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Inhibition of Transglutaminase Activity Reduces Extracellular Matrix Accumulation Induced by High Glucose Levels in Proximal Tubular Epithelial Cells*

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Diabetic nephropathy affects 30–40% of diabetics leading to end-stage kidney failure through progressive scarring and fibrosis. Previous evidence suggests that tissue transglutaminase (tTg) and its protein cross-link product ε(γ-glutamyl)lysine contribute to the expanding renal tubulointerstitial and glomerular basement membranes in this disease. Using an *in vitro* cell culture model of renal proximal tubular epithelial cells we determined the link between elevated glucose levels with changes in expression and activity of tTg and then, by using a highly specific site directed inhibitor of tTg (1,3-dimethyl-2[(oxopropyl)thio]imidazolium), determined the contribution of tTg to glucose-induced matrix accumulation. Exposure of cells to 36 mM glucose over 96 h caused an mRNA-dependent increase in tTg activity with a 25% increase in extracellular matrix (ECM)-associated tTg and a 150% increase in ECM ε(γ-glutamyl)lysine cross-linking. This was paralleled by an elevation in total deposited ECM resulting from higher levels of deposited collagen and fibronectin. These were associated with raised mRNA for collagens III, IV, and fibronectin. The specific site-directed inhibitor of tTg normalized both tTg activity and ECM-associated ε(γ-glutamyl)lysine. Levels of ECM per cell returned to near control levels with non-transcriptional reductions in deposited collagen and fibronectin. No changes in transforming growth factor β1 (expression or biological activity) occurred that could account for our observations, whereas incubation of tTg with collagen III indicated that cross-linking could directly increase the rate of collagen fibril/gel formation. We conclude that Tg inhibition reduces glucose-induced deposition of ECM proteins independently of changes in ECM and transforming growth factor β1 synthesis thus opening up its possible application in the treatment other fibrotic and scarring diseases where tTg has been implicated.

Diabetic nephropathy (DN)[1] accounts for 30–40% of patients requiring renal replacement therapy (1–3). It is characterized by an early thickening of tubular and glomerular basement membranes due to an excessive accumulation of the extracellular matrix and ultimately leads to progressive scarring and fibrosis of the kidney (1–3). It also causes a significant increase in the cardiovascular morbidity and mortality of diabetic patients. The incidence of diabetes and diabetic nephropathy continues to rise worldwide. It is predicted that the number of people with diabetes will continue to increase, reaching 221 million by 2010 (1).

Clinically, DN manifests itself by the onset of continuous microalbuminuria followed by the appearance of persistent proteinuria (2). This is followed by a progressive decline in glomerular filtration rate ultimately leading to end-stage renal failure. Although DN was traditionally considered to be primarily a glomerular disease, it is now widely accepted that the rate of deterioration of kidney function correlates best with the degree of tubulointerstitial fibrosis (3). Consequently, increased research has focused on the role of the tubular epithelial cells, in particular the proximal tubular epithelial cells, in the initiation of fibrosis.

In a recent study using the rat streptozotocin-induced hyperglycemic model of diabetes, we demonstrated elevated levels of the protein cross-linking enzyme tissue transglutaminase (tTg) in the peritubular interstitial space. This change in enzyme distribution was paralleled by an increase in the ε(γ-glutamyl)-lysine protein cross-link (4). We have shown similar changes in fibrotic/scarred human kidney biopsy material (5) and in the rat remnant kidney model of renal scarring (6, 7). In all cases the up-regulation of tTg and its product correlated significantly with increased interstitial fibrosis and scarring. In both animal and human tissues, we demonstrated tubular epithelial cells to be the major source of tTg.

Tissue transglutaminase belongs to a group of calcium-dependent mammalian enzymes that have the capacity to irreversibly cross-link proteins through the formation of ε(γ-glutamyl)lysine bonds (8). In addition to the potential role in kidney scarring, elevated levels of tTg have been associated with a number of other fibrotic diseases, including lung, liver, and heart (9–11), where its ability to cross-link ECM is thought to facilitate their increased deposition (12, 13) as well as their increased resistance to the action of matrix metalloproteinases (7). In addition to this proposed direct effect of tTg on matrix accumulation, the enzyme has also been implicated in the matrix storage and subsequent activation of the fibrogenic cytokine, transforming growth factor β_1 (TGFβ_1), through the cross-linking of the latent TGFβ_1-binding protein (LTBP-1) to

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[1] The abbreviations used are: DN, diabetic nephropathy; tTg, tissue transglutaminase; Tg, transglutaminase; ECM, extracellular matrix; TGFβ1, transforming growth factor β1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PTCs, proximal tubular epithelial cells; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; TMB, 3,3′,5,5′-tetramethylbenzidine; DOC, sodium deoxycholate; MOPS, 4-morpholinepropanesulfonic acid.

the ECM (14) thus facilitating its activation.

Given these potential direct and indirect contributions of tTg to the pathogenesis of DN and the epidemiological evidence, which indicates that the complications of diabetes are related to poor glycemic control (2, 15), it was important to demonstrate a molecular link between elevated glucose levels and changes in the expression and action of tissue transglutaminase. To date, changes in tTg in relation to elevated glucose conditions have only been observed in the pancreatic β cell (16).

The aim of the current study is to investigate the effects of high glucose levels on tTg expression and activity in the well characterized opossum proximal tubular epithelial cell line (OK cells) (17–19). Initial studies were aimed at examining the regulation of tTg expression in response to elevated glucose concentrations, whereas subsequent studies examined how changes in tTg could affect ECM deposition.

We demonstrate that elevated glucose concentrations stimulate *de novo* synthesis of tTg, which is paralleled by an increase in the expression of the ECM proteins fibronectin, collagen III, and collagen IV, with a subsequent increase in the deposition of total collagen and fibronectin. In the time frame of our experiments (96 h) we observed no changes in the expression or presence of TGFβ_1. We demonstrate, for the first time, that the observed increase in the deposition of fibronectin, collagen, and other matrix proteins induced by elevated glucose conditions can be directly linked to their covalent cross-linking via ϵ(γ-glutamyl)lysine bridges.

EXPERIMENTAL PROCEDURES

Experimental Protocol and Conditions—Opossum kidney proximal tubular epithelial cells (OK PTCs) (European collection of cell cultures, Cambridge, UK) were cultured in Dulbecco's modified Eagle's medium containing 6 mM D-glucose (glucose-free Dulbecco's modified Eagle's medium (Life Technologies) plus 1.1g/liter D-glucose), 100 units/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine, and 10% (v/v) fetal calf serum in a humidified atmosphere at 5% (v/v) CO_2.

For experimentation, 6 mM D-glucose was considered equivalent to normal glycemic conditions. Glucose concentrations between 12 and 36 mM were considered to represent the clinical hyperglycemic environment. To achieve this, media was further supplemented with D-glucose to the required concentration with a parallel flask containing an equivalent concentration of L-glucose to control for osmolarity. All experiments were carried out over a 96-h period with the alteration to glucose at the time of plating. To ensure that glucose differentials were maintained throughout the experimental time period, media was assayed for glucose daily using a glucose analyzer (Analox P-GM7 Micro-Stat) and glucose added to compensate for cell usage.

