Role of connective tissue growth factor in the pathogenesis of diabetic nephropathy

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We characterized a rabbit polyclonal antibody raised against human recombinant connective tissue growth factor (CTGF). The antibody recognised a higher molecular mass form (approx. 56 kDa) of CTGF in mesangial cell lysates as well as the monomeric (36–38 kDa) and lower molecular mass forms (< 30 kDa) reported previously. Immunohistochemistry detected CTGF protein in glomeruli of kidneys of non-obese diabetic mice 14 days after the onset of diabetes, and this was prominent by 70 days. CTGF protein is also present in glomeruli of human patients with diabetic nephropathy. No CTGF was detected in either normal murine or human glomeruli. Transient transfection of a transformed human mesangial cell line with a CTGF–V5 epitope fusion protein markedly increased fibronectin and plasminogen activator inhibitor-1 synthesis in cultures maintained in normal glucose (4 mM) conditions; a CTGF-antisense construct reduced the elevated synthesis of these proteins in high glucose (30 mM) cultures. Culture of primary human mesangial cells for 14 days in high glucose, or in low glucose supplemented with recombinant CTGF or transforming growth factor β1, markedly increased CTGF mRNA levels and fibronectin synthesis. However, whilst co-culture with a CTGF-antisense oligonucleotide reduced the CTGF mRNA pool by greater than 90 % in high glucose, it only partially reduced fibronectin mRNA levels and synthesis. A chick anti-CTGF neutralizing antibody had a similar effect on fibronectin synthesis. Thus both CTGF and CTGF-independent pathways mediate increased fibronectin synthesis in high glucose. Nevertheless CTGF expression in diabetic kidneys is likely to be a key event in the development of glomerulosclerosis by affecting both matrix synthesis and, potentially through plasminogen activator inhibitor-1, its turnover.

Key words: fibronectin, fibrosis, non-obese diabetic mice, plasminogen activator inhibitor-1.

INTRODUCTION

Hyperglycaemia is implicated as the major factor initiating the development of diabetic nephropathy (DN) in humans and in animal models of diabetes mellitus [1]. DN is characterized by an accumulation of mesangial extracellular matrix, manifest as glomerulosclerosis, and by the development of tubulointerstitial fibrosis [2]. However, the molecular mechanisms driving these changes are less clear. Previously [3], using a subtraction hybridization technique, we identified upregulated expression of connective tissue growth factor (CTGF) in human mesangial cells (HMCs) exposed to high concentrations of glucose. This was confirmed by Murphy et al. [4]. CTGF is a cysteine-rich member of the CCN family of growth regulators, which includes Cyr61/Cef10, NOV and ELM-1 [5], and is a monomeric 36–38 kDa secreted protein. However, other forms also exist such as a smaller 10–12 kDa product that retains biological activity [6], and 18 kDa and 24 kDa degradation products which have been found in human biological fluids [7]. The human CTGF gene has been mapped to chromosome 6q23. It has been reported to be transcriptionally activated by serum growth factors [8], transforming growth factor β1 (TGFβ1) [9,10], bone morphogenetic protein 2 [11], glucocorticoids such as dexamethasone [12], thrombin [13], lysophosphatidic acid and 5-hydroxytryptamine [14], high glucose and cyclic mechanical strain [15]. The regulation of CTGF gene expression appears to be through *cis*-acting elements in both the promoter [16] and the 3′-untranslated region [17].

The physiological function of CTGF has not yet been fully elucidated. However, it may be involved in the pathogenesis of fibrosis, possibly acting as a mediator of TGFβ1 [18,19]. Increased expression of CTGF has been found in inflammatory bowel disease [20], skin lesions from scleroderma and systemic sclerosis [21,22], renal fibrosis [23,24], liver fibrosis [25], idiopathic pulmonary fibrosis and pulmonary sarcoidosis [26].

To assess the role of CTGF in the pathogenesis of DN, we first investigated its expression in the kidneys of non-obese diabetic (NOD) mice at various times after developing hyperglycaemia, and in non-hyperglycaemic control animals. We also examined CTGF levels in renal biopsy specimens from human DN patients. Secondly, we investigated whether transient overexpression or depleted expression of endogenous CTGF modulated the expression of fibronectin and plasminogen activator inhibitor-1 (PAI-1) in transformed HMC. Thirdly, we investigated whether

Abbreviations used: CRE, cAMP-response element; CTGF, connective tissue growth factor; rCTGF, recombinant CTGF; DN, diabetic nephropathy; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMC, human mesangial cell; NOD, non-obese diabetic; PAI-1, plasminogen activator inhibitor-1; RT, reverse transcriptase; TGFβ1, transforming growth factor β1; THMC, transformed HMC.

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CTGF is the only mediator for the effects of long-term exposure to high glucose or TGFβ1 on fibronectin expression in primary HMC cultures.

MATERIALS AND METHODS

Primary normal adult HMCs (CC-2259, lot 3F1510) from BioWhittaker (Wokingham, Berks., U.K.) were maintained in culture between passages 6–10 as described previously [27]. Normal HMCs were transformed with simian virus 40 large T antigen following the Immortalex kit instructions (Novus Molecular, San Diego, CA, U.S.A.) and were cultured beyond crisis to obtain an immortalized line [transformed HMCs (THMCs)]. The THMCs were maintained in RPMI 1640 growth medium containing 4 mM D-glucose as for primary cells, but the concentration of foetal calf serum (FCS) was reduced to 5 % (v/v), and insulin, transferrin, sodium and selenite were not added. This THMC line responded to high glucose conditions (see the Results section) and behaved very similarly in culture to another transformed line reported previously [28,29].

Recombinant human CTGF, rabbit anti-human CTGF antibody (pAb2), and chicken anti-human CTGF neutralizing antibody (pIgY3), together with their pre-immune sera, were kind gifts from FibroGen (South San Francisco, CA, U.S.A.). An anti-V5 epitope antibody was purchased from ClonTech. Recombinant TGFβ1 was from R & D Systems (Abingdon, Oxfordshire, U.K.). Phosphothioate antisense (TGG GCA GAC GAA CG) and control oligonucleotides (ACC GAC CGA CGT GT) directed to CTGF were designed and manufactured by Biognostik GmbH (Göttingen, Germany), who own the intellectual property rights to the sequences.

Kidneys from NOD mice were kindly provided by Dr Masakazu Hattori (Joslin Diabetes Center, Boston, MA, U.S.A.). The animals had been screened for diabetes [30], the onset of diabetes being defined as the onset of hyperglycaemia. Control kidneys were from non-diabetic NOD mice that were transgenic for an E alpha transgene [31] and were a gift from Professor Anne Cooke (Department of Pathology, University of Cambridge, U.K.). Renal biopsies from three human DN patients were investigated using tissue surplus to requirements for diagnostic histology. Only biopsies for which a diagnosis of diabetic glomerulosclerosis had been confirmed by a renal pathologist using light and electron microscopy were used.

