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# Elevated level of ambient glucose stimulates the synthesis of high-molecular-weight hyaluronic acid by human mesangial cells. The involvement of transforming growth factor β1 and its activation by thrombospondin-1

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**The dysregulation of the metabolism of glycosaminoglycan and protein components of extracellular matrix (ECM) is a typical feature of diabetic complications. High glucose-induced enrichment of ECM with hyaluronan (HA) not only affects tissue structural integrity, but influences cell metabolic response due to the variety of effects depending on the HA polymer molecular weight. TSP-1-dependent activation of TGFβ1 axis is known to mediate numerous matrix disorders in diabetes, but its role concerning HA has not been studied so far. In this work we demonstrated that 30 mM D-glucose increased the incorporation of [$^3$H]glucosamine in high-molecular-weight (> 2000 kDa) HA of medium and matrix compartments of human mesangial cultures. Simultaneously, the synthesis of HA with lower molecular weight and HA degradation were not altered. The cause of the increased high-molecular-weight HA synthesis consisted in the up-regulation of hyaluronan synthase (HAS) 2 mRNA without alterations of the expression of HAS3, which generates HA of lower molecular weight. D-Glucose at 30 mM also stimulated the production of transforming growth factor β1 (TGFβ1), the excessive activation of which was determined by the up-regulation of thrombospondin-1 (TSP-1). The blockage of TGFβ1 action either by neutralizing anti-TGFβ1 antibodies or by quenching the TGFβ1 activation (with TSP-1-derived synthetic GGWSHW peptide) abolished the effect of high glucose on HAS2 mRNA expression and normalized the synthesis of HA. Exogenous human TGFβ1 had the same effect on HAS2 expression and HA synthesis as high glucose treatment. Therefore, we supposed that TSP-1-dependent TGFβ1 activation is involved in the observed high glucose effect on HA metabolism. Since high-molecular-weight HA polymers, unlike middle- and low-molecular weight HA oligosaccharides, are known to possess anti-inflammatory and anti-fibrotic functions, we suppose that the enrichment of mesangial matrix with high-molecular-weight HA may represent an endogenous mechanism to limit renal injury in diabetes.**

**Keywords**: high glucose, hyaluronic acid, mesangial cells, transforming growth factor β1, thrombospondin-1

## INTRODUCTION

Expansion of the glomerular mesangial matrix is a typical feature of diabetic nephropathy, and the elevated blood glucose level is considered to be the main cause of this phenomenon. Numerous *in vivo* and *in vitro* investigations indicated that high ambient glucose level induces dysregulation of the metabolism of the extracellular matrix (ECM) and excessive protein accumulation (McLennan *et al.*, 1999).

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**Abbreviations**: ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hyaluronic acid; HAS, hyaluronan synthase; HMCs, human mesangial cells; HMW, high molecular weight; ITS, insulin, transferrin, sodium selenite; LMW, low molecular weight; MMW, middle molecular weight; PTC, proximal tubular epithelial; TGFβ1, transforming growth factor β1; TSP-1, thrombospondin-1; W-peptide, synthetic GGWSHW peptide; Y-peptide, synthetic GGYSHW peptide.

Besides, it has been shown that elevated level of ambient glucose modulates also the metabolism of non-protein components of mesangial matrix, and, in particular, stimulates the accumulation of hyaluronic acid (HA). Previous works demonstrated increased HA content in glomeruli of diabetic animals (Mahadevan *et al.,* 1995), in high glucose-treated rat mesangial cells (Dunlop *et al.,* 1996; Wang & Hascall, 2004) and in other cell types responsible for normal physiology of kidney (Jones *et al.,* 2001; Takeda *et al.,* 2001). It seems that the effect of elevated glucose level on HA metabolism in mesangial cells has been studied insufficiently, especially taking into account not only the HA role in tissue structural integrity, but its various metabolic effects, which are determined by the polymer molecular weight (Noble, 2002).

In almost all kidney cell types, and in mesangial cells, in particular, high glucose up-regulates the expression and activity of transforming growth factor-β1 (TGFβ1). It is known that virtually all mediators and signaling pathways that have been shown in diabetic nephropathy have also been identified as stimulators of TGFβ1 up-regulation. The blocking of TGFβ1 action by various approaches evidently attenuates the high glucose-induced increase of matrix expression, demonstrating that TGFβ1 is the major mediator of this pathological phenomenon (Ziyadeh, 2004).

The implication of TGFβ1 in high glucose-induced alterations of HA metabolism has been demonstrated only for renal intestinal fibroblasts (Takeda *et al.,* 2001), and Wang and Hascall (2004) gave a hint at a possible participation of TGFβ1 in increased HA production by rat mesangial cells treated with high glucose. However, TGFβ1 is known modulator of HA metabolism in various cell types (Heldin *et al.,* 1989; Haubeck *et al.,* 1995; Breborowich *et al.,* 1996; Ellis & Schor, 1998; Sugiyama *et al.,* 1998; Usui *et al.,* 2000; Reckleis *et al.,* 2001), hence the participation of TGFβ1 in high glucose-induced increase of HA production by mesangial cells seems to be likely.

And, finally, TGFβ1 is secreted in a latent form by virtually all cells, and the changes in its expression will have no biological effect unless mechanisms of its activation are operational (Hugo, 2003). TGFβ1 may be activated in various ways, including proteolysis of the complex which determines latency. However, at high glucose conditions the likelihood of this way of activation is reduced because of the reduced activity of the main enzymes involved in TGFβ1 activation.

As to mesangial cells, it was demonstrated that in high-glucose conditions the activation of TGFβ1 (and, therefore, the accumulation of matrix proteins) is realized by the action of endogenous thrombospondin-1 (TSP-1), the production of which is increased at high glucose conditions (Poczatek *et al.,* 2000; Yevdokimova *et al.,* 2001). Moreover, TSP-1 is believed to be the main physiological activator of endogenous TGFβ in various cells and tissues (Crawford *et al.,* 1998; Hugo, 2003). However, the role of TSP-1-dependent TGFβ1 activation with respect to HA metabolism has not been studied.