For Tg inhibition studies, two highly specific active site-directed, irreversible inhibitors were used. One, 1,3-dimethyl-2[(oxopropyl)thio]-imidazolium (20, 21) was termed compound NTU283. The other, a carboxybenzoyl-glutamylglycine analogue, was termed compound NTU281.[2] Both compounds were synthesized in-house. The inhibitor NTU283 was added to 36 mM D-glucose culture media at the time of plating to a final concentration of 33, 66, or 99 μM. The inhibitor NTU281 was used in the collagen fibrillogenesis assays at a concentration of 250 μM.

Cell Number, Protein, DNA, and Viability Determination—Protein concentrations were determined using protocols based on the Lowry and the bicinchoninic acid assays. DNA was determined using the Burton assay (diphenylamine). Viable cell numbers were measured by the counting of cells using the trypan blue exclusion method. Cell viability/cell leakage was also determined by measuring leakage of lactate dehydrogenase into the cell culture medium (Cytotox 96 viability assay kit, Promega, Southampton, UK).

Quantitation of Transglutaminase—Unless specified, cells were removed with 2 mg/ml trypsin-2 mM EDTA then centrifuged at 300 $\times$ *g* for 5 min, and the resultant pellet was washed in PBS. Cells were resuspended in 250 μl of STE buffer (0.32 M sucrose, 5 mM Tris, 1 mM EDTA) containing protease inhibitors phenylmethylsulfonyl fluoride (1 mM), benzamidine (5 mM), and leupeptin (10 μg/ml) and homogenized on ice.

For extracellular Tg, the media was collected and cells were removed by centrifugation. The media was freeze-dried and re-suspended in 1/10 volume of STE buffer with protease inhibitors prior to analysis for activity and tTg antigen (12).

Transglutaminase Activity—Transglutaminase activity was determined by the Ca^{2+}-dependent incorporation of [1,4-^{14}C]putrescine (Amersham Biosciences) into *N*,*N*′-dimethylcasein as previously described (6). Results were corrected to units/mg of protein (1 unit equals 1 nmol of putrescine incorporated per hour at 37 °C).

tTg Western Blotting—Proteins were separated on a 10% (w/v) polyacrylamide gel and then electroblotted onto Hybond ECL (Amersham Biosciences). The membrane was blocked with 3% nonfat dried milk, 0.05% (v/v) Tween 20 in PBS (milk-Tween-PBS) and then immunoprobed with 0.2 μg/ml of a anti-tTg monoclonal antibody (CUB7402, Stratek Scientific, Luton, UK) at 4 °C overnight. Primary antibody binding was revealed with 1.5 μg/ml goat anti-mouse horseradish peroxidase secondary antibody (Dako, UK) for 1 h at room temperature and visualized using ECL chemiluminescence (Amersham Biosciences). Films were quantified by volume densitometry using a Bio-Rad GS-690 imaging densitometer and molecular analyst version 4 software (Bio-Rad).

tTg ELISA—Ninety-six-well plates were coated with 100 μl of 5 μg/ml fibronectin overnight at 4 °C and blocked as above. Cell homogenates or concentrated culture media (50 μl) were incubated in triplicate for 2 h at room temperature and then washed with milk-Tween-PBS. Wells were then immunoprobed for tTg with 100 μl of 0.2 μg/ml of anti-tTg monoclonal antibody CUB7402 overnight at 4 °C and revealed with 100 μl of 1.5 μg/ml goat anti-mouse horseradish peroxidase-conjugated antibody for 2 h at room temperature using a TMB (3,3′,5,5′-tetramethylbenzidine) substrate. 50 μl of 2.5 M H_2SO_4 was used to stop the reaction, and the absorbance was read at 450 nm.

Immunolocalization of tTg—OK cells were seeded and cultured in 8-well plastic chamber slides (Lab Tech, Scientific Laboratory Supplies, Nottingham, UK), incubated for 96 h under experimental conditions and immunoprobed for tTg, as previously described (22) using 0.6 μg/ml of anti-tTg monoclonal antibody CUB7042 overnight at 4 °C. Binding was revealed using 3 μg/ml goat anti-mouse secondary antibody conjugated to fluorescein and visualized using a Leica TCS-NT confocal laser microscope with excitation at 488 nm and the emissions recorded at 530 nm.

Extracellular Matrix Analysis—For ϵ-(γ-glutamyl)lysine and hydroxyproline analysis, OK cells were grown in 10-cm Petri dishes and solubilized with 1 ml of 0.1% (w/v) sodium deoxycholate-2 mM EDTA (DOC-EDTA). The DOC-EDTA-soluble fraction was kept for protein analysis (BCA assay) while the insoluble residue, remaining on the plate and predominantly representing the ECM, was partially digested with 0.2 mg/ml trypsin/1 mM EDTA and then scraped off. The fraction was then freeze-dried. For tTg and fibronectin analysis, cells were seeded in 96-well plates, and the DOC-EDTA-insoluble fraction was immunoprobed directly on the plate.

ϵ-(γ-Glutamyl)lysine Levels—Freeze-dried proteins were suspended in 0.1 M ammonium carbonate and subjected to exhaustive proteolysis as previously described (23). Digests were freeze-dried again and resuspended in 0.1 N HCl. ϵ-(γ-glutamyl)lysine was analyzed by cation exchange chromatography using an LKB 4151 amino acid analyzer (Amersham Biosciences) using a modification of a lithium citrate buffer method with an Ultrapac 8 cation exchange resin (8 $\pm$ 0.5-μm particle size (23)). The detection of amino acids and peptides was undertaken by a post column reaction with 600 mg/liter *o*-phthalaldehyde/5 ml/liter 2-mercaptoethanol, and the fluorescence was observed at 450 nm after excitation at 360 nm using an LS1 detector (PerkinElmer Life Sciences) with analysis of chromatograms by a Nelson 9000 A-D integrator and software (Nelson Analytical). The amount of ϵ-(γ-glutamyl)lysine in each sample was quantified by standard addition of 1 nmol of ϵ-(γ-glutamyl)lysine dipeptide.

Quantitation of Hydroxyproline—DOC-EDTA-insoluble proteins were acid-hydrolyzed in 6 N HCl overnight at 110 °C in an oxygen-free environment. After freeze drying and re-suspending in H_2O the hydrolysates were assayed for hydroxyproline by derivatization with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole and separation by reverse phase high-performance liquid chromatography (Beckman Instruments, High Wycombe, UK) (24). Quantification was by absorption at 495 nm and peak integration as above.

Extracellular Fibronectin and tTg—DOC-EDTA-insoluble proteins were blocked with milk-Tween-PBS before the addition of either 0.2 μg/ml of the monoclonal anti-tTg antibody CUB7042 or a 1:1000 dilution of rabbit anti-fibronectin (clone IST-1, Sigma) at 4 °C overnight

[2] Patent application number GB0314262.7, June 2003.