RNA extraction and reverse transcriptase (RT)-PCR analysis

Total RNA was extracted from 6×10^6 mesangial cells using the RNAzol B method [AMS Biotechnology (Europe), Abingdon, Oxfordshire, U.K.]. RNA was dissolved in diethyl pyrocarbonate-distilled water, quantified and stored at -70 °C until use. Equal amounts of total RNA (2 μg) from each sample were reverse transcribed into cDNAs using SuperScript II RNase H^+ reverse transcriptase (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) and random primers. Equal amounts (0.5 μl) of the reverse transcription reaction (20 μl) were subjected to PCR amplification in a 100 μl volume containing 10 μl of 10 $\times$ PCR buffer, 16 μl of dNTPs (1.25 mM each), 2 mM $MgCl_2$, 0.5 μM of each specific primer and 1.25 units of Amplitaq DNA polymerase (Gibco BRL). Amplification was started with 5 min of denaturation at 94 °C followed by 27 PCR cycles. Each cycle consisted of 60 s at 94 °C, 60 s at 55 °C and 60 s at 72 °C. The final extension was for 10 min at 72 °C. We co-amplified the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to allow semi-quantificative comparison of PCR products and to confirm equivalent loading of RNAs. The amount of reverse transcription reaction used for the amplification (0.5 μl) was selected as being non-saturating for the PCR product of all genes under investigation after 27 cycles of amplification. The sequences of primers were designed from the published sequences of the human genes and are listed in Table 1. After amplification, 10 μl of each PCR reaction product was electrophoresed through a 1.2 % (w/v) agarose gel containing ethidium bromide (0.5 μg/ml). Gels were scanned using an Epson GT-8000 scanner and Adobe PhotoShop software.

Cloning and sequence analysis

A 1054 bp PCR fragment containing the whole coding region of the CTGF gene as well as 6 bp upstream from the start codon was amplified by RT-PCR (Table 1) and cloned into the pcDNA3.1/V5-His TOPO vector (Invitrogen, Groningen, The Netherlands) in the sense orientation, or into the pTracer-CMV2 vector (Invitrogen) in the antisense orientation.

Transient transfection

THMCs were grown under 4 mM D-glucose conditions. CTGF–V5 constructs (30 μg) or CTGF-antisense constructs (30 μg) were transfected into 5×10^6 THMCs in 0.8 ml of the optimized electroporation buffer, Optimix (EquiBio, Kent, U.K.). For controls, cells were transfected with the vectors without inserts (mock transfections). The protocol was modified slightly from that described previously [28]. Briefly, the cells and plasmid DNA were incubated at room temperature for 3 min prior to electroporation at 260 V, 1050 μF, using an Easyject Optima Electroporation Unit (Floegen, Staffordshire, U.K.). Immediately after the pulse the cell suspension was transferred to a T75 culture flask containing pre-warmed RPMI 1640 medium with 10 % FCS. Transfection efficiency under these conditions was > 50 %. After overnight incubation at 37 °C in 5 % CO_2, cultures

Table 1 Primer sequences for amplifying various transcripts

Abbreviation: ORF, open reading frame.

Transcript	Length of transcript (bp)	Primers		Gene accession number
		Sense	Antisense	
Complete ORF of CTGF	1029	5′-GCCAACCATGACCGCCGCCAG-3′	5′-TGCCATGTCTCCGTACATCTTCCTG-3′	XM004525
CTGF	477	5′-AACTATGATTAGAGCCAACTGCCTG-3′	5′-TCATGCCATGTCTCCGTACATCTTC-3′	XM004525
Fibronectin	639	5′-CGAAATCACAGCCAGTAG-3′	5′-ATCACATCCACACGGTAG-3′	X02761
PAI-1	396	5′-GTATCTCAGGAAGTCCAGCC-3′	5′-TCTAAGGTAGTTGAATCCGAGC-3′	M16006
GAPDH	452	5′-ACCACAGTCCATGCCATCAC-3′	5′-TCCACCACCCTGTTGCTGTA-3′	XM009352

were washed three times with PBS and serum free medium was added. Cells and media were collected after a further 48 h incubation. Secreted recombinant CTGF (rCTGF) was purified from the medium using a Talon metal-affinity resin (ClonTech) or with a heparin affinity column (Sigma–Aldrich, Poole, Dorset, U.K.) according to the manufacturer's instructions.

Western blotting

Conditioned media were centrifuged (500 ***g***, 5 min, 4 °C) to remove any particulate matter before addition of ammonium sulphate (final concentration 20 % w/v). After incubating overnight at 4 °C, samples were centrifuged (14 000 ***g***, 15 min, 4 °C) and the pellet solubilized in reducing SDS/PAGE loading buffer containing a cocktail of protease inhibitors (1 mM EDTA, 1 mM EGTA, 0.2 mM tosyl-lysylchloromethane ('TLCK'), 1 mM *N*-ethylmaleimide, 0.1 mM tosylphenylalanylchloromethane ('TPCK'), and 2 mM PMSF; Sigma). Samples were boiled for 5 min and resolved on 4–12 % gradient gels by SDS/PAGE. Proteins were transferred on to a PVDF membrane filter (Immobilin-P, Millipore) using a BioRad transfer apparatus. Immunodetection was performed essentially as described by Towbin et al. [32]. Bound antibodies were visualized using the enhanced chemiluminescence reagent Luminol (Autogen Bioclear, Calne, Wiltshire, U.K.). Prestained molecular-mass standards (Amersham International) were used to monitor protein migration.

ELISA

Conditioned media collected from cell cultures were diluted 1:15 with 0.05 M sodium carbonate, 0.05 M sodium bicarbonate, pH 9.6 (coating buffer), and 100 μl of each sample was added to the wells of a NUNC microtitre plate (Gibco BRL) in triplicate. Protein was allowed to adsorb passively overnight at 4 °C. Plates were washed three times with PBS/0.05 % (v/v) Tween 20 and blocked with 150 μl PBS/Tween 20 containing 0.5 % (w/v) casein (from bovine milk) for 2 h at 37 °C. After three further washes with PBS/Tween 20, 100 μl (1:3000 dilution) of anti-human fibronectin antibody (Sigma) was added to each well and incubated for 1.5 h at 37 °C. Plates were washed once more and 100 μl of goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3000 dilution; Sigma) was added to each well for 1.5 h at 37 °C. A final wash was followed by development using the colorimetric reagent 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (100 μl) (Sigma). This was dissolved in 100 mM citric acid and 100 mM Na_2HPO_4, pH 4.1, to a final concentration of 0.4 mg/ml, with the addition of 60 μl of H_2O_2 (30 %) per 25 ml buffer immediately prior to use. Plates were read at A_{405} in a Titertek Multiskan plate reader (MCC/340). Serial dilutions of standard fibronectin (Gibco BRL) were included on each plate to generate a standard curve. Each assay was repeated three times.

Immunohistochemistry

Kidneys were snap-frozen and sectioned in a cryostat at 8 μm. After fixation in acetone for 10 min, sections were washed in PBS/0.05 % Tween 20 and pre-incubated in a malate buffer (100 mM maleic acid and 150 mM NaCl,), pH 7.5, containing 2 % blocking reagent (Roche Diagnostics, Lewes, East Sussex, U.K.) and 20 % heat-inactivated FCS for 90 min. Sections were then incubated with the primary rabbit anti-CTGF antibody (1:300 dilution) overnight at 4 °C, after which immunoreaction was detected with FITC-conjugated goat anti-rabbit antibody (1:200 dilution; Sigma). For controls, the anti-CTGF antibody was absorbed with rCTGF (1:3 mol. ratio) prior to incubation with the section. Coverslips were mounted on glass slides with anti-fade mounting media (Vector Laboratories, Peterborough, U.K.) and the sections examined with an Olympus AX70 fluorescence microscope.