Thus, it was of interest to ascertain that an elevated level of ambient glucose stimulates the production of HA by human mesangial cells and, in case of positive results, to verify the supposed participation of TGFβ1 and its activation by TSP-1 in the mediating of high glucose effect on HA metabolism.

To this aim we (a) investigated the incorporation of a radioactive precursor of HA into medium, matrix and cell-associated compartments of mesangial cultures, treated with elevated glucose; (b) studied the molecular size distribution of labeled HA in all compartments, and compared the capacity of mesangial cells to degrade exogenously added HA at normal and elevate glucose levels; (c) determined the level of TGFβ1 secretion and activation, and the secretion of TSP-1 at high glucose conditions; (d) investigated the effects of a TGFβ1 neutralizing antibody, synthetic TSP-derived GGWSHW peptide (which is known to stop TSP-dependent TGFβ1 activation), and exogenous TGFβ1 on HA metabolism; (g) determined the expression of mRNA for hyaluronan synthases (HAS1, HAS2 and HAS3) in human mesangial cells and studied the effect of high glucose, GGWSHW peptide and exogenous TGFβ1 on mRNA expression of these enzymes.

## MATERIALS AND METHODS

**Materials.** Primary normal human mesangial cells (HMC; CC-2259, lot 8F1498) were purchased from BioWhittaker (Walkersville, MD, USA). Fetal calf serum (FCS) was from Gibco BRL (Paisley, UK). RPMI 1640 medium without D-glucose, EMEM and DMEM medium with Glutamax, ITS (insulin, transferrin, sodium selenite), non-essential amino acids, antibiotics, glutamine, D-glucose, bovine serum albumin, trypsin, soybean trypsin inhibitor, heparitinase I, hyaluronate lyase from *Streptomyces hyalurolyticus*, pronase E, monoclonal mouse anti-TSP-antibody, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), blue dextran calibration standard and decorin were from Sigma-Aldrich (Dorset, UK, and Schnelldorf, Germany). Monoclonal mouse anti-TGFβ1 antibody, recognizing human TGFβ1 and TGFβ2, were from Genzyme (Minneapolis, MN, USA). Hyaluronic acid-K-salt was from Fluka Chemical Corp. (Milwaukee, WI, USA), HEALON GV from Pharmacia & Upjohn (Stock-

holm, Sweden), DEAE-cellulose was from Serva (Heidelberg, Germany). Sephacryl S-1000 and D-[1-$^3$H]glucosamine (7.20 Ci/mmol sp. act.) were from Amersham Pharmacia Biotech (Uppsala, Sweden). Human TGFβ1 and TGFβ1 determination immunoassay kit were from R&D Systems (Minneapolis, MN, USA). Human platelet TSP-1 was from Calbiochem-Novabiochem (La Jolla, CA, USA). Secondary peroxidase-conjugated rabbit anti-mouse antibody was from DAKO (High Wycombe, UK). RNAzolB was from AMS Biotechnology (Oxfordshire, UK). RT-PCR kits and 1-kb DNA ladder were supplied by Life Technologies (Gibco BRL, Paisley, UK) and primers by PE-Applied Biosystems (Cheshire, UK). Both the GGWSHW peptide and the control GGYSHW peptide were synthesized in the Advanced Biotechnology Center of Imperial College (London, UK). All other chemicals used were of the highest quality available.

**Cell culture.** Primary human mesangial cells (HMCs) were maintained at 37°C with 5% $CO_2$/95% air in RPMI 1640 medium, containing 4 mM glucose, 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 1 μg/ml amphotericin B, and ITS (insulin, transferrin and sodium selenite at 5 μg/ml, 5 μg/ml, 5 ng/ml, respectively,). Cells were routinely passaged with 1:4 split and were used for experiments at the 7th to 9th passages.

Mink lung epithelial (MvILu) cells were grown in DMEM with Glutamax, supplemented with 10% FCS and antibiotics (as above). MvILu cells were passaged with 1:10 split at 80% confluence.

Human dermal fibroblast culture was initiated in the laboratory by the explant method. The secondary culture was maintained in EMEM supplemented with 10% FCS, 2 mM glutamine, antibiotics (as above), 1% non-essential aminoacids, and subcultured (1:6) every 5–6 days.

**Metabolic labeling and analysis of HA.** The determination of [$^3$H]glucosamine incorporation into HA was carried out as described (Yevdokimova, 2003) Briefly, at the end of incubation, the conditioned medium was aspirated. Cell layers were washed; the wash and medium were combined and designated as a medium (secreted) fraction. Cell layers were processed with 0.025% trypsin/0.01% EDTA, the cells were resuspended and aspirated, the flask was washed with 0.05% trypsin inhibitor, and the cell suspension was combined with wash liquid and centrifuged. The supernatant (trypsin-released material) was designated as a pericellular matrix fraction, whereas the pellet was designated as a cell-associated one. All fractions were digested with pronase E (1.5 mg/ml, 18 h, 50°C), dialyzed, and applied onto DEAE-cellulose columns. Elution was carried out with 0.10 M and 0.28 M NaCl, as described previously (Yevdokimova & Freshney, 1997). The material eluted with 0.10 M NaCl, is the residual glycoproteins, and they were discarded. HA was eluted with 0.28 M NaCl, precipitated with ethanol (the final concentration 75% v/v) and dissolved in water. Aliquots were used for the determination of tritium radioactivity. The identification of [$^3$H]HA was carried out by enzyme degradation of the material after its desalting and concentration on a Centriprep-3 with consequent gel-filtration on Sephadex G-50 and radioactive determination. Material sensitive to hyaluronate lyase (EC 4.2.2.1) and insensitive to heparitinase I (EC 4.2.2.8) was designated as HA. Samples with [$^3$H]HA material were combined, precipitated with ethanol, dissolved in 0.2 ml 100 mM Na-acetate, 10 mM NaCl, 0.025% CHAPS, pH 6.5, and used for the analysis of molecular size distribution. Two samples with similar material ([$^3$H]HA of medium, matrix or cell-associated fractions from cultures treated with 4 mM or 30 mM glucose) were combined and chromatographed on Sephacryl S-1000 column (1 x 30 cm). Elution was carried out at 12 ml/h with the same buffer, and fractions of 0.5 ml were collected. In the initial experiments the material of all fractions was verified with hyaluronate lyase and heparitinase I digestion. The column was calibrated with (a) Healon GV (7000 kDa), (b) blue dextran (2000 kDa), (c) decorin (100 kDa) and (d) [$^3$H]glucosamine hydrochloride (215 Da).