TABLE I
Effect of high glucose on cell proliferation and viability

Extracellular D-glucose	Cell number	Total protein	Total DNA	Intracellular D-glucose	LDH ratio	Trypan blue staining
mM	$\times 10^6$	*mg/plate*	*μg/plate*	*μM*	%	
6	1.45 ± 0.15	0.23 ± 0.06	18.2 ± 1.3	0.87 ± 0.18	96.6 ± 0.4	95.4 ± 2.17
24	1.59 ± 0.17[a]	0.34 ± 0.08[a]	19.9 ± 0.7	1.36 ± 0.19	97.3 ± 0.8	96.6 ± 1.3
36	1.83 ± 0.30[a]	0.38 ± 0.1[a]	22.9 ± 2.3[a]	1.79 ± 0.19	97.4 ± 0.7	94.6 ± 2.8

[a] $p < 0.05$

(22). Primary antibody was revealed with 1.5 μg/ml goat anti-mouse IgG-horseradish peroxidase or goat anti-rabbit IgG-horseradish peroxidase conjugates before the application of 70 μg/ml TMB in phosphate-citrate urea hydrogen peroxide buffer. The reaction was stopped with 2.5 M sulfuric acid, and the absorbance was read at 450 nm.

Measurement of ECM Collagen by ^3H Proline Labeling—Cells were seeded in 10-cm Petri dishes at a density of 3.75×10^6 per dish. Deposited collagens were labeled by the addition of 20 μl of [2,3-^{3}H]proline (1.0 mCi/ml, ICN) at the time of plating. After 96 h the media was removed, and cells were washed with PBS. Cells were then removed with 2 ml of 0.25 M ammonium hydroxide in 50 mM Tris, pH 7.4, at 37 °C for 10 min. The soluble fraction was collected, and protein concentration was determined using the BCA assay. The dishes were washed extensively with increasing volumes of PBS before the ECM was solubilized with 2 ml of 2.5% (w/v) SDS in 50 mM Tris, pH 6.8. The dish was then scraped to ensure complete removal of the ECM, and 200 μl was measured for radioactivity in a scintillation counter.

Collagen Fibrillogenesis Assay—Collagen fibrillogenesis was monitored using a spectrophotometric method (25). Type III collagen (Sigma C3511) was solubilized in 0.2 M acetic acid at a concentration of 5 mg/ml at 4 °C with constant stirring for 24 h. Initiation of fibrillogenesis was by neutralization of the collagen mixture by the addition of 10× Dulbecco's modified Eagle's medium and 0.2 M HEPES buffer to final concentrations of 1× and 0.02 M, respectively. Addition of $CaCl_2$ to 5 mM and dithiothreitol to 5 mM was made immediately after neutralization and before addition of guinea pig liver tissue transglutaminase (Sigma T5398, ~1000 units/mg). Fibril formation after neutralization was monitored by measuring absorbance at 325 nm using a PYE Unicam SP1800 UV spectrophotometer.

mRNA Analysis—Total RNA was extracted from at least 5×10^6 cells using TRIzol™ (Invitrogen) and quantitated by optical density at 260 nm. 15 μg of total RNA was then run on a 1.2% (w/v) agarose/MOPS/formaldehyde gel and then capillary blotted on to Nylon+ (Roche Applied Science) and cross-linked with 70 mJ/cm^2 UV radiation (UV cross-linker, Amersham Biosciences). This was then probed with [α-^{32}P]dCTP random primed labeled (Prime a gene, Promega, UK), sequence-specific DNA probes for tTg (7), collagens III (26) and IV (27), fibronectin (28), and TGFβ1 (29) before exposure to BioMax MS film. The resulting autoradiographs were then quantified by scanning densitometry using a Bio-Rad GS-690 densitometer and Molecular Analyst version 4 software. Transcript size was determined by comparison to RNA molecular weight markers (Promega, UK) using the same analysis package. Values were then corrected for loading using repeat probings with GAPDH.

Detection of Active TGFβ—Active and total TGFβ (heat-activated) was determined using the mink lung epithelial cell assay whereby mink lung epithelial cells are stably transfected with a construct consisting of the TGFβ1 promoter region of plasmin activator inhibitor ligated to the luciferase reporter construct (30). Media (serum-free) from OK cells grown under increased glucose concentrations was collected and applied to mink lung epithelial cells overnight. The media was then removed, and the cells were lysed and assayed for luciferase activity using a luciferase assay kit (Promega) and luminometer (Anthos Lab Tec Instruments, Salzberg, Austria), as per the manufacturer's instructions. Commercially available TGFβ1 was used as a positive control.

Collagen Secretion—Cells were grown for 96 h with [2,3-^{3}H]proline (Amersham Biosciences) added to the culture media at a final concentration of 75 kBq/ml. To determine the total amount of [2,3-^{3}H]proline-labeled proteins present in the media, 25 μl of media was precipitated onto 3MM filter paper using ice-cold 10% trichloroacetic acid, washed three times in 5% trichloroacetic acid and twice in ethanol before counting in a scintillation counter. To determine the collagen component of this, a parallel sample of media was digested with 30 units/ml of chromatography-purified (*i.e.* free of proteases and clostripain) bacterial collagenase (Sigma) for 15 min at 37 °C in 50 mM Tris, pH 7.4, 10 mM $CaCl_2$, 0.2 M NaCl before precipitation with trichloroacetic acid. The overall amount of collagen-labeled material in the media was calculated by subtracting the counts following collagenase digestion from the total labeled protein.

Fluorography of Secreted Collagens—Media from cells grown in 75 kBq/ml labeled proline (Amersham Biosciences) for 96 h was precipitated using 4 volumes of acetone, washed 3×, and then resuspended in 1% (w/v) SDS, 50 mM Tris, pH 7.4, 1 mM EDTA. 50 μl of protein was then separated on 10% (w/v) polyacrylamide gels using an interrupted SDS-PAGE (31, 32) approach to allow clear separation of collagen I and III bands. The gel was then dried under vacuum at 80 °C and exposed to BioMax MS film at −70 °C for 4 weeks.

Data Analysis—All data are shown as means ± S.E. Experimental groups were compared using Student's *t* test with unequal variance. $p < 0.05$ was taken as statistically significant.

RESULTS

Effect of High Glucose on Cell Proliferation and Viability—Increasing the concentration of glucose from 6 to 36 mM in the culture media of OK PTCs over a 96-h period caused a dose-dependent increase in viable cell numbers with no significant changes observed in cell leakage (lactate dehydrogenase ratio) and cell viability (Trypan Blue) (Table I).

Effect of High Glucose on Transglutaminase—Increasing the media concentration of glucose caused a dose-dependent increase in the cellular activity of Tg in OK cells (Fig. 1*a*). This was shown to be due to increases in tTg protein measured by both ELISA (Fig. 1*b*) and Western blotting (Fig. 1*c*), which was mRNA-dependent (Fig. 1*d*). Immunoprobing of cells for tTg showed that large increases in antigen were localized to cell clusters (Fig. 1*e*). Measurement of Tg activity and tTg antigen in the culture media demonstrated that changes in intracellular tTg were carried through to the extracellular environment with Tg activity increasing from 1.65 ± 0.21 to 2.5 ± 0.27 units/10^6 cells and tTg antigen from 1.51 ± 0.07 to 3.3 ± 0.41 ng/10^6 cells as glucose concentration was increased from 6 to 36 mM, respectively.