Quantitative analysis and statistics

Western blots and photographs of DNA gels were scanned with an Epson GT-8000 scanner into Adobe PhotoShop software; the pixel intensity for each band (average pixel intensity multiplied by area) was analysed with the Image software (National Institutes of Health Shareware). Peak areas were measured in arbitrary units. To minimize error either in the scanning procedure or due to variation in band width and thickness, the procedure was repeated three times and the mean value was used. Results for each group of data are given as mean $\pm$ S.E.M. Differences between means were tested with Student's unpaired *t*-test; $P < 0.05$ was accepted as significant.

RESULTS

Expression of a recombinant CTGF-fusion protein and characterization of the anti-CTGF antibody

The full coding sequence of CTGF was cloned into the pcDNA3.1/V5-His TOPO vector and transfected into THMCs. The expressed CTGF-fusion protein contained the V5 epitope which provided an alternative means of immunodetection. The fusion protein was recovered from the medium of transfected cells by heparin-bead affinity purification and examined by SDS/PAGE and Western blotting with anti-V5 antibody (Figure 1A, media/CTGF–V5), or with anti-CTGF antibody (Figure 1B, media/CTGF–V5), or with anti-CTGF antibody which had been pre-absorbed with rCTGF (Figure 1C, media/CTGF–V5). Media from mock-transfected cells were treated in the same way as for transfected cells (Figures 1A–1C, media/mock). Western blotting of affinity-purified fractions with anti-V5 antibody revealed a doublet band of 42–44 kDa, the anticipated size for the fusion protein (Figure 1A, media/CTGF–V5). A minor band (approx. 26 kDa) was also detected and must be a C-terminal product of proteolytic cleavage of the fusion protein (Figure 1A, media/CTGF–V5). The anti-CTGF antibody (pAb2) also detected a large doublet band of approx. 42–44 kDa, together with an additional 36–38 kDa band, the latter being the anticipated size for endogenous CTGF (Figure 1B, media/CTGF–V5). Neither band is detected if the anti-CTGF antibody is first absorbed with rCTGF (Figure 1C, media/CTGF–V5). CTGF–V5 recovered from the culture media by metal-affinity using Talon resin gave the same result when examined by electrophoresis and Western blotting (results not shown).

We conclude that the 42–44 kDa component in the medium is due to secreted CTGF–V5 fusion protein since (i) it is the correct size, (ii) it was detected with both anti-V5 and anti-CTGF antibodies in heparin-affinity fractions (Figures 1A and 1B, media/CTGF–V5) and in Talon-affinity fractions from transfected cells, but not in fractions from mock-transfected cells (Figures 1A and 1B, media/mock), and (iii) it was not detected with pre-absorbed anti-CTGF antibody. Similarly the 36–38 kDa band is attributed to endogenous CTGF on the basis of (i) molecular mass, (ii) detection with anti-CTGF antibody in heparin-affinity fractions from medium of either transfected or mock-transfected cells (Figure 1B, media/mock and media/CTGF–V5), but not with anti-V5 antibody (Figure 1A, media/mock and media/CTGF–V5), (iii) detection with anti-CTGF antibody in these fractions is abolished by pre-absorbing

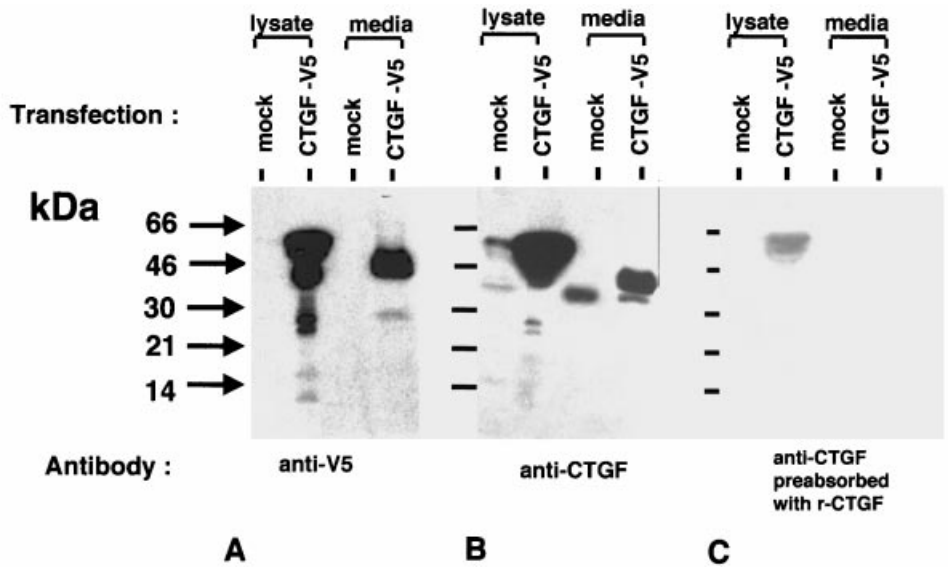

Figure 1 Expression of recombinant CTGF in THMC cultures

THMCs were transfected with a CTGF–V5 construct or were mock transfected, as described in the Materials and methods section. After 48 h culture in serum-free conditions, the cells were lysed in SDS/PAGE loading buffer, and secreted CTGF was purified from the medium using heparin-affinity beads. Samples of equal volume were resolved by SDS/PAGE (4–12% gel) and Western blotted with either anti-V5 antibody (**A**), or rabbit anti-(human-CTGF) antibody (**B**), or with rabbit anti-CTGF antibody pre-absorbed with rCTGF (**C**). (**A**–**C**) First lane, cell lysate from mock-transfected cells; second lane, cell lysate from CTGF–V5-transfected cells; third lane, heparin-affinity purified fraction from culture medium of mock-transfected cells; fourth lane, heparin-purified CTGF fraction from culture medium of CTGF–V5-transfected cells.

the antibody with rCTGF (Figure 1C, media/mock and media/CTGF–V5), (iv) the band is not detected in fractions purified from the medium by Talon-affinity chromatography (results not shown).

Western blotting of the cell lysate of THMCs transfected with pcDNA 3.1/V5-His using anti-V5 antibody (Figure 1A, lysate/CTGF–V5) or anti-CTGF antibody (Figure 1B, lysate/CTGF–V5) indicates that the recombinant 42–44 kDa CTGF-fusion protein is also present intracellularly. As expected, it was not detected in mock-transfected cells (Figures 1A and 1B, lysate/mock). In addition both antibodies detected bands of higher (approx. 56 kDa) and lower (26 kDa and less) molecular mass in the lysate of transfected cells (Figures 1A and 1B, lysate/CTGF–V5). Immunodetection of these bands is either abolished or markedly reduced by prior absorption of the anti-CTGF antibody with rCTGF from Fibrogen (Figure 1C, lysate/CTGF–V5), indicating that they are derived from the CTGF-fusion protein and are not non-specific. The lower molecular mass bands are likely to be proteolytic cleavage products, whereas the prominent 56 kDa band may be a dimer of the fusion protein and a cleavage product or of cleavage products alone. The 56 kDa band cannot be due to cross reaction with another growth factor of the CCN family to which CTGF belongs since it was detected by anti-V5 antibody, as well as by anti-CTGF antibody. Interestingly, endogenous 36–38 kDa CTGF was also detected in the lysate of mock-transfected cells (Figure 1B, lysate/mock), together with the 56 kDa band, indicating that the latter is formed in physiological conditions and is not an artifact of expressing the CTGF–V5 fusion protein.