**Degradation of exogenous [$^3$H]HA by HMCs.** Human skin fibroblasts were used to prepare [$^3$H]HA which was then added to HMC cultures. Fibroblasts were grown to confluence in T75 flasks and incubated with [$^3$H]glucosamine for 72 h in serum-free EMEM. The conditioned medium was removed, [$^3$H]HA was isolated and analyzed, as above. After Sephacryl S-1000 chromatography, HA was pooled according to its radioactive profile. The fractions corresponding to high-molecular-weight (HMW) HA (>2000 kDa) were dialyzed repeatedly against serum-free RPMI 1640 medium. Three milliliters of RPMI 1640 medium, supplemented with 4 or 30 mM glucose, containing approx. 250 000 d.p.m. of [$^3$H]HA were added to HMCs, which had been incubated with 4 or 30 mM glucose, respectively. After 24 h or 72 h the conditioned medium fraction was removed and analyzed, as above.

**Determination of secreted TSP-1, and total and active TGFβ1.** At the end of incubation period the conditioned medium was collected under sterile conditions and centrifuged (3000 × *g*, 10 min). To the samples, assigned to the determination of TSP-1, protease inhibitors were added to final concentrations of 10 mM aminohexanoic acid, 1 mM EDTA, 5 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride, and 1 mM *N*-ethylmaleimide. Samples were stored at –70°C before assay. The determination of TSP-1 was performed by ELISA, as described previ-

ously (Yevdokimova *et al.*, 2001). The optimal dilution of primary and secondary antibodies was found to be 1:1000. The bound antibodies were detected with 2,2′-azinobis-3-ethylbenzthazoline-6-sulphonic acid at 405 nm.

Total and active TGFβ1 levels were determined using the MvILu cell growth inhibition assay as described (Yevdokimova *et al.*, 2001). MvILu cells were seeded at 7–10 × $10^4$ cells/ml in 96-well plates in DMEM with 10% FCS. After 2–3 h the plates were washed, and HMCs-conditioned medium (100 μl) and DMEM with 10% FCS (100 μl) were added to the wells. To measure total TGFβ1, conditioned medium samples were heat-activated for 10 min at 80°C. After 3 days the number of cells was determined by sulphorodamine B assay. A standard curve was constructed for 1 × $10^4$–30 × $10^4$ cells. A further standard curve was set up for each assay with 0.01 to 1 ng/ml of human platelet TGFβ1. Specificity of the assay was proven by ELISA determination of TGFβ1.

**RT-PCR.** Cell layers (4.5–5 × $10^5$ cells) were washed extensively with phosphate-buffered saline at 4°C and solubilized in RNAzolB. Total RNA were extracted, dissolved in diethyl pyrocarbonate-treated water, and stored at –70°C before assay. RNA was quantified by the absorbance at 260 and 280 nm. The samples showed $A_{260}/A_{280} \geq 1.8$.

Equal amounts (2 μg) of DNA-free total RNA from each sample were converted to cDNA by SuperScriptTM II RNase H-reverse transcriptase with random primers in a 20 μl reaction volume. The reverse transcription reaction (0.5 μl) was subjected to PCR amplification, using 2.5 U of *Taq* DNA polymerase in 100 μl reaction volume with 0.5 μM of each dNTP, 0.5 μM of each specific primer, and 1.5 mM $MgCl_2$.

The sequences of the primers were: HAS1 (497 bp): 5′-GCG GGC TTG TCA GAG CTA-3′ — forward, 5′-AGA GCG AGA GAA GCA CCA-3′ — reverse; HAS2 (398 bp): 5′-GTG ATG ACA GGC ATC TCA-3′ — forward, 5′-GCG GGA AGT AAA CTC GA-3′ — reverse; HAS3 (450 bp): 5′-CAG CCT GCA CCA TCG A-3′ — forward, 5′-AGA GGT GGT GCT TAT GGA-3′ — reverse (Stuhlmeier & Pollaschek, 2004). We co-amplified the house-keeping gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to allow semi-quantificative comparison of PCR products and to confirm the equal usage of mRNA. GAPDH (195 bp): 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′ — forward, 5′-TTC TTG GAG GCC ATG TGG GCC AT-3′ — reverse.

The amount of reverse-transcribed cDNA (0.5 μl) used for amplification was determined to be nonsaturating for the PCR product of all genes under investigation. Amplification was started with 3 min of denaturation at 94°C followed by 33 PCR cycles. Each cycle consisted of 60 s at 94°C, 60 s at 62°C and 60 s at 72°C. The final extension was for 10 min at 72°C. 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 photographed, scanned and analyzed with Image software (NIH Image). The results were normalized to the intensity of the GAPDH bands.

**Statistical analysis.** Results were compared using unpaired *t*-test. *P* values of <0.05 were regarded as significant.

## RESULTS

For the experiments HMCs cells were plated at 4–5 × $10^3$ cells/$cm^2$ in T25 flasks and grown to confluence (7–8 days). Confluent cultures of HMC were maintained at 4 mM, 30 mM glucose or 4 mM glucose + 26 mM mannitol (osmotic control) for 48 h. For the first 24 h the medium was supplemented with 10% FCS, and then it was changed to a serum free one, supplemented with 500 ng/ml BSA and 10 μCi/ml [$^3$H]glucosamine for the next 24 h.

The cell density in confluent cultures of HMC maintained at different conditions showed no change over the incubation period and was 1.94 ± 0.06 × $10^4$ cells/$cm^2$ (n = 5) at the beginning of the experiment, 1.97 ± 0.08 × $10^4$ cells/$cm^2$ (n = 12), 2.01 ± 0.05 × $10^4$ cells/$cm^2$ (n = 12) and 1.99 ± 0.09 × $10^4$ cells/$cm^2$ (n = 6) after 48 h incubation at 4 mM, 30 mM glucose or 4 mM glucose + 26 mM mannitol, respectively.

