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Increased activity of the insulin-like growth factor system in mesangial cells cultured in high glucose conditions. Relation to glucose-enhanced extracellular matrix production

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Summary Recent evidence suggests that several growth factors participate in diabetic glomerular disease by mediating increased extracellular matrix accumulation and altered cell growth and turnover leading to mesangial expansion. Transforming growth factor (TGF)-β has been demonstrated to be upregulated both in vivo and in vitro, whereas studies on the activity of the renal insulin-like growth factor (IGF) system in experimental diabetes have provided conflicting results. We investigated the effects of prolonged exposure (4 weeks) of cultured human and rat mesangial cells to high (30 mmol/l) glucose vs iso-osmolar mannitol or normal (5.5 mmol/l) glucose levels on: 1) the autocrine/paracrine activity of the IGF system (as assessed by measuring IGF-I and II, IGF-I and II receptors, and IGF binding proteins); and, in parallel, on 2) TGF-β1 gene expression; 3) matrix production; and 4) cell proliferation. High glucose levels progressively increased the medium content of IGF-I and the mRNA levels for IGF-I and IGF-II, increased IGF-I and IGF-II binding and IGF-I receptor gene expression, and reduced IGF binding protein production. TGF-β1 transcripts and matrix accumulation and gene expression were increased in parallel, whereas cell proliferation was reduced. Iso-osmolar mannitol did not affect any of the above parameters. These experiments demonstrated that high glucose levels induce enhanced mesangial IGF activity, together with enhanced TGF-β1 gene expression, increased matrix production, and reduced cell proliferation. It is possible that IGFs participate in mediating diabetes-induced changes in matrix turnover leading to mesangial expansion, by acting in a paracrine/autocrine fashion within the glomerulus. [Diabetologia (1996) 39: 775–784]

Keywords Insulin-like growth factor-I–II, binding proteins, receptors, transforming growth factor-β, extracellular matrix, mesangial cell, diabetes mellitus.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; GH, growth hormone; HMC, human mesangial cells; IEMA, immunoenzymometric assay; IGF, insulin-like growth factor; IGFr, insulin-like growth factor receptor; IGFBP, insulin-like growth factor binding protein; IRMA, immunoradiometric assay; RMC, rat mesangial cells; SDS, sodium dodecylsulphate; TGF-β, transforming growth factor-β.

A growing body of evidence suggests that hyperglycaemia-induced glomerular injury could be mediated by a group of multifunctional substances which normally regulate tissue remodelling as well as haemodynamics, haemorheology and permeability by acting in a paracrine/autocrine fashion within the glomerulus [1]. These substances have been presumed to play a role in the pathogenesis of enhanced extracellular matrix (ECM) accumulation and altered cell growth and turnover leading to mesangial expansion, the characteristic anatomical lesion of diabetic glomerular disease [2]. An increased production and/or gene expression of transforming growth factor (TGF)-β has been demonstrated both in vivo [3–7] and in vitro [7, 8]. In addition, experimental diabetes was shown

to be associated with increased renal/glomerular synthesis and/or content of other growth factors and cytokines [4], including insulin-like growth factor (IGF)-I [9, 10].

The vast majority of previous observations on the activity of the IGF system in response to hyperglycaemia have been derived from in vivo studies in experimental diabetic animals. These studies consistently showed that the kidney content of IGF-I increases during the first few days following diabetes induction, and returns to normal levels thereafter [9, 10], thus suggesting that IGF-I is implicated in the initial renal hypertrophy associated with diabetes, which predominantly involves the tubular and vascular components. By contrast, IGF-I mRNA [11–13], IGF receptors [11, 14, 15] and binding proteins (IGFBPs) [16–19] showed discordant changes, which may reflect differences in the duration and severity of the diabetic state as well as in the nutritional and developmental conditions. However, these discrepancies may also depend upon the different contributions of the various parts of the kidney and of the circulation to the total kidney levels of IGFs, IGFBPs and IGF binding capacity, reflecting both the endocrine (growth hormone [GH] – dependent) and the paracrine/autocrine actions of IGF-I. In fact, the regulation of the activity of the IGF system is under the control of multiple factors acting both systemically and locally [20], thus resulting in region-specific differences in the expression patterns of IGFs, IGF receptors and IGFBPs in qualitative and quantitative terms, either under normal conditions [21, 22] or in response to hyperglycaemia [19]. Therefore, the results obtained in total kidney samples cannot be extrapolated to the mesangial region, also because the glomeruli (and the mesangium) represent less than 5 % of kidney mass and contain fewer quantities of IGF-I and IGF-I receptor mRNA than medullary collecting ducts [21], thus impacting very little on total kidney levels of IGFs, IGF receptors and IGFBPs. As a consequence, it remains unclear (a) whether the kidney content of IGF-I is actually increased in the more advanced stages of the disease, at the time when significant mesangial ECM accumulation is demonstrable; (b) whether the overall activity of the renal IGF system, including both IGFs, IGF receptors and IGFBPs, is also enhanced; (c) whether the source of IGF-I accumulating in the kidney is renal or extra-renal (GH-dependent); (d) whether IGF content and the paracrine/autocrine activity of the IGF system are increased at the glomerular/mesangial level. We believed that these issues could be addressed more adequately by the use of an in vitro model of cultured mesangial cells exposed to elevated glucose concentrations for a prolonged period of time.