CTGF protein (36–38 kDa) was also detected in the medium of primary HMC cultures, and together with the 26 kDa and 56 kDa product, in lysates of these cells (results not shown). Immunodetection of all these bands was again competed out by prior absorption of anti-CTGF antibody with rCTGF. Thus these bands are formed in primary as well as in transformed HMC cultures.

Table 2 Onset and duration of diabetes in NOD mice

The age of the non-diabetic control mice was 142 days.

Mouse	Age of onset of diabetes (days)	Duration of diabetes (days)
1	119	14
2	158	21
3	177	42
4	163	56
5	153	70
6	197	70

CTGF expression in renal cortex of NOD mice following onset of hyperglycaemia

Sections of renal cortex from six NOD mice with different durations of diabetes (Table 2) and from control non-diabetic mice were immunostained with the rabbit anti-CTGF antibody. There was virtually no CTGF immunostaining in the glomeruli of control mice (Figure 2A), a finding which agrees well with the negligible levels of CTGF mRNA detected in the glomeruli of 5-month-old non-diabetic littermate control mice [15] and with the absence of CTGF transcripts in normal adult rat glomeruli [4]. Tubular epithelial cells showed some immunofluorescence, but this occurred with secondary antibody alone and was therefore non-specific. The onset of hyperglycaemia in NOD mice occurred

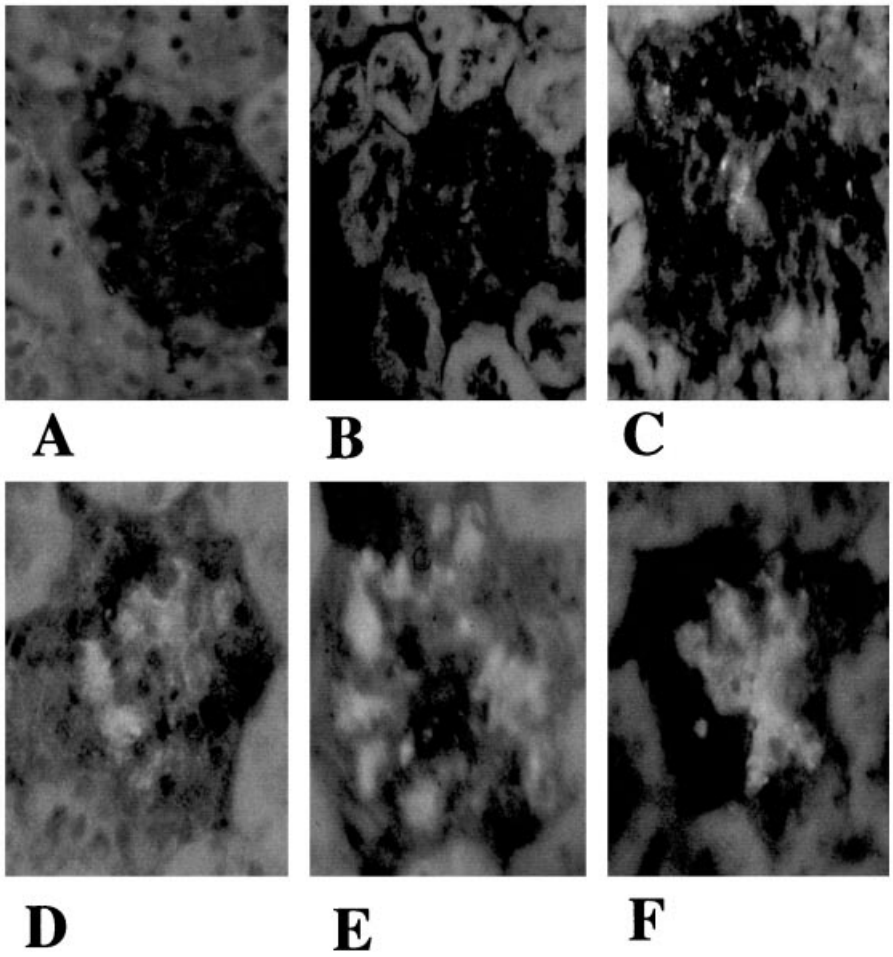

Figure 2 Expression of CTGF in glomeruli of NOD mice

Frozen sections of renal cortex were tested for CTGF expression by immunohistochemistry as described in the text. (**A**) Glomerulus of control non-diabetic mouse aged 142 days. (**B**–**F**) Glomeruli during diabetes of duration 14 days (**B**), 21 days (**C**), 42 days (**D**), 56 days (**E**), and 70 days (**F**) in NOD mice. Details of age of onset of diabetes in NOD mice are given in Table 2. Magnification, ×143.

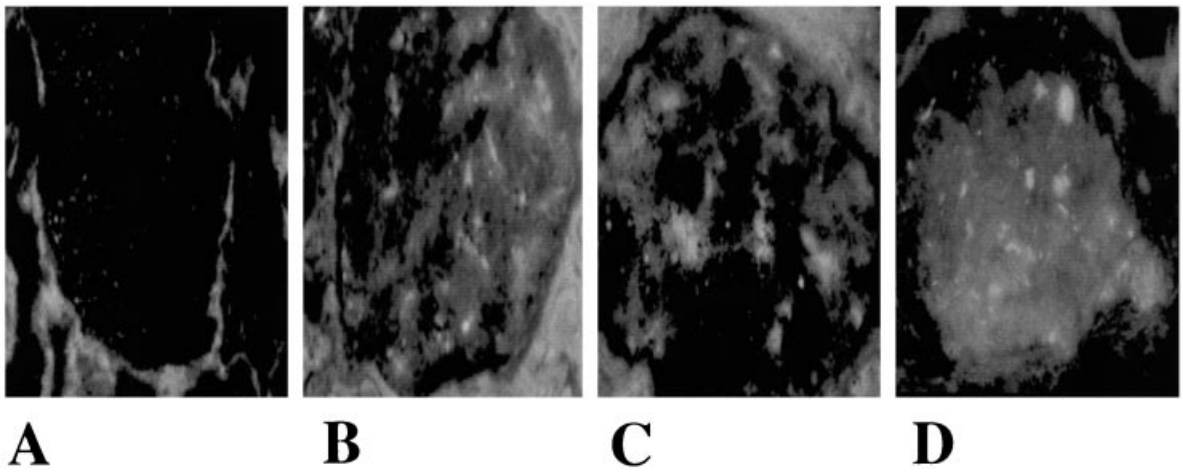

Figure 3 Expression of CTGF in human glomeruli in DN

Immunohistochemical detection of CTGF in glomeruli of frozen sections of normal human kidney (**A**), and of renal biopsies from diabetic patients aged 70 years with early diabetic glomerulosclerosis (**B**), aged 62 years with diffuse diabetic glomerulosclerosis (**C**), and aged 71 years with diffuse diabetic glomerulosclerosis (**D**). Disease classifications were provided by a renal pathologist and were based on wax sections of the same biopsies (results not shown). Magnification, ×143.

