Expression of extracellular matrix molecules in human mesangial cells in response to prolonged hyperglycaemia

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Post-mitotic cultures of human mesangial cells were maintained in media containing 4–30 mM D-glucose for up to 28 days. Changes in mRNA and protein levels for specific macromolecules occurred between 7 and 14 days after initiating hyperglycaemic conditions. Slot blot analysis showed 2–3-fold increases in mRNAs for collagen type I, fibronectin, versican and perlecan, whereas mRNA for decorin was increased by up to 20-fold. Levels of mRNAs for biglycan and syndecan were unaffected by hyperglycaemic culture. Reverse transcriptase PCR (RT-PCR) confirmed that decorin mRNA levels are greatly elevated and also showed increased transcription of the TGF- β 1 gene in hyperglycaemic cultures. Western analysis and ELISA indicated accumulations of collagen types I and III, laminin and fibronectin in the cell layers and media of hyperglycaemic cultures with

increasing time. Type IV collagen did not accumulate in either compartment of hyperglycaemic mesangial cell cultures. Collagen types I, III, and fibronectin did not accumulate in the cell layers of hyperglycaemic human dermal fibroblasts, indicating a cell-specific response in mesangial cultures. Decorin and versican, but not biglycan, were increased in the hyperglycaemic mesangial cell culture media. There were no apparent changes in core proteins for decorin and biglycan in fibroblast media. Transforming growth factor β 1 (TGF- β 1) in hyperglycaemic mesangial cell cultures increased 5-fold after 7 days, but decreased thereafter to only approx. 2-fold after 28 days. The changes in TGF- β 1 mRNA, as detected by RT-PCR, and protein followed one another closely.

INTRODUCTION

Diabetic nephropathy leading to kidney failure is a major complication for a significant number of patients with type I (insulin-dependent) and type II (non-insulin-dependent) diabetes mellitus [1]. Its onset and progression might be delayed in patients with good glycaemic control, suggesting that high concentrations of blood glucose could be involved in either initiating or promoting pathological changes [2]. A major characteristic of diabetic nephropathy is the enlargement of the mesangium, due to accumulation of extracellular matrix (ECM). The degree of mesangial expansion correlates with the loss of renal function [3] as it occurs at the expense of the glomerular capillary lumen and filtration surface [4]. The mesangial matrix is composed of collagens, fibronectin, laminin and proteoglycans, including biglycan, decorin, versican and two heparan sulphate proteoglycans [5–8]. Increased synthesis of various ECM macromolecules has been demonstrated in the glomeruli of diabetic rats and in cultures of rat and calf mesangial cells maintained in media with elevated concentrations of glucose [9–13].

There have been few studies on the effect of hyperglycaemic culture conditions on human mesangial cells (HMCs), although such studies would obviously be pertinent to understanding the development of human diabetic nephropathy. Thus we have investigated the effect of long-term exposure of post-exponential-phase cultures of adult HMCs to elevated concentrations of D-glucose. To determine whether changes in the gene expression and protein production of a number of ECM macromolecules that occur under these conditions are specific to mesangial cells, we also performed similar experiments on cultures of human dermal fibroblasts. Increases in mRNA levels and/or protein for collagen types I and III, fibronectin, laminin and the proteo-

glycans decorin, versican and perlecan were observed in hyperglycaemic mesangial cultures, but not in hyperglycaemic fibroblast cultures. Transforming growth factor β (TGF- β) is a major modulator of expression of extracellular matrix protein genes [14] and has been proposed as an important factor driving fibrosis in the diabetic glomerulus [15]. Both rat and mouse mesangial cells express the TGF- β 1 gene and synthesize the protein product [16,17]. Thus we investigated whether cultures of HMCs express TGF- β 1 and showed that long-term exposure to high concentrations of D-glucose stimulates production of this factor.

MATERIALS AND METHODS

Materials

Special RPMI 1640 medium without D-glucose, minimal essential medium, non-essential amino acids, fetal calf serum and antibiotics were from Gibco (Paisley, Scotland, U.K.). D-Glucose was added to RPMI medium to the required concentrations. Insulin, transferrin, sodium selenite (ITS) and bacterial collagenase (type IV) were from Sigma Chemical Co. (Poole, Dorset, U.K.). Goat anti-(human type III and type IV collagen) antibodies were from AMS Biotechnology (Witney, Oxon., U.K.). Rabbit antibodies against human fibronectin, laminin, and human collagens type I, III, IV, together with human laminin and human fibronectin, were from Gibco. Rabbit anti-(human type I collagen) and anti-(human decorin) antibodies were from Chemicon International (London, U.K.). Affinity-purified polyclonal antibody specific for the mature form of human TGF- β 1 and ultrapure natural human TGF- β 1 protein were from Santa Cruz Biotechnology (Hatfield, Herts., U.K.). Monoclonal antibody to

Abbreviations used: AMV, avian myoblastosis virus; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMC, human mesangial cell; ITS, insulin, transferrin, sodium selenite; RT-PCR, reverse transcriptase PCR; TGF- β , transforming growth factor β ; Tos-Lys-CH₂Cl, tosyl-l-lysylchloromethane; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane.

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vertebrate actin was obtained from Boehringer Mannheim Biochemicals (Lewes, East Sussex, U.K.). The following reagents were gifts: rabbit anti-(human biglycan) (Dr. L. Fisher, NIH, Bethesda, MD, U.S.A.), rabbit anti-(bovine versican) (Dr. D. Heinegård, Lund, Sweden), recombinant decorin (Dr. H. Kresse, Münster, Germany), cDNA clones for human decorin and biglycan (Dr. L. Fisher), human perlecan (Dr. K. Tryggvason, Oulu, Finland), human procollagen type I(α 1) (Dr. R. Boot-Handford, Manchester, U.K.), human fibronectin (Dr. A. R. Korbliht, Buenos Aires, Argentina) and human syndecan-1 (Dr. M. Jalkanen, Turku, Finland). cDNA probes for human laminin B1, β -actin and collagen IV(α 1) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and for versican from Bioquote (York, U.K.).