In this study, we investigated the effects of 4 weeks' exposure of mesangial cells from human (HMC) and rat (RMC) sources to high glucose conditions on the overall autocrine/paracrine activity of the IGF system. In parallel, we assessed TGF-β1 gene expression, to confirm the upregulation of TGF-β synthesis, as well as ECM protein and mRNA levels and cell proliferation, in order to investigate the relationship between previously shown alterations in matrix and cell turnover and changes in growth factor synthesis and action.

Materials and methods

Design. Cells were cultured for 4 weeks (through four-to-six passages) under the following experimental conditions: 1) normal (5.5 mmol/l) glucose; 2) high (30 mmol/l) glucose; 3) 24.5 mmol/l mannitol + 5.5 mmol/l glucose (to match osmolarity of high glucose medium). At each passage, the following parameters were assessed: a) medium content of IGF-I, total IGFBPs and IGFBP-1 and 3, and the ECM components fibronectin, laminin and collagen IV; b) the number and affinity of type I (IGF-Ir) and type II (IGF-IIr) IGF receptors; c) the levels of transcripts for IGF-I, IGF-II, IGF-Ir, TGF-β1, fibronectin, laminin and collagen IV; and d) cell proliferation.

Cell cultures. Glomeruli were isolated using standard sieving techniques either from non-invaded kidney poles from patients nephrectomized for renal cancer [23] or from kidneys obtained from four normal Sprague-Dawley rats [24]. Primary outgrowths of whole glomeruli yielded mostly (95 %) mesangial cells, which were characterized according to morphologic, immunohistochemical and functional criteria, as previously reported [23, 24]. Cells between the third and the tenth passage, originating from three independent cell lines for both HMC and RMC, were plated in 16-mm (for IGF binding and cell proliferation), 35-mm (for IGF-I, IGFBP and ECM accumulation) or 100-mm (for growth factor and ECM gene expression) culture dishes (Falcon, Becton Dickinson, Lincoln Park, N.J. USA). To achieve the same final density at harvest, HMC cultured in high glucose were plated at a higher density than those in iso-osmolar mannitol or normal glucose to account for the observed replicative impairment. Cells were grown to (sub)confluence in Dulbecco's modified Eagle's medium (DMEM, Sigma, St.Louis, Mo., USA), supplemented with 17 % fetal bovine serum, 2 mmol/l L-glutamine, and antibiotics (all obtained from Flow Laboratories, Irvine, Scotland, UK) under the experimental conditions indicated above. Insulin concentration of serum-supplemented medium ranged from 37 to 53 pmol/l, corresponding to values observed in animal models of insulin-dependent diabetes [25]. Subconfluent cells were maintained in serum-free medium for 24–48 h and then used for measuring IGF receptors, and growth factor, IGFBP and ECM production, whereas confluent cells were synchronized by serum deprivation for 48–72 h and then processed for cell proliferation studies.

IGF-I production. Media were lyophylized and reconstituted to obtain 5–20 × concentrated samples. IGF-I concentration in media was measured by RIA [26] using a commercial kit (Nichols Institute, San Juan Capistrano, Calif., USA), after ethanol (87.5 %) – 2 N HCL (12.5 %) extraction of samples followed by neutralization with 0.855 mol/l Tris-base [27]. Selected medium samples were assayed for IGF-I content both before and after ethanol-acid extraction to assess the relative contibution of glucose-induced changes in IGF-I and IGFBP production to free IGF-I levels in culture media. The RIA

procedure was slightly modified by using a 2–5-fold dilution of extracted samples instead of a 15-fold dilution as indicated for the measurement of serum IGF levels.

IGF receptors. Binding studies were performed as previously described by Conti et al. [28]. Cells were incubated for 4 h at 22 °C (based on preliminary experiments showing maximal specific binding at this time and temperature) in binding buffer (in mmol/l:118 NaCl, 5 KCl, 1.2 MgSO4, 100 HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]), 1 % bovine serum albumin, plus sugar concentrations as the correspondent culture medium, pH 7.6) containing 20 000 cpm/well of ^{125}I-IGF-I or IGF-II (2000 Ci/mmol, purchased from Amersham, Amersham, Bucks., UK) and 0–10^{-6} mol/l DNA-recombinant human IGF-I or IGF-II (Calbiochem, San Diego, Calif., USA). Since IGF-II binds to both IGF receptors [20], the IGF-II binding studies were conducted in the presence of 10^{-5} mol/l human recombinant insulin (Novo, Copenhagen, Denmark) displacing labelled IGF-II from IGF-Ir. Monolayers were then washed, solubilized in 0.1 % sodium dodecylsulphate (SDS; Sigma) and counted. Contribution of cell membrane- or plastic-bound IGFBPs IGF binding was assessed by performing binding experiments, for both IGFs, in cell suspensions vs cell monolayers, since mild trypsin treatment removes IGFBPs weakly bound to cell and plastic surfaces [29], and, for IGF-I, in the presence or absence of increasing concentrations (10^{-5}–10^{-10} mol/l) of cold insulin displacing tracer IGF-I from IGF-Ir (to which insulin binds with a 100-times lower affinity than IGF-I [20]), but not from IGFBPs. Conversely, a significant contribution of the insulin receptor to IGF-I binding was eliminated by showing little or no mRNA expression for this receptor by reverse transcriptase-polymerase chain reaction [unpublished observations]. Results of the competition studies were analysed by the computer program LIGAND [30].