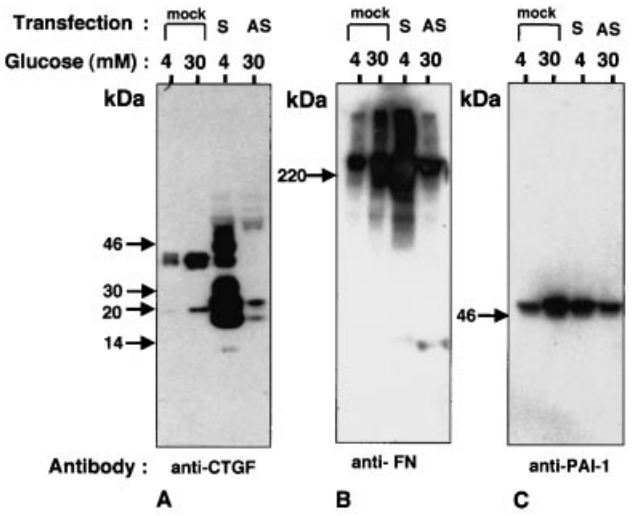

Figure 4 Effect of endogenous CTGF on the expression of fibronectin and PAI-1 in transiently transfected THMC cultures

Equal numbers of cells were either mock transfected (mock) and maintained under 4 mM or 30 mM D-glucose conditions, or were transfected with the CTGF–V5 construct and maintained under 4 mM D-glucose conditions (S), or with the CTGF-antisense construct and maintained under 30 mM D-glucose conditions (AS). Serum-free media were collected after 48 h, precipitated and suspended in equal volumes of sample loading buffer of which one third of the volume was electrophoresed and immunoblotted with anti-CTGF (**A**), anti-fibronectin (FN) (**B**) or anti-(PAI-1) (**C**) antibodies. Results shown are typical of three separate experiments.

between 119–197 days of age in the animals investigated and kidneys were examined 14–70 days after the onset (Table 2). Glomerular CTGF immunostaining was just detectable at the early times after onset of hyperglycaemia (Figure 2B). However, glomerular expression of CTGF increases markedly with the duration of diabetes (Figures 2D–2F). NOD mice develop glomerulosclerosis with advancing diabetes [33], and the pattern of prominent CTGF immunostaining seen in the older animals is suggestive of enhanced expression in mesangial areas.

CTGF expression in renal biopsy specimens of human DN patients

No CTGF immunostaining was observed in the glomeruli of normal human kidney. As with the murine kidney sections, background immunofluorescence occurred in tubular epithelial cells and was due to the secondary antibody. Positive CTGF immunostaining was detected in the glomeruli of three human DN patients (Figure 3). As in the NOD mice, the pattern of staining suggests prominent mesangial expression.

Transient overexpression of CTGF in HMCs and its effect on fibronectin and PAI-1 synthesis

Previous studies have established that exposure of human and rat mesangial cells to high glucose conditions for 2–21 days increases CTGF mRNA levels [3,4,15], and that the addition of exogenous rCTGF to cultures stimulates the synthesis of fibronectin and collagen I [15]. To investigate whether transient overexpression of endogenous CTGF could modulate fibronectin synthesis in human mesangial cells in normal glucose conditions (4 mM), we transfected a mesangial cell line with the CTGF–V5 construct and measured synthesis over the following 48 h. To examine the role of endogenous CTGF in modulating fibronectin synthesis during short-term exposure of HMCs to high glucose (48 h, 30 mM), we transfected cell line cultures with a CTGF-antisense construct to deplete mRNA levels of the growth factor. Control cultures were mock-transfected with empty vector. PAI-1 synthesis was also measured, in addition to fibronectin. After transfection (4 h), cells were washed and transferred to either serum-free medium supplemented with 4 mM D-glucose (CTGF-sense transfects and controls) or with 30 mM D-glucose (CTGF-antisense transfects and controls). After a further 48 h, conditioned media were analysed for fibronectin and PAI-1 by SDS/PAGE and Western blotting, whereas the cells were harvested for RNA extraction and mRNA analysis by RT-PCR.

High glucose conditions upregulated the expression level of CTGF, fibronectin and PAI-1 (Figures 4A–4C, mock/30) in

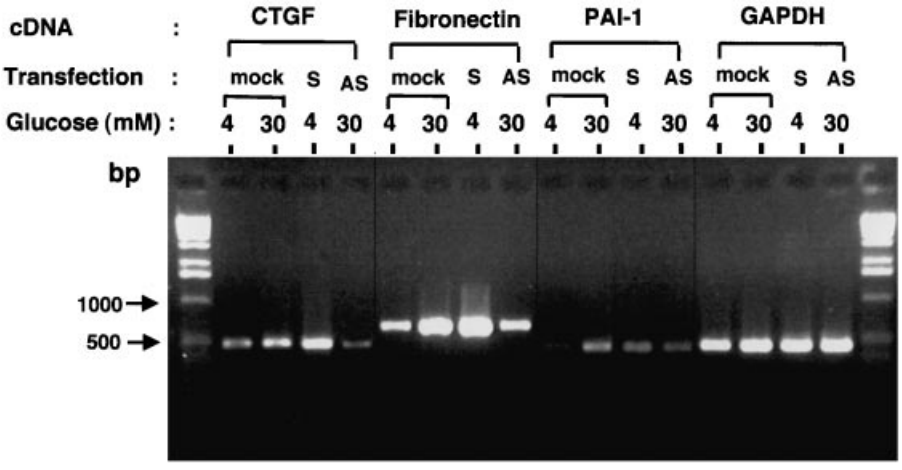

Figure 5 RT-PCR amplification of CTGF, fibronectin, PAI-1 and GAPDH transcripts in transiently transfected THMC cultures

mRNA was extracted from mock-transfected THMCs (mock) maintained under 4 mM and 30 mM D-glucose conditions, or from cells transfected with the CTGF–V5 construct (S) and maintained under 4mM D-glucose conditions, or with the CTGF-antisense construct (AS) and maintained under 30 mM glucose conditions. RT-PCR was performed as described in the Materials and methods section using the primers listed in Table 1.

Table 3 Quantitative assessment of mRNA levels of CTGF, fibronectin, PAI-1 and GAPDH in THMCs transfected with the CTGF–V5 construct or with the antisense (AS) construct

Following RT-PCR, as shown in Figure 5, cDNA bands for CTGF, fibronectin, PAI-1 and GAPDH were quantified with a scanning densitometer. The results shown are the integrated absorbance of each band in arbitrary units and are the means ± S.E.M. for four separate RT-PCR analyses. Results of statistical analysis are given in the text. Three other experiments gave very similar results.

		Integrated absorbance of cDNA band (arbitrary units)							
		CTGF		Fibronectin		PAI-1		GAPDH	
Construct	[D-glucose] (mM)...	4	30	4	30	4	30	4	30
Mock		3141 ± 95	6552 ± 258	10204 ± 288	26298 ± 310	1068 ± 30	4231 ± 154	12636 ± 293	12805 ± 129
CTGF–V5		12472 ± 205	–	46566 ± 791	–	3227 ± 236	–	13028 ± 178	–
CTGF–AS		–	1475 ± 100	–	9190 ± 401	–	2484 ± 218	–	12889 ± 416

mock-transfected THMC compared with 4 mM D-glucose conditions (Figures 4A–4C, mock/4). Thus mock-transfected THMCs respond to high glucose with respect to fibronectin and PAI-1 expression in the same way as primary cultures of normal HMCs [27]. Over-expression of the CTGF gene under 4 mM D-glucose conditions (Figure 4A, S/4) markedly increased the expression level of both fibronectin and PAI-1 proteins (Figures 4B and 4C, S/4). The effect of over-expressing CTGF was more pronounced on secreted fibronectin levels than on secreted PAI-1 levels. Furthermore, depleting CTGF expression with an antisense construct in cells grown under high glucose conditions (Figure 4A, AS/30) prevented the increased expression of both fibronectin (Figure 4B, AS/30) and PAI-1 (Figure 4C, AS/30) when compared with the levels observed in lane mock/30 (Figures 4B and 4C). Thus both fibronectin and PAI-1 protein levels are upregulated directly by endogenously expressed rCTGF in the 4 mM D-glucose cultures and by endogenous native CTGF in high glucose conditions.