Effects of High Glucose on the Extracellular Matrix—Following the removal of cells using a deoxycholate-EDTA solution (DOC-EDTA), the remaining extracellular proteins (*i.e.* ECM) increased in response to glucose from 1.1 ± 0.1 mg/10^7 cells at 6 mM glucose to 2.5 ± 0.2 mg/10^7 cells at 36 mM glucose (Fig. 2*a*). Analysis of fibronectin (Fig. 2*b*) and total collagen (hydroxyproline) (Fig. 2*c*) showed that both these proteins were in part responsible for increased levels of ECM proteins. Northern blot analysis indicated that these increases were mRNA-dependent with specific changes in fibronectin, collagen I and IV mRNA levels (Fig. 2, *d–f*). There was no measurable change in TGFβ1 mRNA or active TGFβ1 present in the cell culture medium (data not shown) suggesting these increases were not mediated via changes in TGFβ1 synthesis.

Analysis of the recovered ECM by reverse phase high-performance liquid chromatography showed a significantly increased level of ε-(γ-glutamyl)lysine cross-linking at high glucose concentrations in comparison to 6 mM glucose-cultured cells where ε-(γ-glutamyl)lysine was not detectable under the conditions used (Fig. 3*b*). Further analysis of the ECM by ELISA for tTg showed a corresponding increase to that of

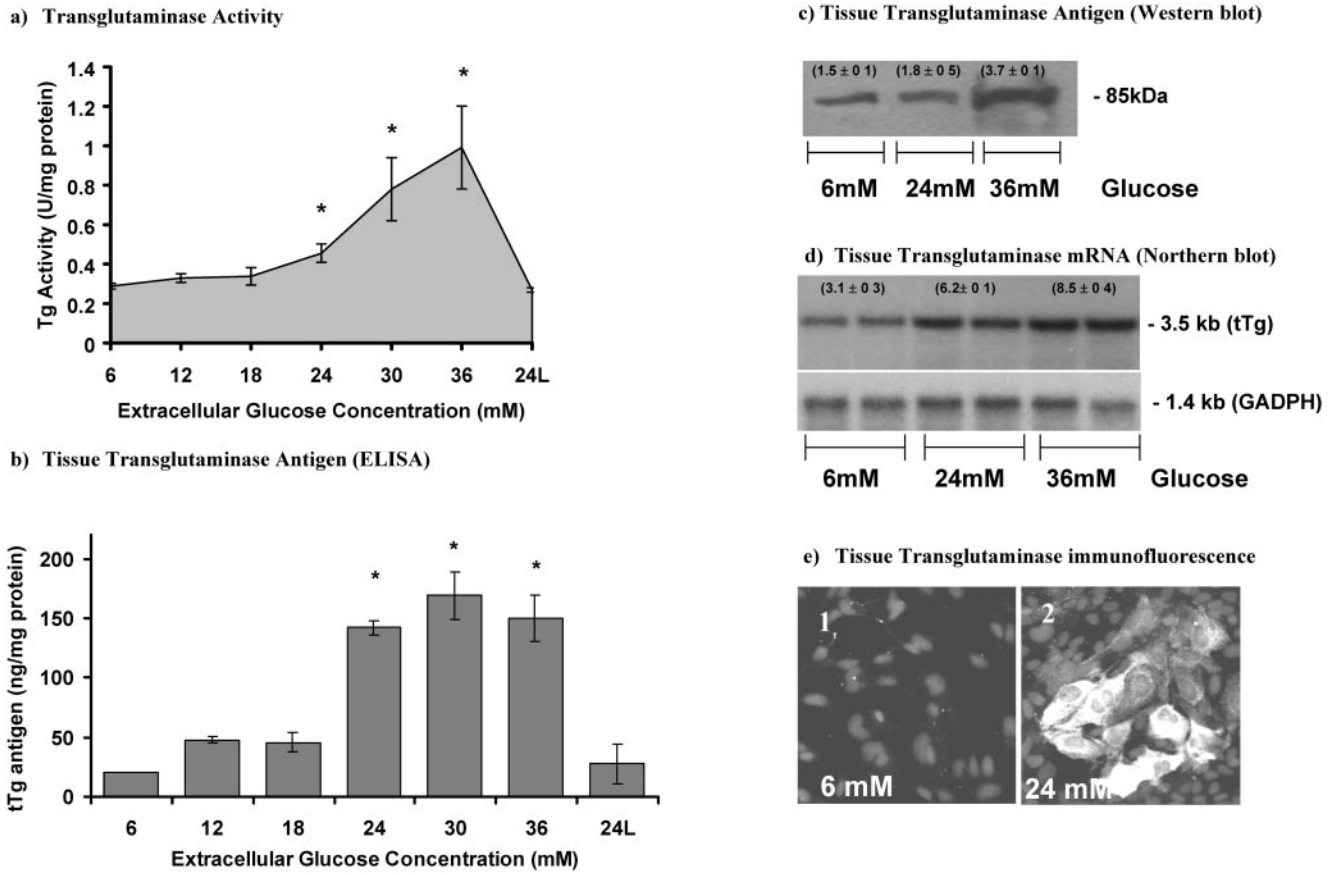

FIG. 1. **The effect of high glucose on transglutaminase levels in OK cells.** OK cells were incubated for 96 h in a range of glucose concentrations and cell homogenates assayed for Tg activity by putrescine incorporation assay (*a*), tTg antigen by ELISA (*b*), and Western blotting (*c*), mRNA by Northern blotting (*d*) and localized using immunofluorescence (*e*) using an fluorescein isothiocyanate label with propidium iodide stained nuclei at 400× magnification. Data in *parentheses* represent volume density measurements, which for protein are corrected for protein loading, and mRNA analysis is corrected for loading using GAPDH. All data represent mean values ± S.E. from at least three separate experiments performed in triplicate.