Isolation and culture of HMCs

Normal renal cortex was obtained from a 51-year-old female patient undergoing nephrectomy for a carcinoma localized in one pole of the kidney. Glomeruli were obtained from the cortex by sieving [18]. Primary cultures of mesangial cells were established from collagenase-treated glomeruli [19]. The growth medium consisted of RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum, 50 i.u./ml penicillin, 50 μ g/ml streptomycin, 300 μ g/ml glutamine and 4 mM D-glucose. ITS (5 μ g/ml, 5 μ g/ml and 5 ng/ml, final concentrations) was added routinely to all culture media. Cells were characterized (D. Woodrow, K. Harper and R. Mason, unpublished work) as mesangial by their morphology and by their immunoreactivity with antibodies against factor VIII (negative), cytokeratin (negative), epithelial membrane antigen (negative), smooth muscle α -actin (positive) and monoclonal antibody F₂25 F3 (supernatant 25; [20]) (positive). The last antibody was raised against cultured HMCs and was a gift from Dr. K. N. Stewart (Aberdeen, Scotland, U.K.). The mesangial cells were passaged with a 1:2 ratio and were stored frozen in liquid nitrogen at the fourth to sixth passage. For experiments, confluent post-exponential-phase cultures of HMCs (passage 6–8) were maintained in culture medium containing 10% (v/v) fetal calf serum and 4 mM (normoglycaemic), 11, 15 or 30 mM (hyperglycaemic) D-glucose for periods of up to 4 weeks. The cultures were not passaged again after exposure to these conditions. Conditioned media were collected every 2 days, centrifuged at 1000 g for 10 min, at 4 °C, and stored at –70 °C before analysis. At the end of each week cultures were washed extensively with PBS and were used either for RNA extraction or for culture for 24 h in glucose-supplemented medium in the absence of serum. After 24 h, conditioned media were collected, centrifuged at 1000 g for 10 min, at 4 °C, and concentrated 10-fold by freeze-drying. Protease inhibitors were added to the reduced volume to give final concentrations of 1 mM EDTA, 1 mM EGTA, 0.2 mM tosyl-lysylchloromethane (Tos-Lys-CH₂Cl), 1 mM N-ethylmaleimide, 0.1 mM tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl) and 2 mM PMSF (Sigma). Media were stored at –70 °C before ELISA analysis. Monolayers were used to study protein expression by Western analysis.

Culture of human fibroblasts

Human dermal fibroblast cells (HF19) were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts., U.K.). The culture medium consisted of minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 50 i.u./ml penicillin, 50 μ g/ml streptomycin, 300 μ g/ml glutamine, 1% (w/v) non-essential amino acids and 5.5 mM D-glucose. For experiments, confluent post-exponential-phase cul-

tures (passage 22) were maintained in culture medium containing 5.5 mM (normoglycaemic), 11 or 30 mM (hyperglycaemic) D-glucose for periods of 1–4 weeks as above.

RNA isolation and gene expression analysis

Total cellular RNA was extracted by a rapid RNA extraction protocol [21]. Northern blot analysis (10 μ g) was used to verify the specificity of all probes that were labelled with ³²P by random priming (oligolabelling kit, Pharmacia LKB Biotechnology, Milton Keynes, U.K.). mRNA expression for selected matrix components was measured by slot blot analysis [22]. RNA (3 μ g per slot) was transferred on to triplicate nylon membranes with a microsample filtration manifold (Schleicher & Schuell) and hybridized with ³²P-labelled cDNA probes by using standard formaldehyde conditions. After overnight hybridization at 42 °C, membranes were washed to 0.5 $\times$ SSC/0.1% SDS, and exposed to X-ray films for between 2 h and 7 days. For rehybridization, the membranes were stripped by washing in 0.1 $\times$ SSC/0.1% SDS, at 90 °C for 5 min and verified for residual bound probe by exposing them to X-ray film for 3 days. The washing procedure was repeated 3 times. Autoradiographs were scanned on a Chromoscan 3 densitometer (Joyce–Loebl) and the hybridization signals for each cDNA probe normalized against the signal for β -actin.

For reverse transcriptase PCR (RT–PCR), total RNA was extracted from 6×10^6 mesangial cells by the RNazol B method (AMS). Samples were dissolved in 20 μ l of DEPC in distilled water and stored at –70 °C until assay. Equal amounts of total RNA (2 μ l) from each sample were first reverse-transcribed into cDNAs by using avian-myeloblastosis-virus reverse transcriptase and the random primers provided in the cDNA cycle kit (Invitrogen, Abingdon, Oxon., U.K.). Equal amounts (2 μ l) of the reverse transcriptase reaction (20 μ l) were in turn subjected to PCR amplification in a 100 μ l volume containing 10 μ l of 10 $\times$ PCR buffer, 16 μ l of dNTPs (10 mM each), 2 mM MgCl₂, 20 μ M of each specific primer and 1.25 units of Amplitaq DNA polymerase. Amplification was started with 5 min of denaturation at 94 °C followed by 30 PCR cycles. Each cycle consisted of 60 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C. The final extension lasted for 10 min at 72 °C in all instances. To quantify PCR products comparatively and confirm the use of equal amounts of the RNAs, we co-amplified a ‘house-keeping’ gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The quantity of reverse transcription reaction used for the amplification (2 μ l) was selected as being non-saturating for the PCR product of GAPDH after 30 cycles of amplification.

The sequences of primers for TGF- β 1 mRNA were designed from the sequence of the human gene: (sense) 5'-GCCCTGG-ACACCAACTATTGCT-3'; (antisense) 5'-AGGCTCCAAA-TGTAGGGGCGAGG-3' (161 bp) [23]. The GAPDH primers were designed from the human gene: (sense) 5'-ACCACAGTCC-ATGCCATCAC-3'; (antisense) 5'-TCCACCACCCTGTTGC-TGTA-3' (450 bp) [24]. The decorin primers were designed from the human gene: (sense) 5'-AGGGCTCCTGTGGCAATT-3'; (antisense) 5'-TCAGATGAGGCGTGTGG-3' (879 bp) [25]. After amplification, 8 μ l of each PCR reaction mix was electrophoresed through a 2% (w/v) agarose gel with ethidium bromide (0.5 μ g/ml). Gels were photographed with a charge-coupled device camera connected to a Macintosh Quadra computer, and analysed with Image software (NIH Shareware).

Proteoglycan extraction and chondroitinase ABC treatment

Conditioned media collected every 2 days were combined and proteoglycans extracted on a DEAE-Sephacel column as de-

Table 1 The effect of D-glucose concentration on mRNA expression of various ECM macromolecules in HMCs

mRNA levels are shown as the ratios of integrated absorbance areas for these mRNAs, divided by the absorbance areas for β -actin mRNA on slot-blot analysis. mRNAs were analysed after 14, 21 or 28 days of culture with 4, 15 or 30 mM D-glucose in the medium. Each analysis is the mean $\pm$ S.E.M. for 10 slots for day 14 and 28 and for 4 slots for day 21. The results were confirmed in two further experiments. * $P < 0.001$; ** $P < 0.005$; *** $P < 0.01$; † $P < 0.05$ compared with 4 mM D-glucose.