We investigated first whether the increased level of glucose modulates the incorporation of $^3$H-glucosamine in HA of the medium, matrix and cell-associated compartments of HMC cultures. It is seen

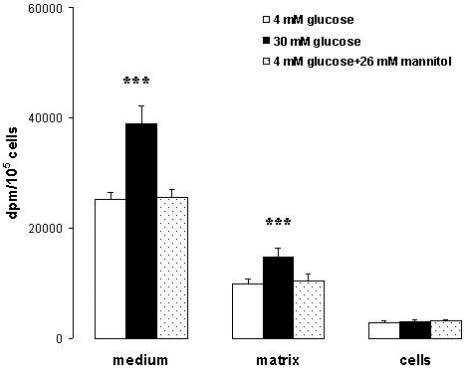


**Figure 1. Incorporation of [$^3$H]glucosamine in hyaluronic acid (HA) of medium, matrix and cell layer compartments of HMC cultures.**
Cells were metabolically labeled with 10 μCi/ml D-[1-$^3$H]glucosamine for 24 h in serum-deprived RPMI 1640 medium, supplemented with 4 mM glucose, 30 mM glucose or 4 mM glucose + 26 mM mannitol. The isolation of [$^3$H]HA is described under Materials and Methods. Data are expressed as mean ± S.E. of 3 independent experiments in triplicate. *** Significant at $P < 0.01$.

(Fig. 1) that the incorporation of [$^3$H]glucosamine was increased (about 1.5-fold) both in the medium and matrix HA of cultures treated with 30 mM glucose in comparison to control ones. No difference was observed in the cell-associated compartment. Cells treated with 4 mM glucose + 26 mM mannitol did not differ from control cells by all indices.

The analysis of [$^3$H]HA molecular size distribution indicated that the increase of HA accumulation in the medium (Fig. 2A) and matrix (Fig. 2B) compartments was caused only by the augmentation of high-molecular-weight (HMW) fraction (>2000 kDa). This fraction of the HA molecules increased from 73.7 ± 2.3% (n = 8) to 85.7 ± 3.0% (n = 8), $P<0.02$, in the medium and from 71.6 ± 2.7% (n = 8) to 83.0 ± 2.7% (n = 8), $P<0.02$) in the matrix, under the action of 30 mM glucose. The levels of middle (between 2000 and 100 kDa, MMW) and low (<100 kDa, LMW) molecular weight HA were not changed.

In the cell-associated compartment of HMC cultures we did not detect any alterations of molecular size distribution (Fig. 2C), the HMW fraction being about 32.5%, the MMW fraction about 11.2% and the LMW fraction about 56.3%, both at 4 mM and 30 mM glucose. Such a distribution of HA molecules with different molecular weights in medium, matrix and cell-associated compartments of HMC cultures could hardly has been caused by alterations of HA degradation under high glucose treatment.

To confirm this suggestion we investigated the functional capacity of HMC cells to degrade exogenously added HMW [$^3$H]HA at 4 mM and 30 mM glucose. There was no difference between the elution profiles of exogenous [$^3$H]HA in the medium of HMC cultures treated or not with 30 mM glucose after 24 h (Fig. 3A) or 72 h (Fig. 3B) of incubation. Thus, the degradation of exogenous HA by HMC cells was the same at 4 mM and 30 mM glucose.

In order to clarify the role of TGFβ1 and its activation by TSP-1 in the high glucose-induced alterations of HA synthesis by HMCs we did the following. At first, we detected the level of total and active TGFβ1 and TSP-1 concentration in the conditioned

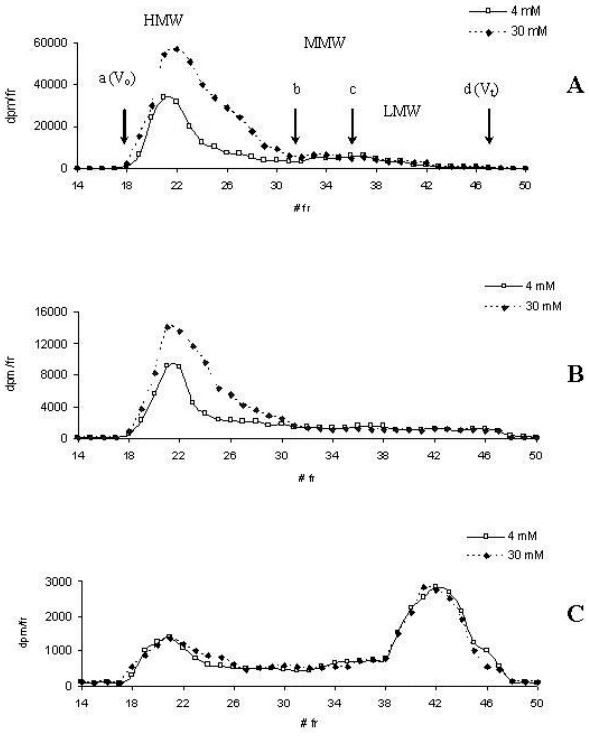


**Figure 2. A typical example of molecular size distribution of [$^3$H]HA in the different compartments of HMC cultures treated with 30 mM glucose.**
Cells were incubated and labeled as in Fig. 1 [$^3$H]HA was isolated from medium (panel A), matrix (panel B) and cell layer (panel C) compartments of HMC cultures and chromatographed on a 1 × 30-cm Sephacryl S-1000 column. Elution was carried out as described under Materials and Methods. Arrows mark the positions of the compounds used for column calibration: a (Healon GV, 7000 kDa) at fraction 18, b (blue dextran, 2000 kDa) at fraction 31, c (decorin, 100 kDa) at fraction 36, and d ([$^3$H]glucosamine hydrochloride, 215 Da) at fraction 47. Three arbitrary size brackets of HA are indicated, high molecular weight (HMW), >2000 kDa; middle (MMW), 100–2000 kDa; and low (LMW), <100 kDa.

