secretion of TGF-β1 by proximal tubular cells. *Am J Pathol* 150:1101–1111, 1997
20. Larkins RG, Dunlop ME: The link between hyperglycaemia and diabetic nephropathy. *Diabetologia* 35:499–504, 1992
21. DeRubertis FR, Craven PA: Activation of protein kinase C in glomerular cells in diabetes. *Diabetes* 43:1–8, 1994
22. Flath MC, Bylander JE, Sens DA: Variation in sorbitol accumulation and polyol-pathway activity in cultured human proximal tubular cells. *Diabetes* 41:1050–1055, 1992
23. Detrisac CJ, Sens MA, Garvin J, Spicer SS, Sens DA: Tissue culture of human kidney epithelial cells of proximal tubule origin. *Kidney Int* 25:383–390, 1984
24. Engval E: *Fibronectin; Methods of Enzymatic Analysis.* Weinhiem, Verlag Chemie, 1986, pp 201–211
25. Donovan KL, Coles GA, Williams JD: An ELISA for the detection of type IV collagen in human urine—Application to patients with glomerulonephritis. *Kidney Int* 46:1431–1437, 1994
26. Baker T, Tickle S, Wasan H, Docherty A, Isenberg D, Waxman J: Serum metalloproteinases and their inhibitors: markers for malignant potential. *Br J Cancer* 70:506–512, 1994
27. Steadman R, Topley N, Jenner DE, Davies M, Williams JD: Type 1 fimbriate *Escherichia coli* stimulates a unique pattern of degranulation by human polymorphonuclear leukocytes. *Infect Immun* 56:815–822, 1988
28. Martin J, Knowlden J, Davies M, Williams JD: Identification and

independent regulation of human mesangial cell metalloproteinases. *Kidney Int* 46:877–885, 1994
29. MANIATIS T, FRITSCH E, SAMBROOK J: *Extraction, Purification, and Analysis of mRNA from Eukaryotic Cells.* Cold Spring Harbor, Cold Spring Harbor Press, 1987
30. O'BRYAN JP, FRYE RA, COGSWELL PC, NEUBAUER A, KITCH B, PROKOP C, ESPINOZA R, LE BM, EARP HS, LIU ET: A transforming gene isolated from primary human myeloid cells, encodes a novel receptor tyrosine kinase. *Mol Cell Biol* 11:5016–5031, 1991
31. BRINKER JM, GUDAS LJ, LOIDL HR, WANG SY, ROSENBLOOM J, KEFALIDES NA, MYERS JC: Restricted homology between human alpha-1 type IV and other procollagen chains. *Proc Natl Acad Sci USA* 82:3649–3653, 1985
32. BERNARD MP, KOLBE M, WEIL D, CHU ML: Human cellular fibronectin: Comparison of the carboxyl-terminal portion with rat identifies primary structural domains separated by hyper variable regions. *Biochemistry* 24:2698–2704, 1995
33. COLLIER TE, SCOTT MW, EISEN AZ, MARMER BL, GRANT GA, GOLDBERG GI: H-ras oncogene-transformed human bronchial epithelial cells secrete a single metalloproteinase capable of degrading basement membrane collagen. *J Biol Chem* 263:6569–6587, 1988
34. DOCHERTY AJP, LYONS A, SMITH BJ, WRIGHT EM, STEPHENS PE, HARRIS TJR: Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. *Nature* 318:66–69, 1985
35. STETLER-STEVENSON WG, BROWN PD, ONISTA M, LEVY AT, LIOTTA LA: Tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA expression in tumour cell lines and human tumour tissues. *J Biol Chem* 265:13933–13938, 1990
36. NEUBAUER A, NEUBAUER B, LIU E: Polymerase chain reaction based assay to detect allelic loss in human DNA: Loss of beta interferon gene in chronic myelogeneous leukemia. *Nucl Acids Res* 18:993–998, 1990
37. CAGLIERO E, ROTH T, MAIELLO M, LORENZI M: Expression of genes related to the extracellular matrix in human endothelial cells. *J Biol Chem* 266:14244–14250, 1991
38. ROY SRS, CAGLERO E, LORENZI M: Over expression of fibronectin induced by diabetes or high glucose: Phenomenon with a memory. *Proc Natl Acad Sci USA* 87:404–408, 1990
39. POULSOM R, KURKINEN M, PROKOP D, BOOT-HANDFORD R: Increased steady-state levels of laminin B1 mRNA in kidneys of long-term streptozotocin-diabetic rats. *J Biol Chem* 263:10072–10076, 1986
40. STETLER-STEVENSON WG: Dynamics of matrix turnover during pathologic remodelling of the extracellular matrix. *Am J Pathol* 148:1345–1350, 1996
41. COHEN MP, SURMA ML, WU VY: In vivo biosynthesis and turnover of glomerular basement membrane in diabetic rats. *Am J Physiol* 242: F385–F389, 1982
42. ASSOIAN RK, FLEURDELYS BE, STEVENSON HC, MILLER PJ, MADTES DK, RAINES EW, ROSS R, SPORN MB: Expression and secretion of type β transforming growth factor by activated human macrophages. *Proc Natl Acad Sci USA* 84:6020–6024, 1987
43. VILLEGER PM, LOTZ M: Differential expression of TGF-beta isoforms by human articular chondrocytes in response to growth factors. *J Cell Physiol* 151:318–325, 1992
44. PHILLIPS AO, TOPLEY N, MORRISEY K, WILLIAMS JD, STEADMAN R: Basic fibroblast growth factor stimulates the release of pre-formed TGF-β1 from human proximal tubular cells in the absence of *de-novo* gene transcription or mRNA translation. *Lab Invest* 76:591–600, 1997
45. WANG JL, NISTER M, RUDLOFF EB, PONTEN J, WESTERMARK B: Suppression of platelet derived growth factor α- and β-receptor mRNA levels in human fibroblast by SV40 T/t antigen. *J Cell Physiol* 166:12–21, 1996
46. CAGLIERO E, MAIELLO M, BOERI D, LORENZI M: High glucose increases TGF-β mRNA in endothelial cells: A mechanism for the altered regulation of basement membrane components? (abstract) *Diabetes* 37:97A, 1988
47. CAGLIERO E, ROTH T, TAYLOR AW, LORENZI M: The effects of high glucose on human endothelial cell growth and gene expression are not mediated by TGF-β. *Lab Invest* 73:667–673, 1995
48. CRAVEN PA, DAVIDSON CM, DERUBERTIS FR: Increase in diacylglycerol mass in isolated glomeruli by glucose from de novo synthesis of glycerolipids. *Diabetes* 39:667–674, 1990
49. BEYER-MEARS A, KU L, COHEN MP: Glomerular polyol accumulation on diabetes and its prevention by oral sorbinil. *Diabetes* 33:604–607, 1984
50. BLEYER AJ, FUMO P, SNIPES ER, GOLDFARB S, SIMMONS DA, ZIYADEH FN: Polyol pathway mediates high glucose induced collagen synthesis in proximal tubule. *Kidney Int* 45:659–666, 1994
51. MOWER P, AYNEDJIAM H, SILVERMAN S, WILKES B, BANK N: Sorbinil prevents glomerular hyper-perfusion in diabetic rats. (abstract) *Kidney Int* 35:433, 1989