IGFBP production. Media were collected in tubes pre-treated with DMEM containing 0.1 % bovine serum albumin at 37 °C for 3 h under agitation (to decrease protein background), lyophylized and reconstituted to obtain 10–20 × concentrated samples. The pattern of IGFBP secretion was evaluated by Western ligand blot analysis [31]. Medium samples were subjected to 12 % SDS-PAGE under nonreducing conditions and proteins were then blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) by a transblotting apparatus (Bio-Rad Laboratories, Richmond, Calif., USA). The nitrocellulose membranes were then incubated for 2 h in Tris-buffer (150 mmol/l NaCl and 10 mmol/l Tris-HCl, pH 7.4) containing 1 % bovine serum albumin, 0.1 % Tween 20, and 2×10^6 cpm of ^{125}I-IGF-II (2,000 Ci/mmol, Amersham). Subsequently, the membranes were washed, dried, and exposed at –70 °C for 3 days to X-Omat RP autoradiography films (Eastman Kodak Co., Rochester, N. Y., USA) with the aid of intensifying screens. Quantification of IGFBPs was performed by scanning densitometry using a GS-670 Imaging Densitometer (Bio-Rad Laboratories). Medium content of IGFBP-3 and IGFBP-1 was assessed by immunoradiometric assay (IRMA) and immunoenzymometric assay (IEMA) using commercial kits from Giuliana Cremascoli Chemicals (Segrate, Milan, Italy) and Medix Biochemica Ab (Kauniainen, Finland), respectively.

ECM accumulation. Serum-free medium to which cells were exposed for 24–48 h contained 0.3 mmol/l ascorbic acid (Sigma) and 0.4 mmol/l β-aminopropionitrile (Sigma), in order to facilitate matrix protein secretion and inhibit ECM assembly, respectively; under these conditions, most of the ECM proteins synthesized are found in the medium, without any change in cell function, in both HMC [32] and RMC [24]. Medium levels of the ECM components fibronectin, laminin and collagen IV were quantified by ELISA, as previously described [24, 32]. Rabbit polyclonal antibodies produced against bovine or rat fibronectin (Calbiochem), human or mouse laminin (B1 chain), and human or mouse collagen IV (NC1 globular domain) were used at 1:10000, 1:72000, 1:2500, 1:2500, 1:5000, and 1:2500 dilutions, respectively.

DNA levels. Results of IGF-I, IGFBP and ECM measurements were normalized per DNA content of monolayers, extracted with 0.5 N NaOH and sonicated [24]. DNA levels were measured fluorimetrically after reaction of cell extracts with 0.6 μmol/l 4,6-diamidino-2-phenylindole (Sigma) [33], as previously described [24, 32].

IGF-I, IGF-II, IGF-Ir, TGF-β1, and ECM gene expression. Total RNA was extracted by the guanidine thiocyanate-phenol-chloroform method [34] using the RNAfast-II kit (Molecular Systems, San Diego, Calif., USA) and quantified by measuring the absorbance at 260 nm; the purity of RNA preparation was confirmed by demonstrating an absorbance 260:280 ratio greater than 1.9. The level of transcripts was measured by Northern blot analysis [35]. Under denaturing conditions 15–35 μg of total RNA per lane were electrophoresed in 1.2 % agarose (Life Technologies, Gaithersburg, Md., USA) gel, transferred onto Hybond-N nylon blotting membranes (Amersham) and fixed under the ultra violet light in Spectrolinker XL-1000 UV Crosslinker (Spectronics Corporation, Westbury, N. Y., USA). Filters were pre-hybridized for 6 h at 42 °C in 50 % formamide (Fluka Chemie AG, Buchs, Switzerland), 5 × SSPE (saline-sodium phosphate-EDTA), 5 × Denhardt's solution, 100 μg/ml of salmon sperm DNA (Sigma), and 0.1 % SDS, then hybridized overnight at 42 °C in the same solution containing 25 ng of cDNA labelled by the random-primer method [36] (Random Primer Labeling Kit Prime-IT II; Stratagene, La Jolla, Calif., USA) and 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) as precursor. The 442 base pairs (bp) *Pst1-RSal1* human [37] or the 505 bp *Sau*3A-NlaIV rat [38] IGF-I cDNA, the 1000 bp *EcoRI* human IGF-II cDNA (American Type Culture Collection, Rockville, Md., USA) [39], the 340 bp *EcoRI* human IGF-Ir cDNA (American Type Culture Collection) [40], the 550 bp *Sma I* human TGF-β1 cDNA [41], the 500 bp *EcoRI* rat fibronectin cDNA [42], the 4500 *HIND III-EcoRI* mouse laminin B1 cDNA [43], the 2600 bp *Pst1* human [44] or the 830 bp *Pst1-Aval* rat [45] α1 collagen IV cDNA, and the 2200 bp *Bam H1* human β-actin cDNA [46] were used as probes. Filters were sequentially washed, then exposed to Hyperfilm-MP autoradiography films (Amersham) with the aid of intensifying screens at –70 °C for 1–7 days. Quantification of specific mRNA levels was performed by scanning densitometry. To account for differences in sample loading, results were normalized to the signal of the 'housekeeping' gene β-actin as well as to the intensity of ethidium bromide-staining of the 18s band of rRNA.

Cell proliferation. Confluent, quiescent cells were exposed for 24 h to 0.5 % or 17 % fetal bovine serum. Cell proliferation was determined by both ^{3}H-thymidine incorporation [24, 32] and crystal violet binding to cell nuclei [47]. In the ^{3}H-thymidine incorporation experiments, cells were then pulsed for 4 h with 1 μCi/ml of methyl-^{3}H-thymidine (25 Ci/mmol, Amersham), and then processed as previously detailed [24, 32]. Results of ^{3}H-thymidine uptake were normalized per protein content of each well, as measured by the Bradford method [48]. In the crystal violet binding experiments, monolayers were fixed

in 1% glutaraldehyde (Sigma) for 15 min, then treated with 0.1% crystal violet (Sigma) for 30 min at room temperature. After removal of unbound dye, crystal violet pigments in the cell nuclei were dissolved in 0.1% aqueous Triton X-100 (Sigma) for 4 h at room temperature, and the absorbance at 600 nm was measured.