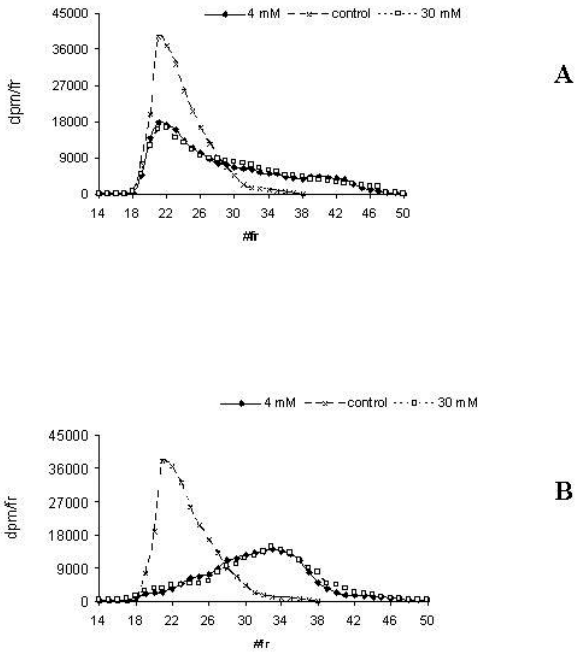


**Figure 3. Incubation of exogenous [$^3$H]HA with HMC cultures.**
Fibroblast-generated HMW [$^3$H]HA (about 250 000 d.p.m.) was added to HMC cultures, which were then incubated in serum-deprived medium at 4 mM or 30 mM glucose for 24 h (panel A) or 72 h (panel B). The HA size distribution in the medium fraction was examined by Sephacryl S-1000 chromatography as described under Materials and Methods. The data were corrected for cell number. The elution profile of the original HMW [$^3$H]HA, which was incubated without cells, is demonstrated for comparison.

**Table 1. Concentrations of total and active TGFβ1 and TSP-1 (ng/ml per $10^4$ cells) in the conditioned medium of human mesangial cells (HMC)**

| | 4 mM glucose | 30 mM glucose | 30 mM glucose + W-peptide | 30 mM glucose + Y-peptide |
|---|---|---|---|---|
| Total TGFβ1 | 1.55 ± 0.06 (n = 6) | 2.21 ± 0.21** (n = 6) | 2.27 ± 0.23** (n = 4) | 2.20 ± 0.19** (n = 4) |
| Active TGFβ1 | 0.074 ± 0.006 (n = 6) | 0.152 ± 0.14** (n = 6) | 0.082 ± 0.008 (n = 4) | 0.159 ± 0.015*** (n = 4) |
| TSP-1 | 6.24 ± 0.61 (n = 6) | 10.01 ± 1.01*** (n = 6) | 11.12 ± 1.11*** (n = 4) | 10.49 ± 1.11** (n = 4) |

HMCs were incubated under 4 mM or 30 mM glucose and under 30 mM glucose + 1 μM synthetic TSP-derived GGWSHW peptide (W-peptide) or GGYSHW peptide (Y-peptide) in RPMI 1640 medium, containing 10% FCS. After 24 h the medium was changed to a serum-free one with the corresponding amounts of glucose and peptides. In 24 h of additional incubation the levels of total and active TGFβ1 were determined by the MvILu cell growth inhibition assay and TSP-1 level was determined by ELISA, as described in Materials and Methods. Data are expressed as means ± S.E. *** Significant at $P<0.01$, ** significant at $P<0.02$, as compared to 4 mM glucose.

medium of HMC cultures under 30 mM glucose. It is seen (Table 1) that a 48 h incubation of HMCs increased the level of total (1.43-fold) and active (2.05-fold) TGFβ1, and the level of TSP-1 (1.6-fold), and corresponded to the results obtained earlier with a more prolonged high glucose treatment (Yevdokimova *et al.*, 2001). Then, we demonstrated that the blockage of TGFβ1 action with an anti-TGFβ1 neutralizing antibody (30 μg/ml) abolished the high glucose-induced alterations of [$^3$H]glucosamine incorporation in medium (Fig. 4A) and matrix HA (Fig. 4B), whereas non-specific IgG was ineffective. We observed also the normalization of the molecular size distribution of medium and matrix [$^3$H]HA with anti-TGFβ1 neutralizing antibody (not shown).

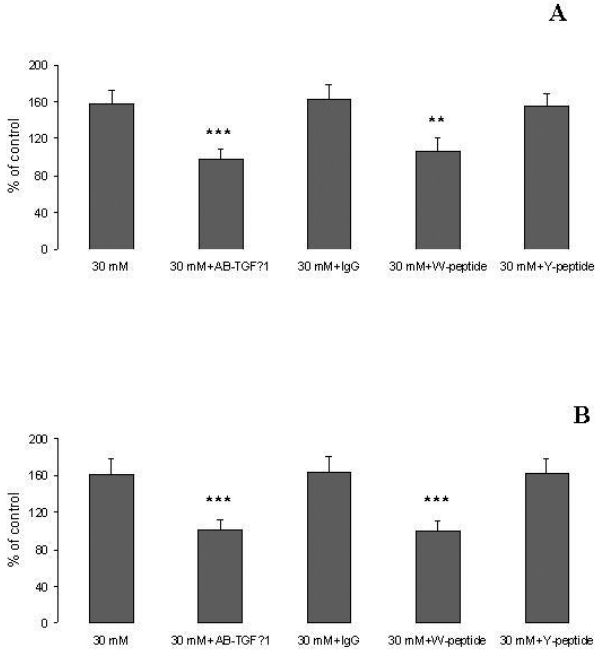


**Figure 4. Effect of the blockage of endogenous TGFβ1 action on the generation of [$^3$H]HA by high glucose-treated HMC cultures.**
The incubation medium, apart from 30 mM glucose, contained: (i) 30 μg/ml of anti-TGFβ1 neutralizing antibody (AB-TGFβ1) or non-specific IgG or (ii) 1.0 μM of the synthetic TSP-derived GGWSHW peptide (W-peptide) or GGYSHW peptide (Y-peptide). The labeling and isolation of [$^3$H]HA from medium (panel A) and matrix (panel B) compartments of HMC cultures were done as described under Materials and Methods. Data represent percentage of the control (4 mM glucose) for each sample and are expressed as means ± S.E. from 3 independent experiments in triplicate. *** Significant at $P < 0.01$, ** significant at $P < 0.02$, as compared to 30 mM glucose.