RT-PCR analysis confirmed that increased CTGF expression occurs in THMCs in high glucose ($P < 0.0001$), or in low glucose after transfection with the CTGF-sense construct ($P < 0.0001$) compared with mock-transfected cells in low glucose (Figure 5 and Table 3). Endogenous CTGF transcripts in high glucose were greatly reduced in cells transfected with the antisense vector ($P < 0.001$) compared with mock-transfected cells in these conditions. Moreover, the level of gene expression for fibronectin and PAI-1 correlated closely with the level of CTGF expression, in each condition ($P < 0.0001$ for fibronectin and $P < 0.001$ for PAI-1). Collectively these results indicate that changes in fibronectin and PAI-1 protein levels in high glucose are due to increased mRNA levels, which are to a significant extent controlled by CTGF.

Role of CTGF in elevated fibronectin synthesis in HMCs exposed long term to high glucose or TGFβ1

Exposure of mesangial cells to high glucose conditions over 14–21 days to simulate chronic hyperglycaemia *in vivo* induces a sustained increase in TGFβ1 synthesis and bioactivity [34,35], and up-regulated expression of this factor is implicated in the development of glomerulosclerosis in DN [36,37]. It has been proposed that the CTGF acts downstream of TGFβ1 to mediate the latter's profibrotic effects [18,19]. To test directly whether increased CTGF expression is wholly responsible for the elevated synthesis of fibronectin in mesangial cells exposed chronically to high glucose (30 mM) or to TGFβ1 (5 ng/ml) in low glucose conditions (4 mM), we co-treated human primary cultures over 14 days with either a CTGF-antisense oligonucleotide, or with a chick anti-CTGF neutralizing antibody (pIgY3). Control cultures were treated with either a control oligonucleotide (see the Materials and methods section) or with chick pre-immune serum. All cultures were maintained in media supplemented with 10% FCS for 14 days, after which they were washed with PBS and exposed to the same conditions, but in the absence of FCS, for the final 24 h. Fibronectin was measured in the conditioned media by ELISA, and RNA was extracted from the cells and used to evaluate the steady state mRNA levels of CTGF and fibronectin by RT-PCR. Some cultures were also treated with rCTGF (40 ng/ml; FibroGen).

Table 4 Role of CTGF in mediating the stimulatory effect of high glucose conditions on fibronectin synthesis in primary HMCs

Primary cultures were maintained under the conditions shown for a period of 14 days. During the last 24 h of the experiment the cultures were maintained in serum-free conditions. Secreted fibronectin protein was measured in the conditioned serum-free media using ELISA, as described in the Materials and methods section. The results represent the mean ± S.E.M. for three separate experiments with triplicate cultures for each condition in each experiment. Results of statistical analysis are given in the text. Abbreviations: Ab, antibody; AS, antisense.

		Fibronectin synthesis (ng/10^4 cells per 24 h)	
Treatment	[D-glucose] (mM)...	4	30
None		9.9 ± 1.6	15.0 ± 1.1
TGFβ1 (5 ng/ml)		12.0 ± 0.9	–
rCTGF (40 ng/ml)		15.0 ± 1.5	–
CTGF-AS oligo (1.6 μM)		–	12.0 ± 0.5
Control-AS oligo (1.6 μM)		–	14.3 ± 0.5
Anti-CTGF Ab (0.4 μg/ml)		–	12.8 ± 0.7
Pre-immune serum (0.4 μg/ml)		–	14.5 ± 0.9
TGFβ1 plus CTGF AS oligo		7.9 ± 0.9	–
TGFβ1 plus control AS oligo		12.2 ± 0.9	–
TGFβ1 plus anti-CTGF Ab		8.2 ± 0.5	–
TGFβ1 plus pre-immune serum		12.0 ± 0.5	–

High glucose conditions increased the level of secreted fibronectin by approx. 50% ($P < 0.002$) compared with that in low glucose conditions, as expected [27] (Table 4). rCTGF added to low glucose cultures also stimulated fibronectin synthesis by 50% ($P < 0.004$, Table 4), a level similar to that observed when serum-starved rat mesangial cells were treated for 48 h with 20 ng/ml of the same rCTGF [15]. In comparison, TGFβ1 only induced a 20% increase in secreted fibronectin in low glucose cultures ($P < 0.05$, Table 4). However, high glucose, and rCTGF

Table 5 Quantitative assessment of the role of CTGF in mediating stimulation of mRNA levels of fibronectin in normal human mesangial cells

Following RT-PCR, as shown in Figure 6, cDNA bands for CTGF, fibronectin and GAPDH were quantified with a scanning densitometer. The results shown are the integrated absorbance of each band in arbitrary units and are the means ± S.E.M. for four separate RT-PCR analyses. Results of statistical analysis are given in the text. Three other experiments gave very similar results. Abbreviations: Ab, antibody; AS, antisense; oligo, oligonucleotide.

		Integrated absorbance of cDNA band (arbitrary units)					
		CTGF		Fibronectin		GAPDH	
Treatment	[D-glucose] (mM)...	4	30	4	30	4	30
None		2179 ± 161	12168 ± 500	8498 ± 349	16892 ± 576	12608 ± 642	13320 ± 431
TGFβ1		10697 ± 542	–	15704 ± 278	–	13320 ± 431	–
rCTGF		12185 ± 211	–	16954 ± 105	–	12946 ± 608	–
CTGF-AS oligo		–	1072 ± 85	–	13674 ± 462	–	13123 ± 349
Control-AS oligo		–	12003 ± 657	–	16060 ± 96	–	12903 ± 209
Anti-CTGF Ab		–	8254 ± 503	–	13853 ± 96	–	12903 ± 209
Pre-immune serum		–	12281 ± 210	–	16874 ± 250	–	12697 ± 514
TGFβ1 plus CTGF-AS oligo		1202 ± 85	–	12168 ± 611	–	13034 ± 265	–
TGFβ1 plus control-AS oligo		12222 ± 801	–	16622 ± 331	–	12762 ± 607	–
TGFβ1 plus anti-CTGF Ab		12074 ± 631	–	13253 ± 291	–	12330 ± 490	–
TGFβ1 plus pre-immune serum		12188 ± 518	–	16030 ± 247	–	12749 ± 510	–