Statistical analysis

Results are expressed as mean ± SD or as mean ± SD of percent variations vs 5.5 mmol/l glucose. To reduce potential type 1 errors related to multiple comparisons, differences between experimental groups were analysed using the Student-Neuman-Keuls test when overall differences, as assessed by one-way analysis of variance, attained statistical significance. All statistical tests were performed on raw data.

Results

IGF-I and IGF-II. In HMC, IGF-I production was progressively enhanced by high glucose culture conditions, whereas it was virtually unchanged in cells grown in normal glucose over the 4-week period (Fig. 1A). In RMC, IGF-I production was also increased by 30 mmol/l glucose, although the trend of these changes was somewhat different from that observed in HMC (Fig. 1B). The increments observed after a 48-h medium collection period (Fig. 1) were more pronounced than those observed after a 24-h medium collection period (data not shown). In both HMC and RMC, high glucose-induced increases vs normal glucose in IGF-I medium content, as measured without previous ethanol-acid extraction, were even greater than those observed in extracted samples (at week 4, + 99 vs + 75% in HMC and + 148 vs + 121% in RMC) attesting to a reduced IGF-I binding capacity of media. In HMC, IGF-I and IGF-II transcripts were also progressively enhanced by 30 mmol/l glucose, whereas no significant change in IGF mRNA levels was observed in cells grown in 5.5 mmol/l glucose over the 4-week period (Fig. 2A and B). In RMC, similar changes in IGF-I mRNA levels were observed (data not shown), whereas no signal for IGF-II mRNA was detected, in keeping with previous reports in rats [21]. Iso-osmolar mannitol did not affect either IGF-I medium content (Fig. 1) or IGF-I and II mRNA levels (data not shown).

IGF receptors. Both IGF-I and IGF-II bound specifically to HMC and RMC. IGFBPs bound to cell and plastic surfaces contributed negligibly to overall IGF binding capacity, as indicated by the findings that total and specific IGF binding to cell suspensions did not differ from those observed in cell monolayers, and cold insulin displaced IGF-I binding with a K_d of 325 ± 75 nmol/l, corresponding to the reported affinity of insulin for the IGF-Ir [28, 49]. Scatchard analysis

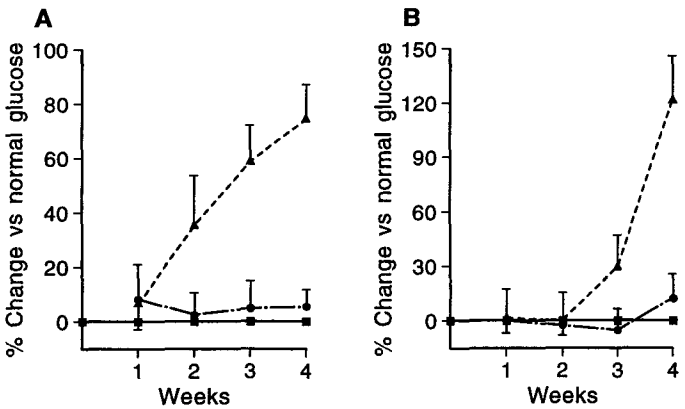

Fig. 1A, B. IGF-I production over 48 h, as assessed by RIA, in (**A**) HMC and (**B**) RMC cultured in high glucose (▲----▲) and iso-osmolar mannitol (●···●) vs normal glucose (■—■) for 4 weeks (n = 9–14 per experimental condition/time). Increases in IGF-I medium content induced by high glucose vs normal glucose were significant at weeks 2 ($p < 0.05$), 3 ($p < 0.01$), and 4 ($p < 0.005$), in HMC, and at weeks 3 ($p < 0.05$) and 4 ($p < 0.005$), in RMC

showed a linear plot, consistent with the presence of one population of receptors for either IGF-I or IGF-II (Fig. 3). The K_d was 4.76 ± 0.61 nmol/l, for IGF-I, and 5.08 ± 1.06 nmol/l, for IGF-II, and the number of sites was 774 ± 19, for IGF-I, and 288 ± 58 fmol/10^6 cells, for IGF-II, in HMC cultured under normal glucose conditions (Fig. 3). While the K_d was not affected by high glucose, the density of IGF-Ir and, in particular, of IGF-IIr was significantly increased in cells grown in 30 mmol/l glucose (to 900 ± 54, $p < 0.05$, and 558 ± 84 fmol/10^6 cells, $p < 0.01$, respectively), compared with those grown in 5.5 mmol/l glucose (Fig. 3). IGF-Ir gene expression was also progressively increased in cells grown in high glucose, but not in those grown in normal glucose (Fig. 2C). Iso-osmolar mannitol did not affect the number and affinity of IGF receptors (Fig. 3) nor IGF-Ir mRNA levels (data not shown).