We investigated next whether the increased production of HA by HMC cultures treated with high glucose was dependent on TSP-1 activation of TGFβ1. The classic mechanism of TGFβ1 activation (Schultz-Cherry *et al.*, 1995) consists in a two-step interaction of TSP-1 molecule with the latent TGFβ1 complex leading to the liberation of active cytokine. The initial step of this process is the binding of the specific amino-acid sequence GGWSHW of the TSP-1 molecule to the mature part of latent TGFβ1 complex. An excess of synthetic GGWSHW peptide (W-peptide) blocks TGFβ1 activation, whereas, synthetic GGYSHW (Y-peptide) is ineffective, and may serve as a control. In a previous work (Yevdokimova *et al.*, 2001) we used this approach, established the optimal concentration of W-peptide (1.0 μM), observed the abolishment of the excessive high glucose-induced activation of TGFβ1, and the normalization of excessive production of ECM proteins by HMC cultures treated with high glucose. The level of total TGFβ1 and TSP-1 did not alter under W-peptide treatment in all the studied range of concentrations (0.01–10 μM). In the present work, we confirmed the rationality of this approach, and demonstrated that the employment of 1.0 μM W-peptide normalized the increased [$^3$H]glucosamine incorporation in medium (Fig. 4A) and matrix (Fig. 4B) HA due to the blockage of TSP-1-dependent TGFβ1 activation (Table 1).

For further verification of the possible role of TGFβ1 in mediating the high glucose effect on HA metabolism we used the incubation of control HMCs with exogenous human TGFβ1. Figure 5A demonstrates the dose dependence of [$^3$H]glucosamine incorporation in matrix HA. Based on these data, we chose 4.0 ng/ml for further work. It is seen that ex-

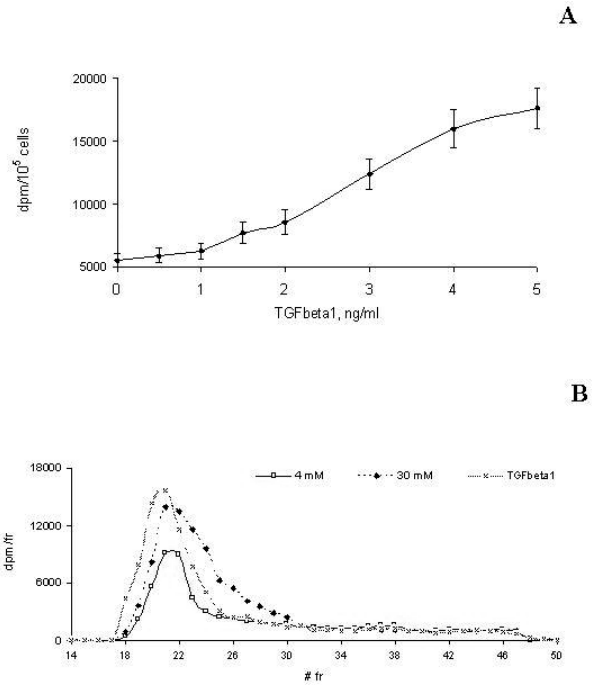


**Figure 5. Effect of exogenous human platelet TGFβ1 on the accumulation and molecular size distribution of [$^3$H]HA in the matrix compartment of HMC cultures.**
**Panel A.** Cells were exposed to increasing concentration of TGFβ1 (0–5 ng/ml) for 48 h. The isolation of [$^3$H]HA from matrix fraction was carried out as described in Materials and Methods. Data are expressed as means ± S.E. of 2 experiments in triplicate. **Panel B**. The [$^3$H]HA was isolated from the matrix compartment of HMC cultures treated with 4 ng/ml TGFβ1 and loaded on Sephacryl S-1000 column. The elution profiles of [$^3$H]HA from control and high glucose-treated cultures are demonstrated for the comparison. The chromatography was carried out as described under Materials and Methods.

ogenous human TGFβ1 increased the incorporation of [$^3$H]glucosamine in matrix HA and altered its molecular size distribution (Fig. 5B), resembling the effect of high glucose. TGFβ1 increased the HMW part of HA molecules to 84.6±4.5% (n=4), not influencing the amount of MMW and LMW HA. A similar elution profile was observed for the chromatography of medium HA, whereas TGFβ1 did not alter the [$^3$H]glucosamine incorporation in the cell-associated fraction of HMC cultures (not shown). Hence, both high glucose and TGFβ1 not only stimulated the HA production, but altered the molecular size distribution, increasing the percentage of HMW molecules in the medium and matrix fraction. This confirmed the likely participation of TGFβ1 in the mediating of high glucose effect on HA metabolism.

To determine if a specific HAS isoform is involved in the high glucose-induced HA synthesis we investigated the expression of mRNAs of HAS1, HAS2 and HAS3 by semi-quantitative RT-PCR. Specific PCR products for HAS2, HAS3 and GAPDH were detected following 28 cycles, whereas a HAS1

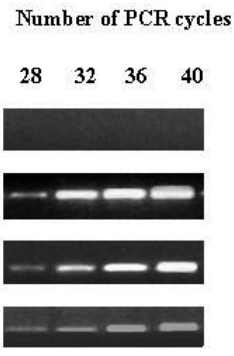


**Figure 6. A typical example of PCR cycle-dependent amount of RT-PCR products of HAS1, HAS2, HAS3 and GAPDH mRNAs.**
The description of RT-PCR experiments is under Materials and Methods.