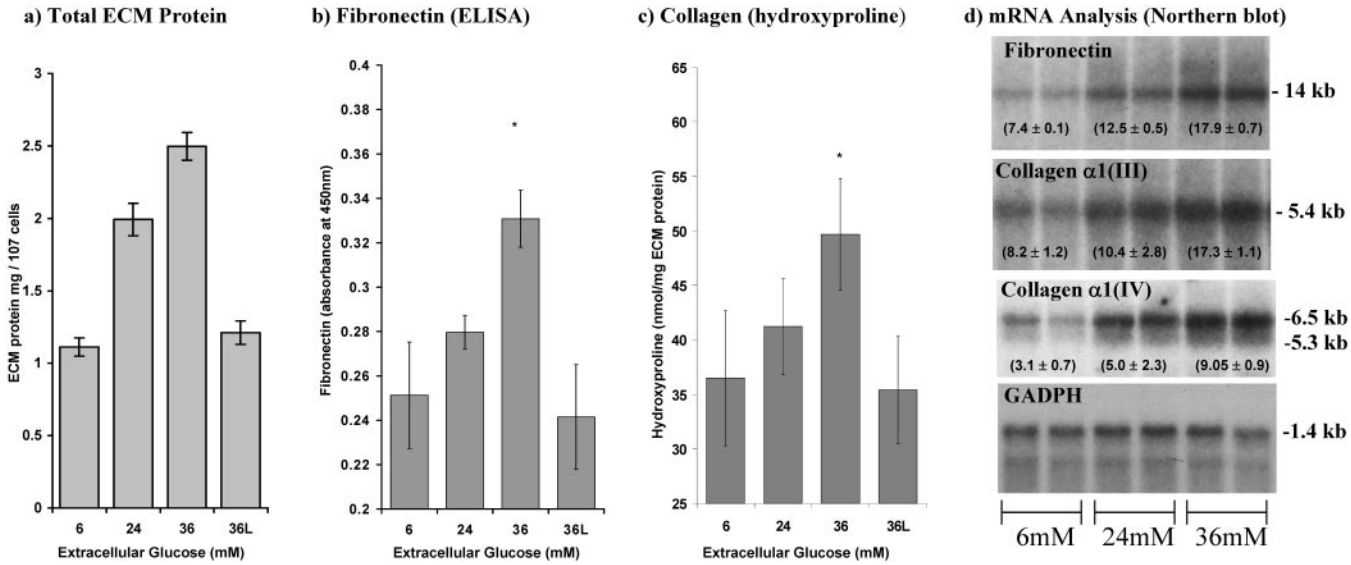

FIG. 2. **The effect of high glucose on the extracellular matrix in OK cells.** OK cells were incubated for 96 h in 6, 24, and 36 mM glucose. Cells were then removed using deoxycholate-EDTA. The insoluble protein remaining on the plate (ECM) was recovered, and the total amount of protein was assayed (Lowry) and expressed per 1×10^7 cells (*a*). Parallel plates were assayed more specifically for fibronectin by ELISA (*b*) and collagen using hydroxyproline (*c*). Northern blot analysis was used to ascertain if any of these changes were mRNA-dependent (*d*) with data in parenthesis representing volume density measurements corrected for loading using GAPDH. All data represents mean values ± S.E. from at least three separate experiments performed in triplicate.

ϵ-(γ-glutamyl)lysine (Fig. 3*a*) indicating that tTg is a strong candidate for the increased cross-linking.

Inhibition of Transglutaminase Activity—To determine if tTg-induced ECM cross-linking affected the accumulation of ECM proteins in response to high glucose in OK cells, cells were incubated in the presence of the specific irreversible site-

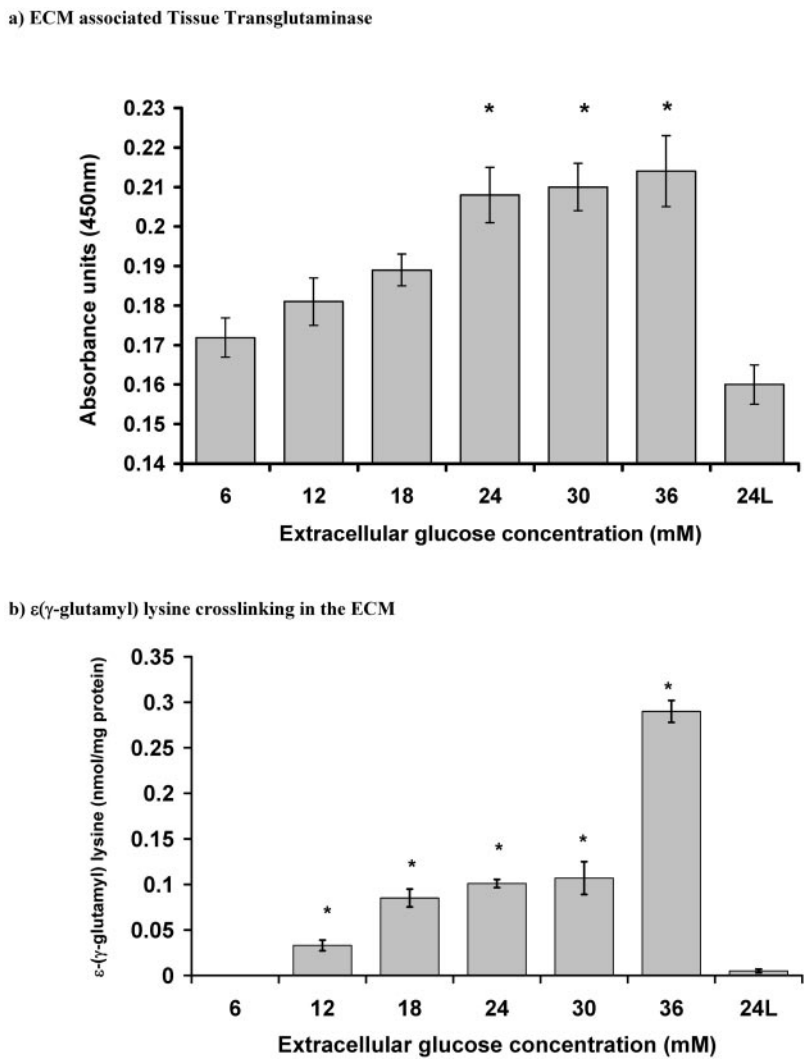

FIG. 3. **Transglutaminase cross-linking in the extracellular matrix in OK cells.** OK cells were incubated in a range of glucose concentrations for 96 h. Cells were then removed using deoxycholate-EDTA. The insoluble protein remaining on the plate (ECM) was then assayed for tTg using a direct ELISA (*a*) or recovered, exhaustively digested, and ϵ(γ-glutamyl)lysine dipeptide cross-link measured by cation exchange on an amino acid analyzer (*b*). All data represent mean values ± S.E. from at least three separate experiments performed in triplicate.

directed Tg inhibitor (NTU283). This led to a dose-dependent decrease in Tg activity from the elevated level observed at 36 mM glucose (Fig. 4*a*). At the highest dose of inhibitor used there was no effect on total viable cell numbers, cell leakage, or cell viability (data not shown) when compared with the respective control cells without inhibitor.

Effect of Tg Inhibition on Glucose-mediated ECM Accumulation—Following removal of OK cells grown in 36 mM glucose with DOC-EDTA, analysis of the recovered ECM showed a corresponding reduction in the level of ϵ-(γ-glutamyl)lysine isodipeptide cross-link per milligram of protein with increasing concentration of Tg inhibitor (Fig. 4*b*). However, even with the highest dose employed, levels could not be normalized to those found in cells grown at 6 mM glucose. The reduction in ϵ-(γ-glutamyl)lysine isodipeptide also corresponded to a reduction in the total amount of ECM protein deposited per cell (Fig. 5*a*). More specific analysis of the ECM for total collagen (hydroxyproline) (Fig. 5, *b–d*) and fibronectin (Fig. 5*e*) showed a corresponding decrease in both these ECM proteins with increasing concentrations of Tg inhibitor 283. To confirm the observed reduction in total collagen deposited, Tg inhibition in a high glucose environment was repeated but using [^{3}H]proline labeling of cells to measure the deposition of collagen in the ECM. Tg inhibition was highly effective in reducing the amount of label incorporated when normalized per cell (Fig. 5*f*).

To rule out the possibility that the Tg inhibitor NTU283 might be affecting transcription of the measured ECM proteins, TGFβ1 or tTg, Northern blot analysis was performed on the inhibitor-treated cells. In no case was any significant alteration in the mRNA levels of collagen III, IV, fibronectin, TGFβ1, or tTg noted (Fig. 6) indicating that the effect of Tg inhibition is not occurring at the transcriptional level.