IGFBPs. Several bands with molecular weights ranging from approximately 40 to 18 kDa were observed in HMC-conditioned media (Fig. 4A), whereas only 3 bands of approximately 40, 24, and 18 kDa were demonstrated in the RMC autoradiograms (Fig. 4B). The doublet-like band of 39–40 kDa was attributed to IGFBP-3, whose production was confirmed by IRMA assessment (Fig. 5). The bands comprised between 33 to 24 kDa could correspond to any of the other IGFBPs, except IGFBP-1, whose levels were below the detection limit of the assay (0.4 ng/ml) even using 40-fold concentrated samples, in keeping with the results of Grellier et al. [22] showing no IGFBP-1 mRNA expression in HMC. The bands of molecular weights below 24 kDa were considered to be the degradation products of IGFBPs [50, 51]. Exposure to high glucose progressively reduced all IGFBP bands

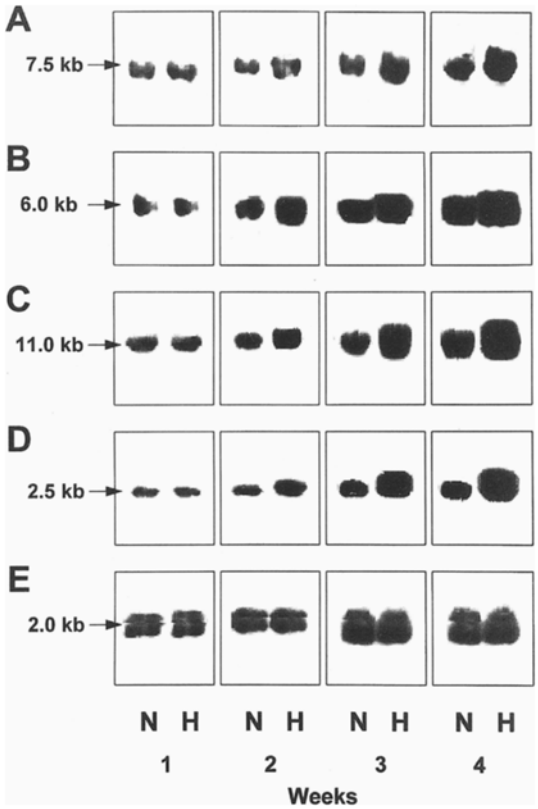

Fig. 2A–E. Steady-state mRNA levels, as assessed by Northern blot analysis, of (**A**) IGF-I, (**B**) IGF-II, (**C**) IGF-Ir, (**D**) TGF-β1 and (E) β-actin gene expression in HMC cultured in high (H) vs normal (N) glucose for 4 weeks (autoradiograms representative of three experiments). The following amounts of total RNA were loaded, for both N and H samples: 25 μg (weeks 1–2) and 35 μg (weeks 3–4) for IGF-I, IGF-II; IGF-Ir, and β-actin; and 15 μg (weeks 1–2) and 25 μg (weeks 3–4) for TGF-β1. After quantification by densitometric analysis, followed by normalization to the β-actin signal (see Fig. 7 for the β-actin signal corresponding to the TGF-β1 autoradiogram), transcripts for IGF-I in cells grown in H were found to be increased by 59, 72 and 133 %, transcripts for IGF-II by 57, 68 and 111 %, transcripts for IGF-Ir by 24, 66 and 115 %, and transcripts for TGF-β1 by 77, 117 and 164 %, at weeks 2, 3 and 4, respectively, compared with cells cultured in N. All these increases were significant at $p < 0.001$, except those in IGF-Ir mRNA at week 2 ($p < 0.05$). No significant change was detected at week 1

in HMC (Fig. 4A) and RMC (Fig. 4B) and IGFBP-3 medium content in HMC (Fig. 5), whereas culture of cells under normal glucose or iso-osmolar mannitol did not significantly affect IGFBP secretion.

TGF-β1. In HMC, TGF-β1 mRNA levels were progressively enhanced by high glucose over the 4-week period (Fig. 2D), whereas they were not significantly affected by either 5.5 mmol/l glucose (Fig. 2D) or iso-osmolar mannitol (data not shown). Similar results were obtained in RMC (data not shown).

Extracellular matrix. In HMC, ECM accumulation was progressively increased by high glucose, but not

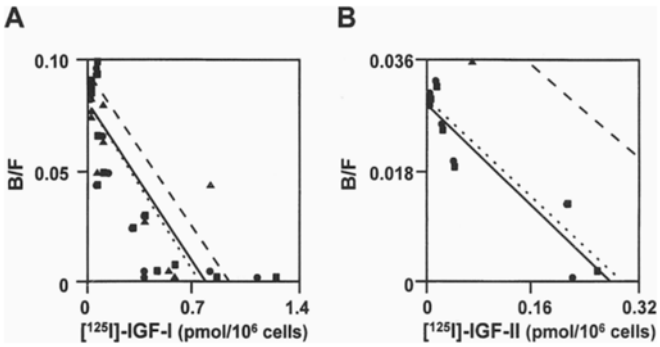

Fig. 3A, B. Scatchard analysis of (**A**) [^{125}I]-IGF-I and (**B**) [^{125}I]-IGF-II binding displaced by cold IGF-I (0–10^{-6} mol/l) and IGF-II (0–10^{-6} mol/l) + insulin (10^{-5} mol/l), respectively, to HMC cultured in high glucose (▲----▲) and iso-osmolar mannitol (●· · · ·●) vs normal glucose (■—■) for 4 weeks (n = 8 per experimental condition and peptide concentration)

by normal glucose or iso-osmolar mannitol. ECM content of high glucose media was significantly increased vs normal glucose media, starting at week 2 and persisting throughout week 4 (Fig. 6). These changes were associated with significant increases in the mRNA levels for fibronectin, laminin and collagen IV in cells grown in 30 mmol/l glucose vs those cultured in 5.5 mmol/l glucose (Fig. 7) and iso-osmolar mannitol (data not shown). In RMC, high glucose produced similar alterations in ECM protein and mRNA levels (data not shown), as reported previously [24, 52].