[D-Glucose] (mM) ...	Type I collagen			Fibronectin		
	4	15	30	4	15	30
14 days	0.29 $\pm$ 0.05	0.36 $\pm$ 0.07	0.57 $\pm$ 0.05†	0.16 $\pm$ 0.02	0.37 $\pm$ 0.04***	0.41 $\pm$ 0.01*
21 days	0.30 $\pm$ 0.02	0.36 $\pm$ 0.06	0.59 $\pm$ 0.05**	0.33 $\pm$ 0.01	0.71 $\pm$ 0.04*	0.80 $\pm$ 0.01*
28 days	0.14 $\pm$ 0.02	0.85 $\pm$ 0.10	0.90 $\pm$ 0.10**	0.29 $\pm$ 0.01	0.71 $\pm$ 0.15	0.99 $\pm$ 0.01*
[D-Glucose] (mM) ...	Perlecan			Decorin		
	4	15	30	4	15	30
14 days	0.15 $\pm$ 0.02	0.63 $\pm$ 0.07†	0.57 $\pm$ 0.07†	0.09 $\pm$ 0.01	0.04 $\pm$ 0.04†	0.43 $\pm$ 0.10†
21 days	0.17 $\pm$ 0.01	0.55 $\pm$ 0.01*	0.61 $\pm$ 0.01*	0.05 $\pm$ 0.01	0.56 $\pm$ 0.13*	0.59 $\pm$ 0.04*
28 days	0.12 $\pm$ 0.01	1.05 $\pm$ 0.05**	0.74 $\pm$ 0.04**	0.04 $\pm$ 0.01	0.63 $\pm$ 0.07*	1.10 $\pm$ 0.04*
[D-Glucose] (mM) ...	Biglycan			Syndecan		
	4	15	30	4	15	30
14 days	0.66 $\pm$ 0.12	0.74 $\pm$ 0.14	0.63 $\pm$ 0.08	0.52 $\pm$ 0.08	0.62 $\pm$ 0.08	0.57 $\pm$ 0.10
21 days	0.48 $\pm$ 0.06	0.55 $\pm$ 0.03	0.54 $\pm$ 0.04	0.50 $\pm$ 0.11	0.64 $\pm$ 0.15	0.62 $\pm$ 0.16
28 days	0.59 $\pm$ 0.10	0.69 $\pm$ 0.13	0.80 $\pm$ 0.11	0.52 $\pm$ 0.12	0.81 $\pm$ 0.19	0.71 $\pm$ 0.14

scribed by Glossl et al. [26]. To identify proteoglycan core proteins, chondroitin and dermatan sulphate side chains were removed by treating 0.5 mg of proteoglycan overnight at 37 °C with 0.1 unit of chondroitinase ABC (Sigma) in 0.1 M Tris/HCl, pH 8.0, containing 0.03 M sodium acetate.

Western blotting

Samples were solubilized in reducing SDS/PAGE loading buffer containing a cocktail of protease inhibitors (1 mM EDTA, 1 mM EGTA, 0.2 mM Tos-Lys-CH₂Cl, 1 mM *N*-ethylmaleimide, 0.1 mM Tos-Phe-CH₂Cl and 2 mM PMSF). Samples were boiled for 5 min and resolved on gradient SDS/PAGE [3–20 % (w/v) gel] [27]. Loads applied to the lanes of SDS/PAGE gels were standardized for cell number in any one experiment, enabling quantitative comparisons to be made between lanes. Proteins were transferred on to poly(vinylidene difluoride) membrane filter (Immobilin-P; Millipore, Bedford, U.K.) with a Bio-Rad transfer apparatus. Immunodetection was performed essentially as described by Towbin et al. [28]. Bound antibodies were revealed by peroxidase-conjugated IgGs and 4-chloro-1-naphthol (Boehringer Mannheim Biochemicals). Prestained molecular-mass standards (Amersham International, Amersham, Bucks., U.K.) were used to monitor protein migration.

ELISA

An ELISA for TGF- β 1 was performed by the method of Voller et al. [29]. Briefly, NUNC microtitre plates (Gibco) were coated overnight at 4 °C with serial dilutions of standard TGF- β 1 in 50 mM carbonate buffer at pH 9.6. Non-specific proteins were blocked by incubating wells for 1 h with 1 % (w/v) BSA in PBS at 37 °C. For human anti-TGF- β 1, the optimal dilution was determined as 1/100, whereas for the secondary antibody it was determined as 1/800. The bound antibodies were detected with the substrate 2,2'-azino-bis(3-ethylbenzthiazoline 6-sulphonic

acid (Kirkegaard and Perry Laboratories). Absorbance was read at 405 nm.

Inhibition ELISA assay

The method of Rennard et al. [30] was used with slight modification. Briefly, optimal protein coating concentration and primary and secondary antibody dilutions were determined for each ECM molecule as described by Voller et al. [29]. Protein standard or sample (60 μ l) was incubated overnight at 4 °C with the same volume of the relevant primary antibody diluted with PBS containing 0.5 % BSA. A 100 μ l portion of this reaction mixture was then transferred to a well in a 96-well NUNC microtitre plate. Wells were precoated overnight with optimal dilution of proteins in 50 mM carbonate buffer at pH 9.6. This was determined as (per well): 300 ng of type I collagen, 2 μ g of type III collagen, 400 ng of type IV collagen, 200 ng of laminin, 100 ng of fibronectin and 4 μ g of recombinant decorin. The plate was incubated at 37 °C for 60 min. The wells were then washed three times with washing buffer (PBS with 0.1 % Tween-20). Peroxidase-conjugated secondary antibody (100 μ l) was added to each well and incubated for 60 min at 37 °C. Wells were washed three times again, and bound antibodies were detected as above.

RESULTS

HMC cultures plated at low density grew satisfactorily in normoglycaemic (4 mM D-glucose) conditions. At passage 6–8, cultures that were confluent and just post-exponential phase were transferred to media containing 4, 15 or 30 mM D-glucose. Cell numbers in confluent cultures of mesangial cells maintained in different glucose concentrations showed little change over the next 28 days (results not shown).