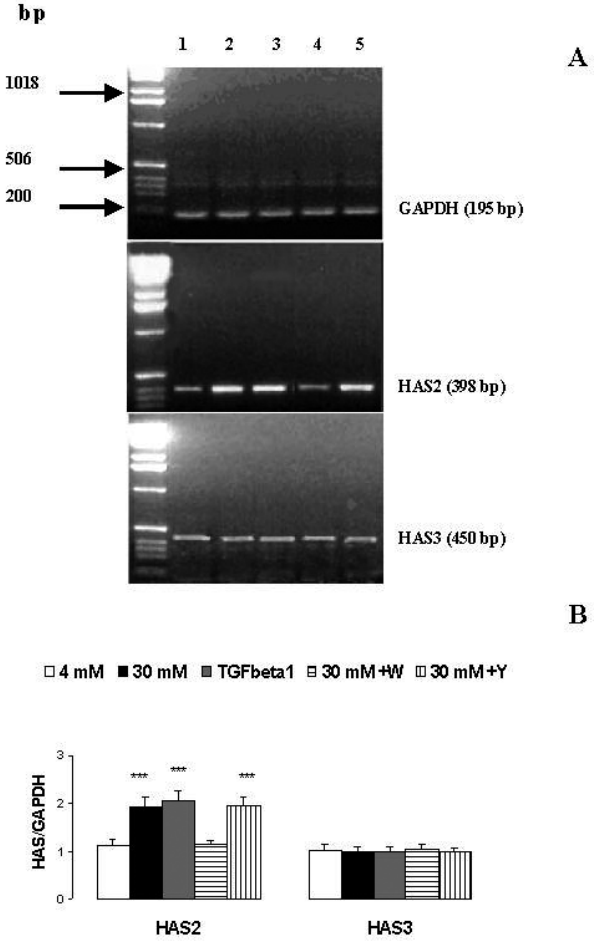


**Figure 7. Expression of GAPDH, HAS2 and HAS3 mRNAs of HMC cultures measured by RT-PCR.**
**Panel A.** An example of RT-PCR product electrophoresis: lane **1,** 4 mM glucose; lane **2,** 30 mM glucose; lane **3,** exogenous TGFβ1; lane **4,** 30 mM glucose + W-peptide; lane **5,** 30 mM glucose + Y-peptide. The description of HMCs incubation and RT-PCR experiments are under Materials and Methods. **Panel B.** The ratio of the fluorescence of HAS2 and HAS3 bands to the fluorescence of GAPDH bands at different experimental conditions. Data are expressed as means ±S.E. of 3 independent experiments in duplicate. ***Significant at $P<0.01$ compared to 4 mM glucose.

PCR product could not be detected even after 40 cycles. The expression of HAS1 mRNA has previously been demonstrated in human fibroblast-like synoviocytes by Stuhlmeier and Pollaschek (2004) and in human dermal fibroblasts by us (not shown) to confirm the efficacy of the primers used.

The linearity of amplification of PCR products for HAS2, HAS3 and GAPDH was observed throughout 28–40 cycles (Fig. 6). We used 33 cycles for further investigations, terminating the reaction in an early log-phase of product accumulation.

The expression of HAS2 mRNA was increased in cells exposed to 30 mM glucose and to exogenous human TGFβ1, comparatively to control cells (Fig. 7A). A densitometric analysis of HAS2 bands, corrected for GAPDH bands (Fig. 7B), showed an increase of 1.72-fold and of 1.82-fold, for 30 mM glucose and TGFβ1, respectively. The addition of the blocking peptide W to high glucose medium abolished the up-regulation of HAS2 mRNA expression, whereas the control peptide Y was ineffective. The expression of HAS3 mRNA did not change under either of the treatments used.

## DISCUSSION

HA is a linear polymer of glucuronic acid and *N*-acetylglucosamine disaccharide with alternating β-1,4 and β-1,3 glycosidic bonds (Laurent & Fraser, 1992). HA is synthesized by the HA synthases (HAS1, HAS2 and HAS3) at the inner side of the plasma membrane, and directly secreted into the extracellular space. It is composed, on average, of 10000–30000 disaccharide units, with a molecular mass about 4000–10000 kDa. HAS1 and HAS2 produce HA chains of a similar large size (>2000 kDa), whereas HAS3 synthesizes shorter chains (100–300 kDa) (Tammi *et al.,* 2001; Noble, 2002). The expression of each of these enzymes is cell-type-specific, and each enzyme is regulated differentially in response to external stimuli (Jacobson *et al.,* 2000). HA is cleaved into fragments of low and intermediate molecular weight by specific hyaluronidases, or *via* the action of reactive oxygen species. The regulation of hyaluronidase activity and the expression of their various isoforms is poorly understood, although it is one of the most important points in the HA role for tissue homeostasis, because HA degradation products have biological functions distinct from those of the native HMW polymer (Noble, 2002).

Some HA functions are connected to its role as an integral and central part of ECM of various tissues. Its unique physicochemical properties are involved in the regulation of cell behavior due to the ability of influencing the cell environment *via* the interaction with cells and other components of ECM. Besides, HA and its oligosaccharides may directly affect cell function through binding to its receptor (CD44) and altering specific gene expression.

At present, it is obvious that HA is essential for tissue homeostasis (Tammi *et al.,* 2001) and for kidney in particular. Nevertheless, although the implication of HA in the regulation of protein and water balance in the kidney was detected already in the fifties (Ginetzinsky, 1958) and many recent works (Wells *et al.,* 1993; Hascall *et al.,* 2004; Asselman *et al.,* 2005) demonstrated an association between HA metabolism and renal injury diseases, the role of this link is not completely clear yet. As to the development of diabetic nephropathy, the comprehension of HA importance began, possibly, from the observation about increased HA synthesis within the glomeruli of diabetic rat kidney (Mahadevan *et al.,* 1995).

In the present work we demonstrated that high glucose stimulates the accumulation of HA in the medium and matrix compartments of HMC cultures without any alterations in the cell-associated compartment. The increase of [$^3$H]glucosamine incorporation in medium and matrix HA was the same, and was caused only by the incorporation in high-molecular-weight molecules, hence, the redistribution of HA molecules between medium and matrix did not take place. Besides, the ability of mesangial cells to degrade exogenous HA did not depend on the glucose concentration in the medium. Therefore, we consider that high glucose did not stimulate HA degradation in human mesangial cells. This result is in line with the observed formation of pericellular HA cable-like structures in high glucose-induced rat mesangial cells (Wang & Hascall, 2004). An increased synthesis of HA due to high glucose was demonstrated also in another work dealing with rat mesangial cells (Dunlop *et al.,* 1996), for primary cultures of human renal proximal tubular epithelial cells (PTC) and an immortal cell line HK-2 obtained from PTCs (Jones *et al.,* 2001), and for a normal rat kidney fibroblast cell line (Takeda *et al.,* 2001).