Cell proliferation. In HMC, the measured indices of cell proliferation were reduced by high glucose (Fig. 8). In fetal bovine serum-stimulated cells, the incorporation of ^{3}H-thymidine was reduced by 20 and 21 % (Fig. 8A), while crystal violet binding to cell nuclei decreased by 10 and 17 % (Fig. 8B) vs normal glucose, at week 1 and 4, respectively. Neither parameter was affected by iso-osmolar mannitol (Fig. 8). As previously reported [24], high glucose did not affect proliferation of cultured RMC (data not shown).

Discussion

No information was available prior to our study with respect to IGFs, IGF receptors and IGFBPs in mesangial cells exposed to elevated glucose concentrations. The novel observation that IGF-I production and IGF-Ir number and mRNA levels are increased in mesangial cells cultured in high glucose is consistent with the previous findings that IGF-I synthesis is constitutively enhanced in mesangial cells from non-obese diabetic mice [53] and that IGF-Ir (and insulin receptors) are upregulated in mesangial cells from *db/db* mice, compared with their control

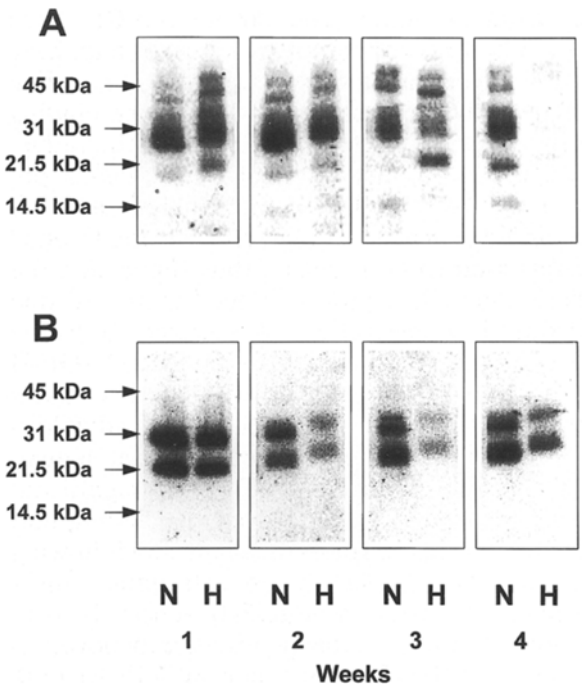

Fig. 4A, B. IGFBP production pattern, as assessed by Western ligand blot analysis, of (**A**) HMC and (**B**) RMC cultured in high (H) vs normal (N) glucose for 4 weeks (autoradiograms representative of three experiments). After quantification by densitometric analysis, IGFBP production was found to be unchanged at week 1, and reduced by 22, 36 and 57 %, in HMC, and by 19, 44 and 55 %, in RMC, at weeks 2, 3 and 4, respectively, in cells grown in H, compared with cells cultured in N. These decreases were significant at weeks 3 ($p < 0.01$) and 4 ($p < 0.001$)

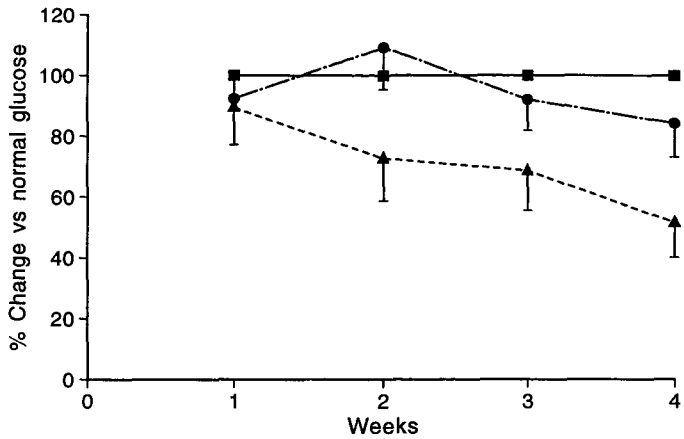

Fig. 5. IGFBP-3 production, as assessed by IRMA, in HMC cultured in high glucose (▲----▲) and iso-osmolar mannitol (●···●) vs normal glucose (■—■) for 4 weeks ($n = 6$–8 per experimental condition/time). Decreases in IGFBP-3 medium content induced by high glucose vs normal glucose were significant at weeks 3 ($p < 0.05$) and 4 ($p < 0.01$)

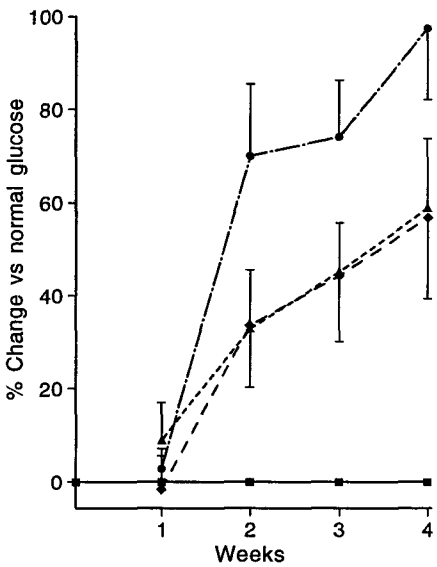

Fig. 6. Medium accumulation over 48 h of the ECM components fibronectin (●···●), laminin (◆----◆) and collagen IV (▲----▲), as assessed by ELISA, in HMC cultured in high glucose vs normal glucose for 4 weeks ($n = 9$–12 per experimental condition/time). Increases in ECM accumulation induced by high glucose vs normal glucose were significant at weeks 2–4 ($p < 0.001$ for fibronectin at all time points; $p < 0.005$, at week 2, and $p < 0.001$, at weeks 3 and 4, for laminin and collagen IV)

littermates *db/m* mice, and that their mRNA levels are further enhanced by high glucose [54].