Hyperglycaemic conditions selectively affected the level of mRNA expression of some ECM macromolecules relative to β -

Figure 1 The effect of D-glucose concentration on mRNA expression of TGF- β 1, GAPDH and decorin in HMCs

Cultures were maintained in media containing 4, 11 or 30 mM D-glucose for periods of 1–4 weeks. RT–PCR was performed as described in the Materials and methods section and 8 μ l of each PCR reaction mix was electrophoresed on a 2% (w/v) agarose gel containing ethidium bromide.

Table 2 Quantification of cDNAs amplified from HMC cultures maintained with different concentrations of D-glucose

Cultures were maintained in medium containing 4, 11 or 30 mM D-glucose for periods of up to 28 days before analysis, as shown in Figure 1. The RT–PCR products corresponding to TGF- β 1, decorin and GAPDH were separated on an agarose gel and the bands quantified with a scanning densitometer. The results shown are the integrated absorbance of each band in arbitrary units and are the means $\pm$ S.E.M. for three separate RT–PCR analyses; they do not represent the analysis in Figure 1 alone. * $P < 0.001$; ** $P < 0.005$; *** $P < 0.01$ compared with 4 mM D-glucose.

[D-glucose] (mM) ...	TGF- β 1		
	4	11	30
7 days	208.3 $\pm$ 4.6	766.3 $\pm$ 47.8*	1211.3 $\pm$ 56.0*
14 days	290.6 $\pm$ 6.0	691.0 $\pm$ 46.5	1077.0 $\pm$ 13.0*
21 days	301.3 $\pm$ 9.0	471.3 $\pm$ 12.6	702.7 $\pm$ 12.0*
28 days	311.3 $\pm$ 34.0	470.7 $\pm$ 10.5	580.3 $\pm$ 67.0
[D-glucose] (mM) ...	GAPDH		
	4	11	30
7 days	951.6 $\pm$ 38.5	1009.7 $\pm$ 70.0	945.7 $\pm$ 31.6
14 days	927.0 $\pm$ 48.2	974.3 $\pm$ 65.4	999.7 $\pm$ 50.8
21 days	935.3 $\pm$ 12.7	899.3 $\pm$ 25.4	923.3 $\pm$ 13.0
28 days	983.7 $\pm$ 11.6	961.0 $\pm$ 21.9	1011.0 $\pm$ 90.7
[D-glucose] (mM) ...	Decorin		
	4	11	30
7 days	202.7 $\pm$ 4.3	243.0 $\pm$ 24.3	324.3 $\pm$ 73.2
14 days	213.0 $\pm$ 6.5	312.7 $\pm$ 16.5	513.3 $\pm$ 20.8*
21 days	261.7 $\pm$ 14.0	344.0 $\pm$ 29.3	587.0 $\pm$ 53.6**
28 days	241.3 $\pm$ 6.0	439.3 $\pm$ 74.8	696.7 $\pm$ 90.0***

actin mRNA (Table 1). These changes first occurred 7–14 days after exposing the cells to increased concentrations of glucose. mRNAs for collagen type I, fibronectin, perlecan and versican (results not shown) were increased by 3–6-fold. The most

Figure 2 The effect of D-glucose concentration on the amount of matrix protein accumulated in HMC layers

Cells were grown to confluence in 4 mM D-glucose and maintained subsequently in the presence of different glucose concentrations (4, 11 and 30 mM) for 1, 2, 3 and 4 weeks. After washing, monolayers were solubilized in the presence of SDS and volumes equivalent to 4500 cells subjected to gradient SDS/PAGE (3–20% gel) electrophoresis. Proteins were electroblotted on to nitrocellulose membrane and probed for: (a) β -actin; (b) type I collagen; (c) type III collagen; (d) type IV collagen; (e) laminin; (f) fibronectin. Standard preparations of each matrix protein were run on one side of the gel. The result shown is representative of three separate analyses.

pronounced increase occurred for decorin mRNA levels which increased by over 20-fold after 28 days of exposure (Table 1). Laminin mRNA was increased approx. 2-fold after 14 days of exposure to hyperglycaemic conditions, but thereafter no consistent changes were seen (results not shown). Hyperglycaemic culture had no detectable effect on the expression of some other ECM molecules such as biglycan or on the cell membrane proteoglycan syndecan-1 (Table 1). Thus long-term exposure of HMCs to hyperglycaemic conditions increases the transcription of a number of specific genes coding for ECM proteins. TGF- β 1 induces an 8–10-fold increase in the synthesis of both decorin and biglycan in rat mesangial cells [31]. Therefore we used RT–PCR to investigate whether prolonged culture of HMCs in high concentrations of glucose (11 and 30 mM) is associated with increased gene transcription of TGF- β 1. The effect of these conditions on decorin expression was also confirmed by RT–PCR while GAPDH was co-amplified as a ‘housekeeping’ gene transcript. The results show that elevated D-glucose markedly increased the expression of TGF- β 1 mRNA at 7 days. Moreover,

Table 3 The effect of D-glucose concentration on the amount of matrix protein released into the culture medium by HMCs

Serum-free media were collected for 24 h after exposure of cultures to 4, 11 and 30 mM D-glucose for up to 28 days, as described in the Materials and methods section. Sample volumes equivalent to 10^5 cells per 24 h were analysed by inhibition ELISA. The results are expressed in ng per 24 h per 10^5 cells and represent the means $\pm$ S.E.M. for three independent experiments. * $P < 0.001$; ** $P < 0.005$; *** $P < 0.01$; † $P < 0.05$ compared with 4 mM D-glucose.