To rule out any possible effects of the Tg inhibitor on collagen secretion [^{3}H]proline was used to label collagen in OK cell cultures grown at 30 mM glucose for 96 h with or without Tg inhibitor NTU283. The total counts adjusted per milligram of cellular protein for the collagenase-degradable protein found in the medium was 309,665 ± 29,604 for the 30 mM glucose medium without inhibitor and 334,183 ± 29,604 with inhibitor obtained from three separate experiments. Because these values are not significantly different, it can be concluded that the Tg inhibitor NTU283 does not affect collagen deposition through its effects on cell secretion. Interestingly, when these values for secreted collagen are compared with the amount of collagen actually deposited (Fig. 5*f*), it can be seen that only a low percentage of the collagen found in the medium (~0.6%) is actually deposited. To confirm the findings that the Tg inhibitor does not affect collagen secretion, fluorography was undertaken on the labeled proteins found in the medium of treated cells following separation of proteins by SDS-PAGE. Despite the presence of multiple bands shown by fluorography (not shown), it was possible to observe particularly intense bands that ran parallel to purified collagens (α1)III and (α1,2)IV, with weaker bands that could be associated with collagen I chains.

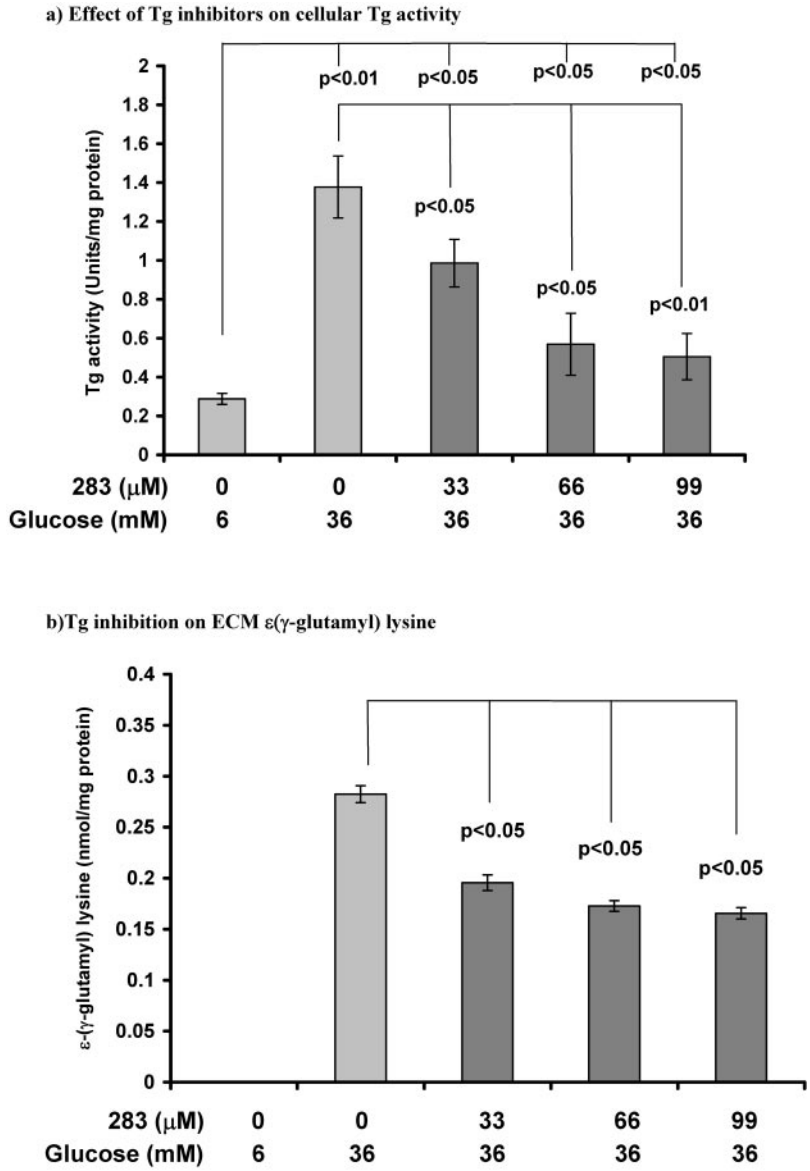

FIG. 4. **Ability of compound 283 to inhibit Tg activity and prevent ECM cross-linking.** OK cells were incubated in 36 mM glucose for 96 h in the presence of Tg inhibitor 283 to a final concentration of 33, 66, or 99 μM. Cells were then harvested, and the cellular Tg activity was measured using a putrescine incorporation assay (*a*). In parallel plates, cells were removed using deoxycholate-EDTA, and the insoluble protein remaining on the plate (ECM) was recovered, exhaustively digested, and $\epsilon(\gamma$-glutamyl)lysine dipeptide cross-link measured by cation exchange on an amino acid analyzer (*b*). All data represent mean values ± S.E. from at least three separate experiments performed in triplicate.

There was no obvious difference in either the banding pattern or intensity between cells grown in 30 mM glucose with or without NTU283 (data not shown).

Effect of Transglutaminase on in Vitro Type III Collagen Fibrillogenesis—To determine whether Tg could affect the deposition rate of collagen into the ECM, an *in vitro* assay of collagen fibril formation was performed. The addition of Tg to neutralized type III collagen *in vitro* resulted in an increase in the rate of fibril formation in a dose-dependent manner (Fig. 7). A significant increase ($p < 0.05$) in the final level of turbidity indicative of fibril formation was also noted with Tg treatment. Inhibition of transglutaminase activity using the Tg inhibitor confirmed that these effects were solely due to Tg activity.

DISCUSSION

Although the etiology of DN is multifactorial, it is now recognized that the degree of glycemic control will play a major role in the rate of progression of kidney scarring (33). Furthermore, it is becoming apparent that the progression of renal dysfunction correlates best with the degree of interstitial fibrosis rather than glomerular pathological changes (34). In previous studies using both animal models (4) of DN and the analysis of human biopsy material (5), we demonstrated that the Ca^{2+}-dependent protein cross-linking enzyme tTg may contribute to the pathological changes occurring in the renal interstitium by increasing matrix deposition either directly through the cross-linking of matrix proteins (7, 13) and/or indirectly through its ability to activate matrix associated TGFβ_1 (22). As a consequence, we have now investigated how elevated glucose concentrations may alter proximal tubular cell (PTC) function in relation to tTg expression and activity and how this in turn may affect changes in the ECM. In the well characterized OK PTC cell line, we demonstrated that elevated glucose levels maintained over a 96-h period lead to a mRNA-dependent increase in tTg activity and antigen. Importantly, as previously observed in other cells expressing high levels of tTg (12, 33), the increase in tTg expression resulted in an increase in the enzyme translocated into the extracellular space by a regulated but unknown process (tTg does not contain a conventional leader sequence for secretion via the Golgi/endoplasmic reticulum route) (35, 36, 37), because no loss of cell viability or increased cell leakage was induced by incubation with high glucose. This increase in tTg seems to be directly related to the stress imposed by the elevated glucose and not due to the effects of up-regulated TGFβ1, a known fibrogenic cytokine capable of inducing tTg expression (38, 39). Over the 96-h period no changes in TGFβ1 expression or biologically active