This effect is D-glucose-specific and not due to an osmotic effect, as the replacement of D-glucose by mannitol (in our investigations) or L-glucose does not cause a similar response.

According to our data, the cause of the increased production of HMW HA consisted in the up-regulation of HAS2 mRNA expression without alterations of the mRNA for HAS3, which produces HA of lower molecular weight. These data are in agreement with the response of PTCs and HK-2 cells to elevated glucose level (Jones *et al.,* 2001).

It should be noted that we were not able to detect the expression of HAS1 mRNA at either normal or high glucose conditions. HAS1 mRNA was undetectable also in PTCs and HK-2 cells (Jones *et*

*al.,* 2001; Selbi *et al.,* 2004; Asselman *et al.,* 2005), and in Madine-Darby canine kidney cells (Asselman *et al.,* 2005). Therefore, the synthesis of high molecular weight HA in kidney seems to be determined mainly by HAS2, which almost obligatorily is involved in HA metabolic alterations in various renal pathological processes (Göransson *et al.,* 2004), including diabetic nephropathy.

Human TGFβ1 up-regulated the expression of HAS2 mRNA and had no effect on HAS3 expression. We already mentioned that the regulation of HAS genes is poorly understood and is cell-type specific (Jacobson *et al.,* 2000). Nevertheless, it should be noted that the influence of TGFβ1 mainly leads to the up-regulation of HAS2 or/and HAS1 mRNAs, whereas HAS3 remains unaltered, or even is down-regulated. For example, TGFβ1 up-regulated: (a) both HAS1 and HAS2 in human skin cells (Sugiyama *et al.,* 1998); (b) HAS1 in human mesothelial cells (at the level of protein expression also) (Jacobson *et al.,* 2000), in human synovial (Recklies *et al.,* 2001; Oguchi & Ishiguro, 2004) and vascular endothelial cells (Suzuki *et al.,* 2003); (c) HAS1 with simultaneous down-regulation of HAS3 in human synovial cells (Stuhlmeier & Pollaschek, 2004); (d) HAS2 in rabbit fibroblasts from the synovial membrane of the temporomandibular joint (Tanimoto *et al.,* 2004), in bovine corneal endothelial cells (Usui *et al.,* 2000) and meshwork cells (Usui *et al.,* 2003) (for both at the level of protein also). Besides, bone morphogenic protein-7 (BMP-7), which belongs to the TGFβ family, up-regulated HAS2 mRNA, had no effect on HAS3 expression and increased the synthesis of only HMW HA in HK-2 cells (Selbi *et al.,* 2004). These investigations allow suggesting that TGFβ1 is able to enhance the synthesis of only HMW HA molecules by selective up-regulation of HAS1 and HAS2.

According to our results, TGFβ1 indeed increased the incorporation of [$^3$H]glucosamine only in HMW HA of mesangial cells, and its effect was similar to the action of high glucose, which up-regulated the expression of HAS2 mRNA, did not influence HAS3 mRNA and stimulated the generation of high-molecular-weight HA. These results lend credit to an involvement of TGFβ1 in the high glucose-induced alterations of HA metabolism, the more so as an anti-TGFβ1-neutralizing antibody completely abolished the effect of high glucose on HA accumulation and molecular size distribution in the medium and matrix compartments of mesangial cultures. An involvement of TGFβ1 in the modulation of HA synthesis by high glucose was demonstrated for renal intestinal fibroblasts (Takeda *et al.,* 2001), and our results confirmed those data.

The blockage of TSP-dependent activation of TGFβ1 with W-peptide normalized the high glucose-induced expression of HAS2 mRNA and simultaneously normalized the increased HA production.

It seems that such an effect of the blockage of TSP-1-dependent TGFβ1 activation not only supports the proposed involvement of TGFβ1 in the high-glucose action on HA metabolism in HMC cultures, but suggests an important role of endogenous TSP-1 with respect to regulation of HA metabolism, as it was recently demonstrated for the inflammation process in TSP-1-deficient mice (Kuznetsova *et al.,* 2005).

As to the role of the increased generation of HA in HMCs treated by high glucose, it remains not clear whether this phenomenon provides a promotional, passive or defensive function in the development of diabetic nephropathy. On the one hand, HA-enriched mesangial ECM stimulates monocyte and macrophage adhesion (Wang & Hascall, 2004), formally promoting the inflammatory response and progression of diabetic nephropathy. On the other hand, HA cable-like structures, which bind monocytes, keep these cells away from the proinflammatory adhesion molecules (ICAM-1/VCAM-1) on the surface of mesangial cells. Hence, they may prevent the interaction of mesangial ICAM-1/VCAM-1 with the corresponding leukocyte counter-receptors and generation of the inflammatory cytokines (van Kooten *et al.,* 1999). Moreover, it should be noted that ICAM-1-deficient mice with streptozotocin diabetes demonstrated decreased macrophage infiltration, albumin excretion, glomerular hypertrophy, mesangial ECM expansion and even down-regulation of TGFβ1 and collagen IY compared with diabetic wild type mice (Okada *et al.,* 2003). This observation suggests that the limitation of the direct contact between mesangial cells and monocytes/macrophages due to cell-associated HA cables (which mainly contain HMW HA) may in fact slow down the development of diabetic nephropathy, despite the commonly held belief that diabetic nephropathy is not an inflammatory condition.

Besides, HMW HA inhibits the activity of TGFβ1 (Locci *et al.,* 1995) and stimulates the secretion of tissue inhibitors of metalloproteinases (Yasui *et al.,* 1992), therefore revealing anti-fibrotic functions.

Thus, it seems that high-molecular-weight HA may provide a defensive role, representing an endogenous mechanism to limit renal injury and the development of diabetic nephropathy.

Overall, our results indicate that high glucose treatment of human mesangial cells leads to an increased synthesis of HA of high molecular weight. This effect is determined by the up-regulation of HAS2 and is mediated *via* the TSP-1-dependent activation of TGFβ1.

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