The observation that mRNA levels for TGF-β1 are enhanced by high glucose confirms previous reports by other investigators showing increased TGF-β gene expression both in human and animal diabetes [3–7] and in mesangial cells cultured in high glucose [7, 8], associated with increased protein levels and/or biological activity [7, 8]. The trend and the extent of upregulation of TGF-β1 mRNA expression was similar to those of IGFs, thus indicating that glucose-induced changes in the production of these growth factors occur in parallel.

This is the first report of mesangial cells of human source showing a progressive enhancement of medium accumulation and gene expression of the ECM components of basement membranes in response to prolonged exposure to high glucose concentrations. This enhancement of ECM production in HMC is consistent with the increase in medium and/or cell content as well as with the enhanced gene expression of these ECM components reported by us in RMC [24, 51] and by other investigators in mesangial and other vascular cells cultured under elevated glucose concentrations [55–58].

Unlike RMC [28], HMC showed reduced DNA synthesis/cell proliferation in response to high glucose, in accordance with reports from Nahman et al. [55] in HMC and from Lorenzi et al. [59] in human endothelial cells, thus suggesting that species differences may explain this discrepancy.

The finding that iso-osmolar mannitol did not affect ECM (and growth factor) production and cell proliferation demonstrates that the effects of high

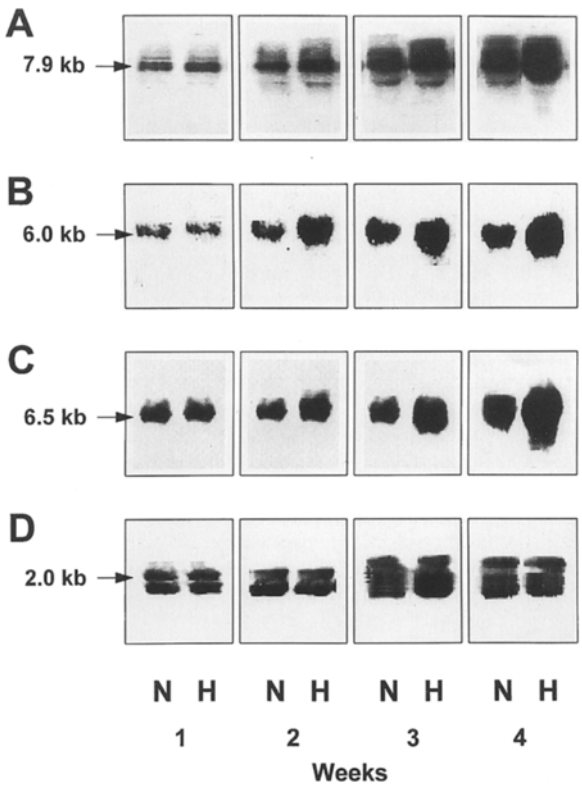

Fig. 7A–D. Steady-state mRNA levels, as assessed by Northern blot analysis, of (**A**) fibronectin, (**B**) laminin, (**C**) collagen IV and (**D**) β-actin gene expression in HMC cultured in high (H) vs normal (N) glucose for 4 weeks (autoradiograms representative of three experiments). The following amounts of total RNA were loaded for both N and H samples: 15 μg (week 1–2) and 25 μg (week 3–4). After quantification by densitometric analysis followed by normalization to the β-actin signal, transcripts for fibronectin in cells grown in H were found to be increased by 73, 93 and 148 %, transcripts for laminin by 37, 54 and 99 %, and transcripts for collagen IV by 50, 79 and 106 %, at weeks 2, 3 and 4, respectively, compared with cells cultured in N. All these increases were significant at $p < 0.001$, except those for laminin at weeks 2 ($p < 0.05$) and 3 ($p < 0.01$) and for collagen IV at week 2 ($p < 0.01$). No significant change was detected at week 1

glucose are not mediated by increased osmolarity, in keeping with our previous results in RMC [24] and with results of Cagliero et al. [58] in umbilical vein endothelial cells.

This study provides the first experimental evidence that the activity of the mesangial IGF system is enhanced in response to elevated glucose concentrations independently of GH. The overall activation of the IGF system is the consequence of complex but coordinate changes resulting in enhanced IGF action. These changes consist of increased production and/or gene expression of IGF-I and IGF-II, accompanied by increased IGF binding to monolayers and reduced IGF binding capacity of media, due to upregulation of IGF receptors and downregulation of IGFBPs, respectively. In fact, the impact of the decreased IGFBP production upon IGF action cannot be inferred from this study. The various IGFBPs have been described as either potentiating or impairing IGF action depending upon several factors, including proteolytic cleavage, dephosphorylation, and binding to cell surface or to ECM [60]. However, the reduction in IGFBP production involved all bands observed at the Western ligand blot analysis and the overall binding capacity of high glucose-conditioned media appeared to be reduced, thus supporting the hypothesis that IGF action is indeed increased due to a reduced sequestration of secreted IGFs by IGFBPs. The significance of the enhanced IGF-II gene expression in response to high glucose remains unclear since the role of this peptide in the adult kidney is uncertain, despite the evidence that human subjects continue to produce IGF-II in significant amounts after birth [21]. Moreover, the finding that IGF-IIr number was increased dramatically following exposure to elevated glucose concentrations is difficult to interpret in terms of IGF-II action. In fact, the peripheral effects of this peptide are believed to be mediated by IGF-Ir rather than by IGF-IIr [20]. Since this receptor has been involved in the degradation of IGFs [20], it may be speculated that the enhanced IGF-IIr density is a compensatory mechanism for removing the increased IGFs and eventually for binding the excess of free peptides resulting from the reduced IGFBP levels.