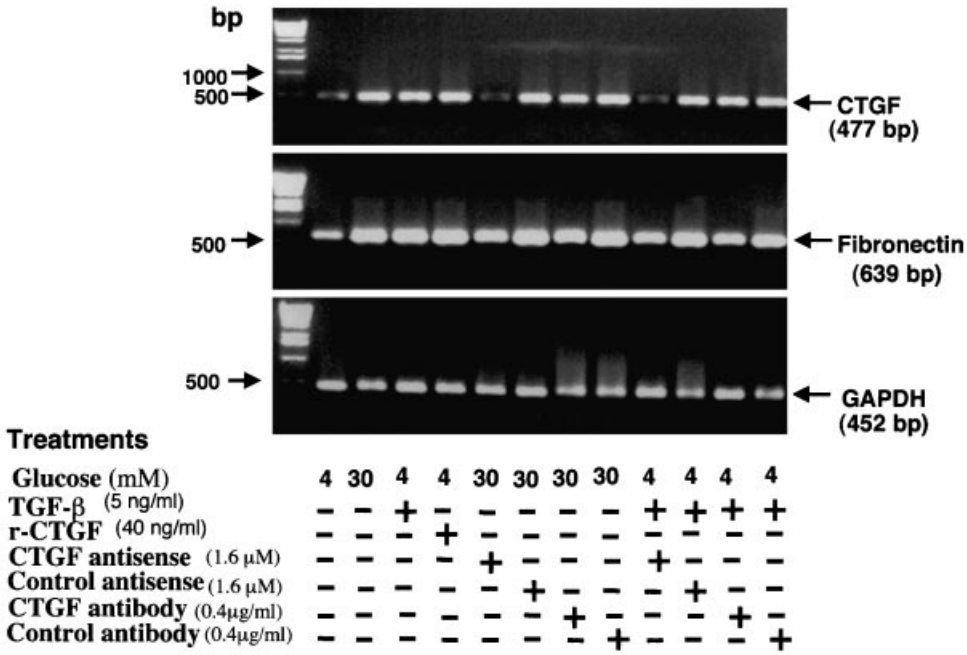

Figure 6 RT-PCR amplification of CTGF, fibronectin and GAPDH transcripts in normal HMCs

RNA was extracted from primary mesangial cells maintained under the following conditions for 14 days: 4 mM D-glucose, 30 mM D-glucose, 4 mM D-glucose plus TGFβ1 (5 ng/ml), 4 mM D-glucose plus rCTGF (40 ng/ml), 30 mM D-glucose plus CTGF antisense or control antisense oligonucleotide (1.6 μM), 30 mM D-glucose plus CTGF neutralizing antibody (0.4 μg/ml) or pre-immune serum (0.4 μg/ml), 4 mM D-glucose and TGFβ1 (5 ng/ml) plus CTGF antisense or control antisense oligonucleotide (1.6 μM), 4 mM D-glucose and TGFβ1 (5 ng/ml) plus CTGF neutralizing antibody or CTGF pre-immune serum (0.4 μg/ml). RT-PCR was performed as described in the Materials and methods section using the primers listed in Table 1.

and TGFβ1 supplements to low glucose conditions, all induced similar levels of CTGF and fibronectin mRNAs compared to low glucose alone (Figure 6 and Table 5; $P < 0.0001$ for all).

When high glucose cultures were treated continuously with CTGF-antisense oligonucleotide, CTGF mRNA levels fell to only 50% of those recorded in low glucose cultures (Figure 6 and Table 5; $P < 0.0001$) and to less than 10% of those in high glucose control cultures. However, the fibronectin mRNA pool in high glucose cultures was only reduced by approx. 20% in the presence of the CTGF-antisense oligonucleotide (Table 5; $P < 0.0001$) and secreted fibronectin levels were still approx. 25% higher than in 4.0 mM glucose-maintained cultures (Table 4; $P < 0.003$). Thus increased CTGF expression does not appear to be the only factor driving increased fibronectin expression in primary cultures of HMCs exposed long term to high glucose conditions. The control oligonucleotide had negligible effects on the CTGF or fibronectin mRNA pool sizes, or on the level of secreted fibronectin.

Interestingly, the chick anti-CTGF antibody brought about a partial reduction in the CTGF mRNA pool size in high glucose cultures (approx. 32%), although it remained increased by 4-fold over that in low glucose conditions (Table 5). This result suggests that at least some newly synthesized CTGF must be exported from the cells and act in an autocrine manner on the cells to stimulate further CTGF transcription. Treatment with the anti-CTGF antibody also appeared to reduce the fibronectin mRNA pool size by about 20% in high glucose cultures (Table 5, but difference not significant in Student's *t*-test), and reduced stimulation of secreted fibronectin protein levels by 44% in such cultures (Table 4; $P < 0.02$). Thus only part of the elevation in fibronectin synthesis in high glucose conditions can be attributed to increased CTGF leaving the cell and acting via an autocrine manner to induce new fibronectin gene expression and protein synthesis.

Treating TGFβ1-stimulated cultures with antisense-CTGF oligonucleotide not only abolished any increase in the CTGF transcript pool, but reduced it to less than that found in cells maintained in 4.0 mM D-glucose alone (Figure 6 and Table 5; $P < 0.0001$). This effect was similar to the effect of the antisense oligonucleotide on the high glucose cultures (Table 5). In contrast, treating TGFβ1-stimulated 4.0 mM cultures with anti-CTGF antibody had no effect on the CTGF mRNA pool size whereas, as described above, such treatment reduced it partially in high glucose-treated cells (Table 5). Since controls (oligonucleotide or pre-immune serum) had no effect in either situation, this suggests that high glucose induces factors in addition to TGFβ1 which modulate the CTGF mRNA pool size.

Both the antisense-CTGF oligonucleotide and the anti-CTGF antibody completely abolished the stimulatory effect of TGFβ1 on secreted fibronectin protein levels (Table 4; $P < 0.0004$ and $P < 0.0001$ respectively), even though they only partially reduced the stimulatory effect of the growth factor on the fibronectin mRNA pool sizes (Figure 6 and Table 5; $P < 0.0001$ for oligonucleotide and $P < 0.03$ for antibody). The result of the CTGF-antisense treatment shows that the fibronectin mRNA pool size is not wholly dependent on elevated CTGF expression in TGFβ1-stimulated cultures, although clearly fibronectin protein synthesis is dependent. CTGF may also induce expression of a factor which is required to achieve increased fibronectin synthesis.

Overall these results support a hypothesis in which high levels of glucose stimulate the expression of CTGF. The latter acts downstream to amplify its own expression in an autocrine loop, but is only partially responsible for inducing up-regulation of fibronectin expression in these conditions. Interestingly, although CTGF-antisense and anti-CTGF antibodies have a similar effect on the fibronectin mRNA pool size in cultures in high glucose, or in low glucose conditions supplemented with TGFβ1, both strategies have a more pronounced effect relatively in reducing fibronectin protein levels in the culture medium of TGFβ1-treated cells than they do with the high glucose-treated cells (Table 4).

DISCUSSION

In the present study we aimed to assess whether CTGF is up-regulated at the protein level in the diabetic glomerulus *in vivo*, and whether the factor is solely responsible for the increased synthesis of the matrix protein, fibronectin, in mesangial cells exposed long term to high glucose or elevated TGFβ1 levels *in vitro*. To investigate the former, we had first to biochemically characterize a polyclonal antibody for immunochemical detection of CTGF in tissues. To directly test the role of CTGF gene expression in the response of mesangial cells to high glucose and TGFβ1 levels, we adopted an antisense strategy to successfully knock out CTGF mRNA in these conditions. We also compared the effects of the antisense strategy with those of treating cells with a chick anti-CTGF neutralizing antibody. These complementary approaches have provided new information about CTGF, showing that: (1) it is present in mesangial cell cultures in a high molecular mass form, in addition to the monomeric form and as low molecular mass peptides derived from it; (2) increased levels of CTGF protein are present in murine and human diabetic glomeruli; (3) whereas increased expression of CTGF alone is sufficient to up-regulate fibronectin production, it can only partially account for the elevated level of synthesis of the matrix protein during long-term exposure of mesangial cells to high glucose; (4) increased expression of CTGF stimulates increased expression and synthesis of PAI-1.