[D-Glucose] (mM) ...	Type I collagen			Type III collagen		
	4	15	30	4	15	30
7 days	122.3 $\pm$ 5.8	111.3 $\pm$ 7.8	131.0 $\pm$ 3.2	52.0 $\pm$ 3.0	60.3 $\pm$ 0.8	74.3 $\pm$ 2.9***
14 days	164.0 $\pm$ 14.7	296.0 $\pm$ 12.9**	275.0 $\pm$ 16.0***	63.7 $\pm$ 2.0	93.3 $\pm$ 2.7*	91.3 $\pm$ 3.5**
21 days	183.3 $\pm$ 5.9	312.3 $\pm$ 12.2*	305.7 $\pm$ 5.4*	66.0 $\pm$ 1.5	106.7 $\pm$ 7.6***	138.0 $\pm$ 3.2*
28 days	196.7 $\pm$ 11.6	327.7 $\pm$ 9.0*	410.7 $\pm$ 7.4*	65.3 $\pm$ 2.9	90.7 $\pm$ 1.8**	104.3 $\pm$ 3.4*
[D-Glucose] (mM) ...	Type IV collagen			Laminin		
	4	15	30	4	15	30
7 days	3.5 $\pm$ 0.2	3.0 $\pm$ 0.3	3.5 $\pm$ 0.3	0.098 $\pm$ 0.003	0.124 $\pm$ 0.01	0.105 $\pm$ 0.02
14 days	3.3 $\pm$ 0.3	3.6 $\pm$ 0.2	3.8 $\pm$ 0.2	0.124 $\pm$ 0.005	0.149 $\pm$ 0.005	0.148 $\pm$ 0.005
21 days	3.7 $\pm$ 0.4	3.8 $\pm$ 0.1	4.1 $\pm$ 0.3	0.144 $\pm$ 0.001	0.176 $\pm$ 0.007	0.186 $\pm$ 0.007
28 days	3.6 $\pm$ 0.1	4.1 $\pm$ 0.3	4.8 $\pm$ 0.1	0.148 $\pm$ 0.003	0.226 $\pm$ 0.032†	0.268 $\pm$ 0.025†
[D-Glucose] (mM) ...	Fibronectin					
	4	15	30			
7 days	0.076 $\pm$ 0.004	0.083 $\pm$ 0.006	0.089 $\pm$ 0.005			
14 days	0.093 $\pm$ 0.003	0.120 $\pm$ 0.005	0.150 $\pm$ 0.010**			
21 days	0.107 $\pm$ 0.008	0.246 $\pm$ 0.039*	0.288 $\pm$ 0.003*			
28 days	0.155 $\pm$ 0.003	0.300 $\pm$ 0.007*	0.307 $\pm$ 0.003*			

the response was dependent on glucose concentration (Figure 1). However, the level of mRNA in hyperglycaemic cultures decreased over the 28 days of exposure (Table 2), whereas there was no significant change in the level of TGF- β 1 mRNA extracted from cells maintained in 4 mM glucose. GAPDH mRNA showed little change under all conditions. In contrast with TGF- β 1, decorin mRNA level increased progressively over the 28 days of exposure to elevated D-glucose (Table 2).

To study the effect of elevated D-glucose concentration on the levels of matrix proteins, the cell layer and conditioned media of cultures were assessed by Western blotting and ELISA respectively. Prolonged exposure of HMC cultures to high concentrations of D-glucose (11 and 30 mM) had no effect on β -actin accumulation in the cell layer (Figure 2a). Hyperglycaemic conditions stimulated the accumulation of type I and III collagen (Figures 2b and 2c), but not of type IV collagen (Figure 2d). Laminin and fibronectin also accumulated in the cell layer in response to prolonged exposure to hyperglycaemic conditions (Figures 2e and 2f). The results for type IV collagen suggest that accumulation of the other matrix proteins in response to hyperglycaemia is a selective phenomenon. Similar experiments were performed in the cell layers of confluent cultures of human dermal fibroblasts. No actin, type I or type III collagen or fibronectin accumulates in the cell layer of hyperglycaemic fibroblast cultures (results not shown).

Collagen types I, III and IV, laminin and fibronectin were released into the medium of mesangial cell cultures. Changes in the medium content of these matrix proteins with hyperglycaemic culture conditions (11 and 30 mM D-glucose) correlate with the changes in their cell-layer levels (Table 3). There was little change in the type IV collagen level compared with the increases seen for the other proteins under hyperglycaemic conditions.

Proteoglycans were mostly secreted into the media. Media from cultures exposed to normoglycaemic or hyperglycaemic conditions for 4 weeks were pooled and their proteoglycans

Glucose Concentration

Figure 3 The effect of D-glucose concentration on the level of proteoglycans released into the medium of cultures of HMCs

Cells were grown under the conditions described in the Materials and methods section. Conditioned media were collected every 2 days for 28 days, pooled, and proteoglycans extracted on a DEAE-Sephacel column. Volumes equivalent to 2000 cells were subjected to gradient SDS/PAGE (3–20% gel) with (+) or without (–) prior digestion with chondroitinase ABC. Western blots are shown for decorin (a), biglycan (b) and versican (c). The results shown are representative of three separate analyses.

Table 4 The effect of D-glucose concentration on the level of decorin and TGF- β 1 secreted by HMCs

Serum-free media were collected for 24 h after exposure of cultures to 4, 11 and 30 mM D-glucose for up to 28 days as described in the Materials and methods section and were analysed for decorin and TGF- β 1 by ELISA. The results are expressed in ng per 24 h per 10^4 cells and are means $\pm$ S.E.M. for three independent experiments. * $P < 0.001$; ** $P < 0.005$ compared with 4 mM D-glucose.

[D-Glucose] (mM) ...	Decorin			TGF- β 1		
	4	15	30	4	15	30 mM
7 days	212.5 $\pm$ 7.2	215.0 $\pm$ 17.5	222.3 $\pm$ 5.2	2.65 $\pm$ 0.11	5.06 $\pm$ 0.06*	11.35 $\pm$ 0.64*
14 days	173.3 $\pm$ 14.5	229.0 $\pm$ 5.5	230.0 $\pm$ 15.3	2.99 $\pm$ 0.06	6.28 $\pm$ 0.39*	9.14 $\pm$ 0.31*
21 days	240.0 $\pm$ 5.8	298.3 $\pm$ 1.7	526.6 $\pm$ 37.0**	4.00 $\pm$ 0.40	5.97 $\pm$ 0.80**	6.50 $\pm$ 0.17*
28 days	221.6 $\pm$ 6.0	534.1 $\pm$ 18.3*	1163.3 $\pm$ 31.0*	4.00 $\pm$ 0.16	4.84 $\pm$ 0.13**	7.43 $\pm$ 0.22*

extracted as described in the Materials and methods section. Western analysis of the proteoglycans before and after digestion with chondroitinase ABC was performed. Native proteoglycans do not penetrate the polyacrylamide gel. Chondroitinase digestion removes chondroitin or dermatan sulphate chains and allows the core proteins to migrate through the gel. The Western analysis showed that both decorin (core protein molecular mass 45 kDa) and versican (core protein molecular mass 400–500 kDa; [32]) were present in increased amounts in the hyperglycaemic mesangial cultures (Figures 3a and 3c). In contrast, biglycan seems to decrease under hyperglycaemic conditions (Figure 3b). No increase in either decorin or biglycan level was detected in hyperglycaemic fibroblast cultures (results not shown).