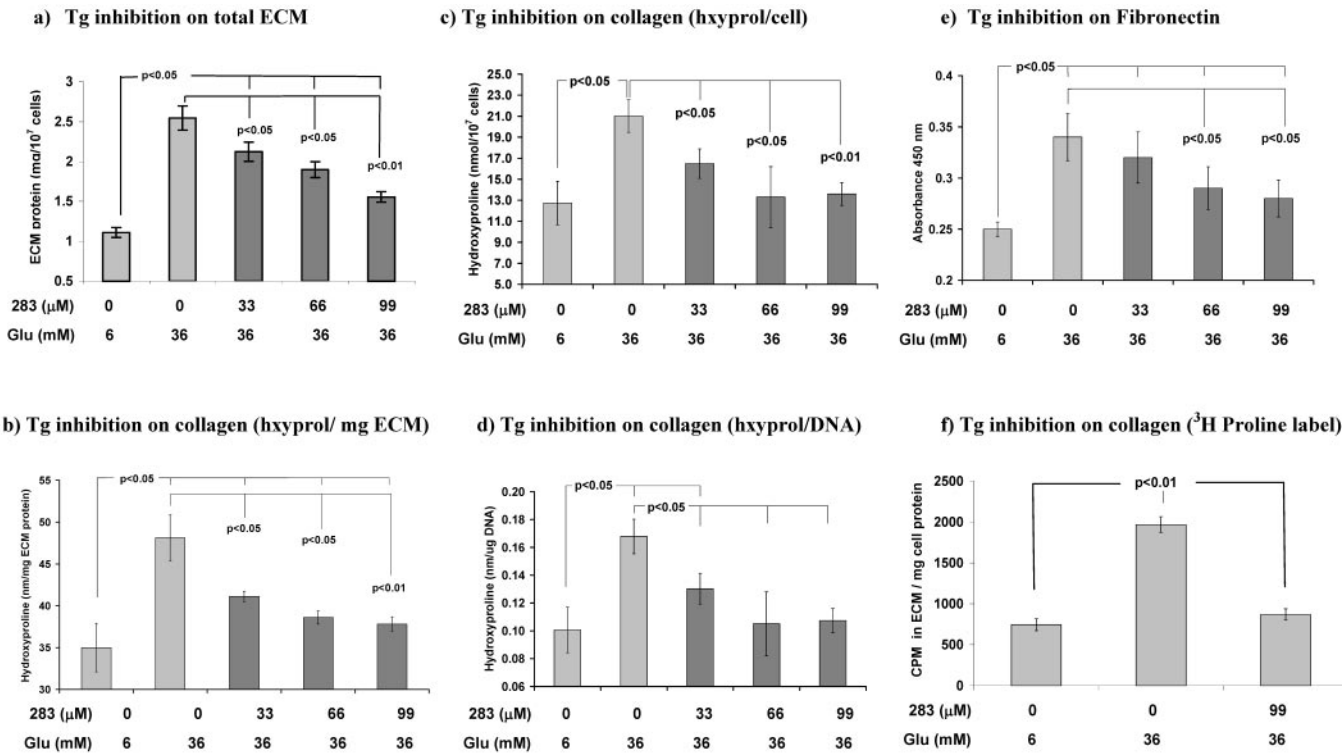

FIG. 5. **Effect of Tg inhibition on glucose-induced ECM levels.** OK cells were incubated in 36 mM glucose (*Glu*) for 96 h in the presence of Tg inhibitor 283 to a final concentration of 33, 66, or 99 μM. Cells were removed using deoxycholate-EDTA, the insoluble protein remaining on the plate (ECM) was recovered, and the total amount of protein was assayed (Lowry) and expressed per 1×10^7 cells (*a*). Parallel plates were assayed more specifically for collagen (hydroxyproline (hxyprol)) (*b–d*) and fibronectin (*e*). Hydroxyproline was normalized to ECM protein (*b*), cell number (*c*), and DNA (*d*). Changes in collagen were confirmed using additional plates grown in the presence of [^{3}H]proline whereby the cells were removed with ammonium hydroxide, and the recovered ECM scintillation was counted and expressed per milligrams of cell protein (*f*). All data represents mean values ± S.E. from at least three separate experiments performed in triplicate.

cytokine were detectable in the OK cells under the conditions that we used. This is in agreement with studies using human proximal tubular epithelial cells, which demonstrated that prolonged exposure to high glucose of 7 days was required to increase TGFβ1 production (40). Changes in TGFβ1 were therefore not responsible for the observed increases in the expression of fibronectin, collagen III, and collagen IV, which was reflected in the parallel increases in total ECM, collagen, and fibronectin deposited in cells exposed to high glucose.

Importantly, these glucose-induced changes in ECM deposition correlated well with the increase in the matrix-associated $\epsilon(\gamma$-glutamyl)lysine covalent cross-link, suggesting a direct involvement of tTg in matrix accumulation. In previous studies, we have demonstrated that increased expression of tTg in Swiss 3T3 fibroblasts leads to an increase in the deposition and cross-linking of fibronectin and the latent TGFβ_1 binding protein (22). We have also shown that exogenous addition of tTg to primary human dermal fibroblasts leads to an increase in the accumulation of matrix-associated collagen (12).

To test whether the glucose-induced increase in the deposition of total ECM and total collagen in the OK cells were directly related to the Tg-mediated cross-linking observed, the specific site-directed irreversible inhibitor of transglutaminase 1,3-dimethyl-2[(oxopropyl)thio]imidazolium ($IC_{50} \sim 50$ μM) was included in the culture medium during exposure of cells to high glucose. Inclusion of the inhibitor (33–99 μM) led to a dose-dependent inhibition of tTg activity measured in cell homogenates with ~70% inhibition obtained at 99 μM. Of relevance, the concentration of Tg-mediated $\epsilon(\gamma$-glutamyl)lysine cross-link found in the ECM was reduced by around 50% when cells were incubated with the inhibitor. This inhibition of Tg-mediated cross-linking was reflected by comparable reductions in total ECM protein and fibronectin confirming the importance of Tg-mediated cross-linking in ECM accumulation. An even greater inhibitory effect (around 90%) was observed in total ECM-associated collagen, the major protein associated with scarring, when measured by two independent methods. The finding, that the site-directed inhibitor of Tg, at the concentrations used, had no effect on the transcription of collagen III, collagen IV, and fibronectin or on the secretion of collagen into the extracellular environment, indicates that its mode of action in reducing levels of ECM is unlikely to be mediated via either reducing the expression of these proteins or by affecting their secretion.

Our results therefore indicate a key role for Tg-mediated cross-linking in the pathological events associated with increased ECM deposition induced by elevated glucose in OK PTCs thus providing the molecular link between hyperglycemia and elevated tTg activity. Apart from factor XIII A subunit and the prostate transglutaminase for which there is no associated evidence within renal proximal tubular cells, tissue transglutaminase is the only known mammalian transglutaminase to be secreted into the extracellular space. The identification of increased levels of tTg in the ECM and medium of OK PTCs after exposure to elevated glucose also confirms the involvement of tTg as the key transglutaminase responsible for the changes in the ECM that we observe. In human dermal fibroblasts, we demonstrated that exogenous addition of tTg not only increased collagen deposition, but it also slowed down its rate of turnover (12), which is in part due to the increased resistance of cross-linked collagen to proteolytic degradation (7). Using *in vitro* studies, we have demonstrated that tTg-mediated cross-linking of collagen I can lead to ~0.5 mol of $\epsilon(\gamma$-glutamyl)lysine incorporated into 1 mol of collagen I monomer (42). In the present study we demonstrate that tTg-mediated cross-linking of collagen III can increase both the rate