After expressing a rCTGF–V5-fusion protein in THMCs, monomers and bands of higher and lower molecular mass were present in cell cultures. The same bands were detected by both the anti-V5 antibody and the rabbit anti-rCTGF antibody from FibroGen, and binding was eliminated by pre-absorption of the latter by rCTGF. The lower molecular mass bands are likely to be cleavage products of CTGF containing modules IV, or III and IV from the C-terminal end of the protein, as reported previously for other systems [6,7]. We speculate that the higher molecular mass band (56 kDa) could be a complex formed between CTGF and one of its smaller cleavage products or another protein. A similar high molecular mass band was present in cell lysates of mock-transfected THMCs and of primary HMC cultures, so it is formed physiologically *in vitro* and is not an artefact due to the CTGF-fusion protein.

This study is the first to demonstrate that increased levels of CTGF protein are present in the glomerulus in both murine and human diabetes. Hardly any CTGF immunostaining was present in the glomeruli of either normal human or control NOD mouse kidney, although Ito et al. [23] detected CTGF mRNA in visceral and some parietal epithelial cells. The amount of CTGF immunostaining correlated with the duration of diabetes in NOD mice and was prominent 70 days after the onset of diabetes. This may reflect the increase in TGFβ1 expression that occurs after a short period of hyperglycaemia (3–7 days) in this diabetic mouse model [36]. Thus it is clear that glomerular CTGF expression is upregulated at the protein level in diabetes, as well as at the mRNA level, as reported by Ito et al. [23] and others [4,15]. This is an important finding since increases in CTGF-transcript level are not always accompanied by increased amounts of protein, as was found when rat mesangial cells were mechanically stretched [15].

Transient overexpression of CTGF in THMCs, or addition of rCTGF to primary HMCs, confirmed that CTGF is a strong inducer of fibronectin [4,15] and showed that it is an inducer of PAI-1 in mesangial cells. The latter is a significant finding since we have shown previously [38] that increased expression of PAI-1 occurs in primary mesangial cells exposed to long-term high glucose conditions, and that it is accompanied by loss of tissue-plasminogen activator activity. Since tissue plasminogen activator is an important activator for the matrix metalloproteinases which are responsible for matrix turnover, upregulation of PAI-1 by CTGF is likely to contribute significantly to mesangial matrix accumulation and glomerulosclerosis in the diabetic glomerulus.

As described in rat mesangial cells [15], rCTGF induced endogenous CTGF expression in HMCs, suggesting an autocrine amplification loop in this cell type. Also, as expected, TGFβ1 stimulated expression of CTGF mRNA and synthesis of fibro-

nectin in HMCs maintained in low glucose conditions. However, although long-term exposure to high glucose increases levels of TGFβ1 mRNA, protein and bioactivity [34,35] and stimulated an increase in the CTGF mRNA pool size, CTGF alone does not account fully for the increase in fibronectin synthesis in these conditions. CTGF mRNA levels were almost eliminated in high-glucose cultures by treatment with the CTGF-antisense oligonucleotide, but fibronectin mRNA and protein levels were only partially reduced. Similarly interrupting the putative autocrine action of CTGF with a neutralizing antibody only partially reduced fibronectin synthesis in high glucose conditions. This indicates that signalling pathways in addition to the TGFβ1–CTGF pathway are involved in upregulating mesangial cell fibronectin expression in primary mesangial cells exposed to long-term high-glucose conditions.

TGFβ1 is secreted as an inactive form in a complex with the latency-associated peptide. We have shown that TGFβ1 activation in high glucose conditiond depends entirely on interaction of its latent complex with thrombospondin-1 [35], a known activator of the complex [39]. Blocking the thrombospondin-1/TGFβ1-activation mechanism in high glucose mesangial cell cultures prevented any increases in fibronectin mRNA or protein [35], inferring that upregulated expression of fibronectin is totally dependent on TGFβ1 in such conditions. Thus if signalling pathways other than the TGFβ1–CTGF pathway are involved in upregulation of fibronectin in high glucose, they must nevertheless involve TGFβ1. It seems possible that if one pathway mediating the effects of TGFβ1 is markedly inhibited, for example the CTGF pathway by the antisense oligonucleotide treatment, an alternative pathway may partially compensate for this.

Activation of TGFβ1-responsive genes appears to be mediated by several different pathways. Thus, TGFβ1-stimulated synthesis of PAI-1 can be mediated via the Smad-signalling cascade [40–42], but stimulation of fibronectin synthesis in a human fibrosarcoma-derived cell line occurs independently of Smad4 [43]. TGFβ1 stimulated fibronectin through the c-Jun N-terminal kinase pathway in these cells. Interestingly, high glucose conditions and TGFβ1 stimulated fibronectin gene expression in HMCs through a cAMP-response element (CRE) in its promoter [44,45]. Induction of fibronectin transcription in HMC is due primarily to the phosphorylation of CRE-binding protein, which binds to this element [45]. In mesangial cells TGFβ1 stimulates protein kinase A activation via the phosphorylation and degradation of inhibitory molecules that interact with the catalytic subunit of protein kinase A [45]. Overexpressing the inhibitory molecules in mesangial cells attenuates TGFβ1-induced stimulation of fibronectin mRNA expression. Moreover addition of H-89, the protein kinase A specific inhibitor, does not affect Smad-2 phosphorylation, but completely inhibits TGFβ1-induced cAMP-response-element-binding protein phosphorylation in mesangial cells and hence inhibits TGFβ1-induced stimulation of fibronectin gene expression [46].

It should be noted that TGFβ1-independent pathways augment TGFβ1-dependent ones by stimulating increased expression of some matrix proteins in high glucose conditions. Thus upregulated expression of decorin in such conditions is driven predominantly from a CRE-like response element in the P1 promoter of the gene [28]. Although this element is responsive to TGFβ1, neutralizing anti-TGFβ1 antibodies were only able to partly suppress increased decorin transcription when P1-promoter activity was stimulated by high glucose, inferring the presence of TGFβ1-independent signals in these conditions. In contrast, increased fibronectin expression in mesangial cells exposed to high glucose was completely suppressed by either TGFβ1-neutralizing antibodies [34] or by inhibiting activation of TGFβ1 in high glucose [35].

In summary, our results strongly support a role for CTGF in the pathogenesis of DN. Antagonism of CTGF function could possibly attenuate, but not completely block, progression of the glomerulosclerosis in this disease. Such an approach may, however, avoid the possible pro-carcinogenic danger associated with the blockade of TGFβ1 [47], which has been proposed as another therapeutic strategy for DN [48].

We thank the Medical Research Council (U.K.) and Diabetes U.K. for financial support, and Dr. Masakazu Hattori and Professor Anne Cooke for NOD mouse and control mouse kidneys. N.Y. is grateful to the Wellcome Trust for a Travelling Research Fellowship and X.J.L. acknowledges support from an Overseas Research Studentship Award and the Special Trustees of Charing Cross Hospital.

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Received 2 February 2001/15 May 2001; accepted 16 July 2001