It is noteworthy that the apparent molecular mass of biglycan core protein (65 kDa) is larger than that for decorin, although the predicted sizes from their cDNA sequences should be similar [33]. This apparent difference has been observed previously, although the magnitude was not as great as in the present experiment [34]. The increased release of decorin by mesangial cells in response to hyperglycaemic conditions was confirmed by ELISA (Table 4). TGF- β 1 released into the medium of HMCs exposed to normoglycaemic or hyperglycaemic conditions is also shown in Table 4. The antibody used in the ELISA for TGF- β 1 detects the mature form of the growth factor. There is a marked increase in the release into the medium of this form of TGF- β 1 by 7 days in hyperglycaemic cultures. Although levels are higher in hyperglycaemic cultures over 28 days than in normoglycaemic cultures, they nevertheless decrease with increasing time.

DISCUSSION

The expansion of the mesangium in diabetic nephropathy involves hypertrophy of its cells without an apparent increase in their number but with a marked increase in their extracellular matrix. The mechanism of mesangial matrix expansion is not understood but is likely to involve several interacting factors including genetic background, glomerular haemodynamic changes and hyperglycaemia [35,36].

In previous studies Ayo et al. observed increases in the levels of mRNA and protein for fibronectin, laminin and collagen IV in rat mesangial cultures grown in 30 mM D-glucose compared with 10 mM D-glucose over 1–4 weeks [10,37]. These effects were not seen in the presence of 30 mM mannitol, indicating that they were not due to increased osmolarity alone. Silbiger et al. [32,40] grew rat mesangial cells on Millipore filters in media containing either 11 or 28 mM D-glucose for periods of up to 8 weeks. They found no difference in total ^3H -labelled protein synthesis, but a small decrease (28%) in incorporation of [^3H]proline into collagenase-degradable macromolecules in 28 mM glucose. Total

proteoglycan accumulation, as assessed by glucuronate assay, was also unchanged in the two culture conditions [32]. Although these assays are less specific than those used by Ayo et al. [10,37], they suggest that the response to high glucose conditions might depend in part on the environment in which the mesangial cells are grown. However, both groups of investigators used 10 or 11 mM D-glucose as the baseline for comparisons with 30 mM D-glucose culture conditions. In our experience with cultures of HMCs, 11 mM D-glucose has significant stimulatory effects on the transcription and synthesis of specific matrix macromolecules when compared with 4 mM D-glucose culture conditions. Our experimental design also differs in that the human cells were grown in 4 mM D-glucose (normoglycaemic conditions) until passage 6–8. Only then were post-exponential-phase cultures transferred to 4, 11, 15 or 30 mM D-glucose media. Thus our results indicate the effect of hyperglycaemic conditions on matrix synthesis by cells that are undergoing very little cell division. This state is more akin to that in the mesangium *in vivo*.

Pugliese et al. [13] grew rat mesangial cells in normoglycaemic (5.5 mM) and hyperglycaemic conditions through several passages. Synthesis of fibronectin, laminin and collagen IV was increased in the hyperglycaemic cultures but not in cultures in which the osmolarity had been increased to the same level by the addition of mannitol. Collagen types I and III were not increased in the hyperglycaemic cultures [13]. Type IV collagen synthesis is also increased in hyperglycaemic cultures of calf mesangial cells [12]. There have been few previous studies with HMCs, although it has been reported that both 50 mM glucose and 50 mM mannitol have similar effects in stimulating increases in mRNA and protein levels for fibronectin in human cultures [38]. Thus hyperosmolarity might be involved in the response of HMCs to hyperglycaemia, whereas it does not seem to be a factor in the response of rat mesangial cells.

Our results indicate that HMCs increase production of collagen types I and III, fibronectin and laminin when exposed to even moderately elevated concentrations of D-glucose (11 mM) and this response is even more pronounced under high-glucose conditions (30 mM). In contrast, collagen type IV levels are not increased. Whereas the increases in fibronectin and laminin are similar to those occurring in rat mesangial cells exposed to high-glucose conditions [13], the increases for collagen types I and III and lack of change for collagen type IV are different. As mentioned above, levels of collagen types I and III remained unchanged, whereas collagen type IV levels increased in the rat cultures. These differences suggest that the mechanism of increased deposition of specific matrix macromolecules in the diabetic mesangium might differ in different species. For example, hyperglycaemia could stimulate enhanced synthesis of fibronectin in both rat and human *in vivo*. The same might be true for type

IV collagen in diabetic rat, but increased amounts of this protein in the human diabetic mesangium might accumulate by other mechanisms. Decreased turnover of type IV collagen could be one such mechanism.

We have shown previously that confluent cultures of human cells synthesize biglycan, decorin, versican and two heparan sulphate proteoglycans [5,39]. The present work shows that when HMCs are exposed to increased concentrations of D-glucose they show increased levels of mRNA for decorin, versican and perlecan. Biglycan and syndecan mRNA levels are not affected. There was an apparent decrease in biglycan at the protein level. No differences were found in the levels of biglycan mRNA in rat mesangial cells cultured in 11 and 28 mM D-glucose [40].

Increased protein levels of both decorin and versican occurred in hyperglycaemic cultures of HMCs. For decorin the increase was approx. 6-fold in cultures maintained in 30 mM glucose for 28 days compared with the level in normoglycaemic cultures. Because decorin is known to interact with several other proteins and to modulate their function [34], elevations of this magnitude might have biological effects if they occurred in hyperglycaemia *in vivo*. Decorin interacts with fibrillar collagens and decreases the diameter of fibrils formed [41,42]. It binds to fibronectin [43,44] and might disrupt cell attachment to fibronectin [45]. It is also a ligand for thrombospondin and inhibits cell attachment to that protein. Overexpression of decorin in Chinese hamster ovary cells inhibits their proliferation [46]. This seems to be due to decorin's binding to TGF- β [47] and acting as a negative regulator of the growth factor [48].