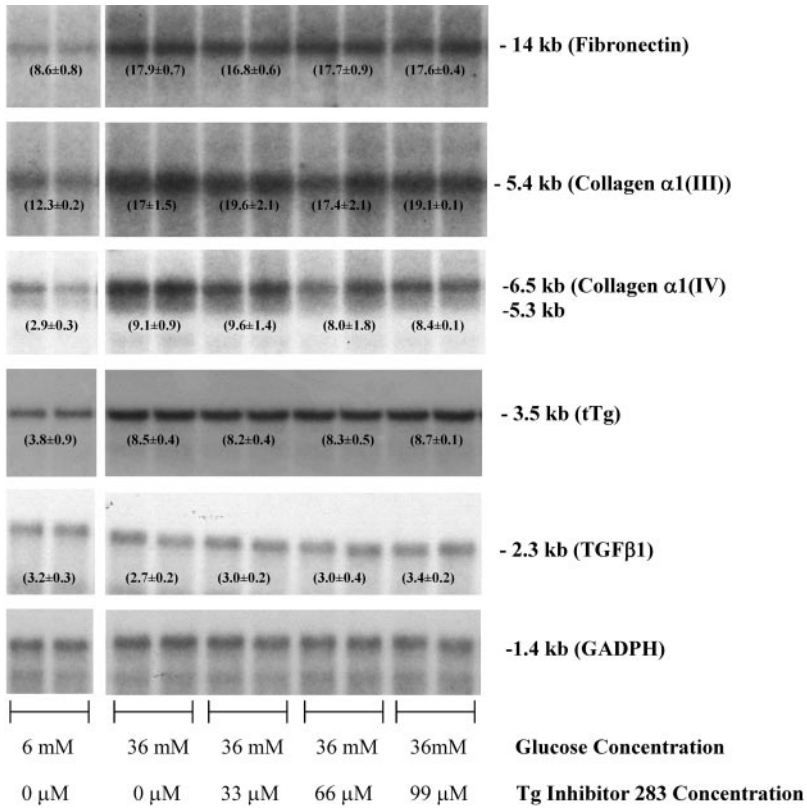

FIG. 6. **Effect of Tg inhibition on tTg, TGFβ1, and ECM protein mRNA expression.** OK cells were incubated in 36 mM glucose for 96 h in the presence of Tg inhibitor 283 to a final concentration of 33, 66, or 99 μM. Cells were harvested, and total RNA was extracted and subjected to Northern blot analysis using specific [^{32}P]dCTP random-primed cDNA probes. Representative autoradiographs are shown for collagens III and IV, fibronectin, tTg, and TGFβ1. Repeat probings using GAPDH acted as a loading control. Arbitrary volume density values corrected for GAPDH are shown in *parentheses*.

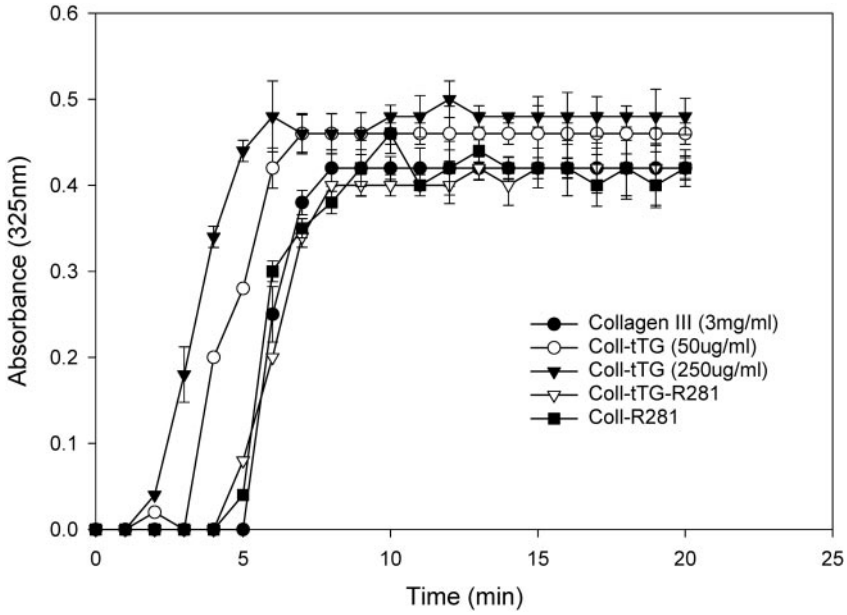

FIG. 7. **Effect of tTg on type III collagen fibrillogenesis.** Collagen (3 mg/ml) was neutralized as outlined under "Experimental Procedures" and then treated with 0, 50, or 250 μg/ml tTg. Transglutaminase inhibitor (R281 500 μM) was used to confirm that the effects were due to transglutaminase activity. The absorbance at 325 nm was measured using a PYE Unicam SP1800 UV spectrophotometer. The temperature was controlled at 25 °C using a Techne C-85A circulator. Results are from the average of three independent experiments.

and amount of collagen fibril formation. The speed at which this occurs, *i.e.* over 5 min, suggests that very little cross-link is required. This is confirmed by the finding that in the high glucose-treated OK PTCs ~ 0.3 nmol of ε(γ-glutamyl)lysine was found per milligram of total ECM. However, this value is likely to vary quite considerably in the cell culture, because enzyme expression as defined by immunofluorescence was much more concentrated in specific areas after glucose exposure. Assuming collagen III is the preferred collagen substrate as demonstrated by others (43) and that this protein makes up around 25% of the total ECM, then this value equates to around 1 cross-link per 2–3 collagen molecules of 300,000 Da. However, the ability of the site-directed inhibitor of tTg activity to reverse this effect both *in vitro* and in the cell model, confirms that transamidation is essential for any effect on fibril formation to occur. Importantly, transglutaminase-mediated cross-linking of collagen is able to increase both the rate and level of its deposition as well as reducing its rate of degradation thus tipping the balance toward collagen accumulation.

tTg-mediated cross-linking can therefore affect both the rate of ECM deposition as well as its clearance; either of which can account for the reduction in ECM levels following Tg inhibition. Laurent *et al.* (44, 45) suggested that ECM components are synthesized in excess of requirements, a finding that is confirmed by our studies when analyzing the amount of collagen secreted into the culture medium compared with that which is

deposited. Hence the rate of deposition of available components is crucial to ECM levels. Clearly an enzyme capable of cross-linking proteins leading to their increased deposition and proteolytic stability would incorporate more proteins into the available matrix by non-conventional routes. Work by Kleman *et al.* (13) also showed that tTg could stabilize fibrils of collagen V and XI independently of lysyl oxidase further indicating that tTg can provide such a route. This is likely to be applicable to other collagens and ECM components that are tTg substrates.

In conclusion, when taken together our data strongly support the hypothesis that increased expression of tTg in renal proximal epithelial cells induced by elevated glucose is a key contributory factor in the pathological events associated with the progression of interstitial fibrosis and scarring in diabetic nephropathy. As such this enzyme presents itself as a potential therapeutic target for the treatment of this disease and other fibrotic and scarring conditions where tTg has been implicated (9–11). The availability of specific inhibitors makes this task closer to clinical translation.

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Inhibition of Transglutaminase Activity Reduces Extracellular Matrix Accumulation Induced by High Glucose Levels in Proximal Tubular Epithelial Cells

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