The proposal that IGF-I plays a role in the pathogenesis of both early and end-stage glomerular lesions occurring in diabetes has been prompted by the observations that human and experimental diabetes of recent onset is associated with renal enlargement and increased glomerular filtration rate [61], that mice transgenic for GH or GH releasing hormone develop marked glomerular hypertrophy and sclerosis with morphological features similar to those observed in diabetic glomerulosclerosis [62], and that octreotide prevents early kidney enlargement [63] and long-term-glomerular lesions and albuminuria [64] in diabetic rats. However, the report of reduced circulating levels of GH and IGF-I associated with a transient increment of renal IGF-I content in diabetic rats [9] has highlighted the importance of changes in local rather than systemic (GH-dependent) IGF-I in the pathogenesis of initial renal hypertrophy.

The parallelism between changes in the activity of mesangial IGF system and changes in ECM production observed in this study supports the hypothesis that the activation of this system, together with upregulation of TGF-β [3–8] and of other growth factors and cytokines [4], may play a role in modulating the enhanced ECM accumulation characterizing the more advanced stages of diabetes and leading to mesangial expansion. This view is further supported by the demonstration of the ability of both IGFs in stimulating matrix production [32, 65] and by the

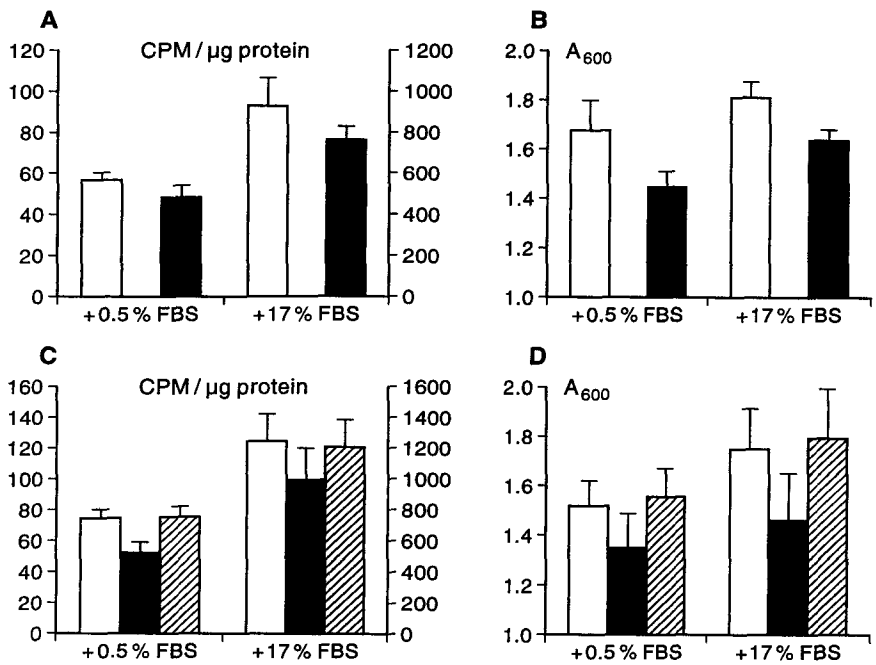

Fig. 8 A–D. ^{3}H-thymidine incorporation (A and C) and crystal violet binding to cell nuclei (B and D) in HMC cultured in high glucose (■) and iso-osmolar mannitol (▨) vs normal glucose (□) for 1 week [top] or 4 weeks [bottom], serum-deprived for 48–72 h, then exposed to 0.5 or 17 % fetal bovine serum (FBS) for 24 h (n = 8–16 per experimental condition/time). In FBS-stimulated cells, the reduction induced by high glucose vs normal glucose in ^{3}H-thymidine incorporation was significant at week 4 ($p < 0.05$), while the decrease in crystal violet binding to cell nuclei was significant at weeks 1 and 4 ($p < 0.01$)

observation that TGF-β interacts synergistically with the IGF system [66]. However, the conclusive evidence of the role of IGFs in the altered ECM turnover induced by hyperglycaemia remains to be provided. As far as the reduced cell turnover is concerned, it may be speculated that this abnormality is mainly related to the enhanced production of TGF-β, which has been previously shown to either decrease or increase mesangial cell proliferation with prevailing inhibitory action at higher doses [67], rather than to the enhanced activity of the IGF system. In fact, the stimulation of mesangial cell proliferation by IGF-I was shown to be suppressed by high glucose [68].

In conclusion, these experiments showed that high glucose conditions induce enhanced mesangial IGF activity, together with increased TGF-β1 gene expression and ECM protein and mRNA levels and with reduced cell proliferation. Based on these results, it may be speculated that changes in IGF-I and IGF-II synthesis and action participate in mediating changes in matrix turnover underlying diabetes-induced mesangial expansion.

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