In initial experiments we were unable to detect elevated mRNA levels for TGF- β 1 in hyperglycaemic cultures of HMCs by slot-blot analysis [49]. However, the present results from RT-PCR experiments and ELISA clearly show increased levels of TGF- β mRNA and protein in the hyperglycaemic cultures. TGF- β accumulates slowly in the mesangium of rats with streptozotocin-induced diabetes and is present in human diabetic glomerulosclerosis [15,50]. The growth factor stimulates the expression of many ECM proteins at both the mRNA level and protein level [51], including collagen types I and III [52–55], fibronectin [54], heparan sulphate proteoglycan [56], versican [57] and decorin [31] in a variety of cell types. Thus it has been suggested that TGF- β plays a causative role in diabetic mesangial expansion [15] and one possibility is that the changes in matrix protein levels induced by culture of our HMCs under high-glucose conditions are mediated by TGF- β . However, TGF- β alone markedly increased biglycan expression in the human osteosarcoma cell line MG-63, but had little effect on decorin expression [58]. In rat mesangial cells both biglycan and decorin expression were increased 10-fold in response to TGF- β [31]. The effect of TGF- β on the synthesis of these two proteoglycans in HMCs is not known, so the connection between elevated TGF- β and decorin levels in hyperglycaemic cells is unclear.

It is noteworthy that in our experiments TGF- β 1 mRNA and protein levels are maximally stimulated after 7 days of hyperglycaemic culture and that they are less elevated after prolonged exposure to high glucose (21–28 days). In contrast, the accumulation of protein for collagen types I and III, laminin and fibronectin is negligible after 7 days of hyperglycaemic culture but markedly elevated after 14–28 days. Decorin mRNA and protein levels are similarly unchanged after 7 days but greatly increased after 21–28 days of exposure to high-glucose conditions. Thus these changes are delayed compared with the pattern that might be predicted if their accumulation is due solely to enhanced transcription under the stimulus of TGF- β 1. Moreover, TGF- β 1 has been defined as a potent signal for up-regulating production

of biglycan in virtually all cells [34] with the exception of myofibroblasts [59]. As noted above, biglycan synthesis remained unchanged in our hyperglycaemic cultures. In separate experiments, when we added TGF- β 1 to normoglycaemic cultures of HMCs, mRNA levels for biglycan increased during the following 48 h, after which they returned to pretreatment levels (K. Harper and R. M. Mason, unpublished work).

Any consideration of TGF- β as a signal mediating the effects of high-glucose conditions on mesangial cells must take into account whether the factor, even though present, is able to act on cells. TGF- β is secreted in an inactive form and must be converted to the active peptide *in vivo*, possibly by the action of a proteolytic enzyme [60]. The small leucine-rich proteoglycans decorin and biglycan both bind TGF- β and might compete with its receptors for it as a ligand [47]. Administering recombinant decorin to rats with anti-Thy 1.1 glomerulonephritis, a disorder mediated by TGF- β [31], suppressed mesangial changes and indicates that the proteoglycan can act as an inhibitor of the growth factor's actions *in vivo* [61]. Because biglycan accounts for approx. 45%, and decorin for approx. 10%, of the total 35 S-labelled proteoglycans made by HMCs cultured in standard RPMI 1640 medium (11 mM glucose) [5], it is likely that these proteoglycans compete for TGF- β *in vitro*. The greatly elevated level of decorin at late stages in chronic hyperglycaemic cultures would increase the competition for binding and sequestering the growth factor away from its receptors. Furthermore present evidence indicates that TGF- β receptors I and II must act together to generate a signal [62]. However, TGF- β 1 down-regulates the expression of TGF receptor II in rat mesangial cells [63]. Thus before concluding that TGF- β is the main factor driving the enhanced expression of matrix proteins in hyperglycaemic HMC cultures, it will be necessary to show that the factor is biologically active and that functional receptors for it are expressed.

Clearly the mechanism by which long-term hyperglycaemic culture induces increased transcription and accumulation of matrix proteins is complex. It has some specificity for mesangial cells because these changes do not occur in fibroblasts. Hyperosmolarity might play a part in the mechanism [38], but we do not as yet have results to support this. Potentially, changes in the activity of proteolytic enzymes in turning over matrix proteins could contribute to their accumulation in hyperglycaemic conditions.

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REFERENCES

- Pirart, J. (1978) *Diabetes Care* **1**, 168–188
- Lasker, R. D. (1993) *New Engl. J. Med.* **329**, 1035–1036
- Mauer, S. M., Steffes, M. W., Ellis, E. N., Sutherland, D. E. R., Brown, D. M. and Goetz, F. C. (1984) *J. Clin. Invest.* **74**, 1143–1155
- Steffes, M. W., Østerby, R. W., Chavers, B. and Mauer, S. M. (1989) *Diabetes* **38**, 1077–1081
- Thomas, G. J., Mason, R. M. and Davies, M. (1991) *Biochem. J.* **277**, 81–88
- Kashgarian, M. and Sterzel, R. B. (1992) *Kidney Int.* **41**, 524–529
- Abrass, C. K., Peterson, C. V. and Raugi, G. J. (1988) *Diabetes* **37**, 1695–1702
- Nerlich, A. and Schleicher, E. (1991) *Am. J. Pathol.* **139**, 884–899
- Fisher, E., McLennan, S. V., Tada, H., Hefferman, S., Yue, D. K. and Turtle, J. R. (1991) *Diabetes* **40**, 371–376
- Ayo, S. H., Radnik, R. A., Glass, II, W. F., Garoni, J. A., Rampt, E. R., Appling, D. R. and Kreisberg, J. I. (1991) *Am. J. Physiol.* **260**, F181–F191
- Williams, B. and Schrier, R. W. (1992) *Diabetes* **41**, 1464–1472
- Danne, T., Spiro, M. J. and Spiro, R. G. (1993) *Diabetes* **42**, 170–177
- Pugliese, G., Pricci, F., Pugliese, F., Mene, P., Lenti, L., Andreani, D., Galli, G., Casini, A., Bianchi, S., Rotella, C. M. and DiMario, U. (1994) *Diabetes* **43**, 478–490
- Massague, J. (1990) *Annu. Rev. Cell Biol.* **6**, 597–641

- 15 Yamamoto, T., Nakamura, T., Noble, N. A., Ruoslahti, E. and Border, W. A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1814–1818
- 16 Nicolic-Paterson, D. J., Ramm, H. C., Yu, Y. and Atkins, R. C. (1992) *Kidney Int.* **4**, 1033
- 17 Kaname, S., Uchida, S., Ogata, E. and Kurokawa, K. (1992) *Kidney Int.* **42**, 1319–1327
- 18 Striker, G. E., Killen, P. D. and Farin, F. M. (1980) *Transplant. Proc.* **12**, no. 3, suppl. 1, 88–99
- 19 Bernik, M. B. (1969) *Nephron* **6**, 1–10
- 20 Stewart, K. N., Roy Chaudhury, P., Lumsden, L., Jones, M. C., Brown, P. A., Macleod, A. M., Haites, N. E., Simpson, J. G. and Power, D. A. (1991) *J. Pathol.* **163**, 265–272
- 21 Boehm, T., Buluwela, L., Williams, D., White, L. and Rabbitts, T. H. (1988) *EMBO J.* **7**, 2011–2017
- 22 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 23 Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B., Sporn, M. B. and Goeddel, D. V. (1985) *Nature (London)* **361**, 701–704
- 24 Ercolani, L., Florence, B., Denaro, M. and Alexander, M. (1988) *J. Biol. Chem.* **263**, 15335–15341
- 25 Krusius, T. and Ruoslahti, E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7683–7687
- 26 Glossl, J., Beck, M. and Kresse, H. (1984) *J. Biol. Chem.* **259**, 14144–14150
- 27 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 28 Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 29 Voller, A., Bidwell, D. E. and Bartlett, A. (1976) in *Manual of Clinical Immunology* (Rose, N. and Fishman, H., eds.), pp. 506–512, American Society for Microbiology, Washington, DC
- 30 Rennard, S. I., Berg, R., Martin, G. R., Foidart, J. M. and Robey, P. G. (1980) *Anal. Biochem.* **104**, 205–214
- 31 Border, W. A., Okuda, S., Languino, L. R. and Ruoslahti, E. (1990) *Kidney Int.* **37**, 689–695
- 32 Silbiger, S., Crowley, S., Shan, Z., Brownlee, M., Satriano, J. and Schlondorff, D. (1993) *Kidney Int.* **43**, 853–8642
- 33 Fisher, L. W., Termine, J. D. and Young, M. F. (1989) *J. Biol. Chem.* **264**, 4571–4576
- 34 Kresse, H., Hausser, H. and Schonherr, E. (1993) *Experientia* **49**, 403–416
- 35 Seaquist, E. R., Goetz, F. C., Rich, S. and Barbosa, J. (1989) *New Engl. J. Med.* **320**, 1161–1165
- 36 O'Donnell, M. P., Kasiske, B. L. and Keane, W. F. (1988) *FASEB J.* **2**, 2339–2347
- 37 Ayo, S. H., Radnik, R. A., Garoni, J. A., Glass, W. F. and Kreisberg, J. I. (1990) *Am. J. Pathol.* **136**, 1339–1348
- 38 Nahman, Jr., N. S., Leonhart, K. L., Cosio, F. G. and Herbert, C. L. (1992) *Kidney Int.* **41**, 396–402
- 39 Thomas, G. J., Bayliss, M. T., Harper, K., Mason, R. M. and Davies, M. (1994) *Biochem. J.* **302**, 49–56
- 40 Silbiger, S., Schlondorff, D., Crowley, S., Rosenberg, L., Choi, H., Hatcher, V. and Gordon, P. (1993) *Diabetes* **42**, 1815–1822
- 41 Vogel, K. G. and Trotter, J. A. (1987) *Collagen Relat. Res.* **7**, 105–114
- 42 Vogel, K. G., Koob, T. J. and Fisher, L. W. (1987) *Biochem. Biophys. Res. Commun.* **148**, 658–663
- 43 Schmidt, G., Hausser, H. and Kresse, H. (1991) *Biochem. J.* **280**, 411–414
- 44 Winnemoller, M., Schmidt, G. and Kresse, H. (1991) *Eur. J. Cell Biol.* **54**, 10–17
- 45 Lewandowska, K., Choi, H. U., Rosenberg, L. C., Zardi, L. and Culp, L. A. (1987) *J. Cell Biol.* **105**, 1443–1454
- 46 Yamaguchi, Y., Mann, D. and Ruoslahti, E. (1988) *Nature (London)* **336**, 244–246
- 47 Hildebrand, A., Romaris, M., Rasmussen, L. M., Heinegård, D., Twardzik, D. R., Border, W. A. and Ruoslahti, E. (1994) *Biochem. J.* **302**, 527–534
- 48 Yamaguchi, Y., Mann, D. and Ruoslahti, E. (1990) *Nature (London)* **346**, 281–284
- 49 Harper, K. and Mason, R. M. (1994) *Biochem. Soc. Trans.* **22**, 184S
- 50 Nakamura, T., Fukui, M., Ebihara, I., Osada, S., Nagaoka, I., Tomino, Y. and Koide, H. (1993) *Diabetes* **42**, 450–456
- 51 Roberts, A. B., McCune, B. K. and Sporn, M. B. (1992) *Kidney Int.* **41**, 557–559
- 52 Ignatz, R. A. and Massague, J. (1986) *J. Biol. Chem.* **261**, 4337–4345
- 53 Fine, A. and Goldstein, R. H. (1987) *J. Biol. Chem.* **262**, 3897–3902
- 54 Varga, J., Rosenbloom, J. and Jimenez, S. A. (1987) *Biochem. J.* **247**, 597–604
- 55 Lawrence, R., Hartmann, D. J. and Sonenshein, G. E. (1994) *Am. Soc. Biochem. Mol. Biol.* **269**, 9603–9609
- 56 Nugent, M. A. and Edelman, E. R. (1992) *J. Biol. Chem.* **267**, 21256–21264
- 57 Schonherr, E., Jarvelainen, H. T., Sandell, L. J. and Wight, T. N. (1991) *J. Biol. Chem.* **266**, 17640–17647
- 58 Breuer, B., Schmidt, G. and Kresse, H. (1990) *Biochem. J.* **269**, 551–554
- 59 Meyer, D. H., Krull, N., Didier, K. L. and Gessner, A. M. (1992) *Hepatology* **16**, 204–216
- 60 Roberts, A. B. and Sporn, M. B. (1990) in *Handbook of Experimental Pharmacology* (Sporn, M. B. and Roberts, A. B., eds.), pp. 419–472, Springer-Verlag, Heidelberg
- 61 Border, W. A., Noble, N. A., Yamamoto, T., Harper, J. R., Yamaguchi, Y., Pierschbacher, M. D. and Ruoslahti, E. (1992) *Nature (London)* **360**, 361–364
- 62 Lin, H. Y. and Lodish, H. F. (1993) *Trends Cell Biol.* **3**, 14–19
- 63 Choi, M. E., Kim, E. G., Huang, Q. and Ballermann, B. J. (1993) *Kidney Int.* **44**, 948–958