Modulation of neutral protease expression in human mesangial cells by hyperglycaemic culture

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We report on the effect of prolonged hyperglycaemic (11 and 30 mM D-glucose) culture conditions on human mesangial cell matrix metalloproteinases (MMPs), plasminogen activators and their inhibitors. The results indicate that hyperglycaemic conditions modulate the potential proteolytic activity of the enzymes secreted by confluent cultures of these cells. Gelatinase A (MMP-2) activity was always higher in cultures maintained under hyperglycaemic than under normoglycaemic conditions (4 mM D-glucose). In contrast, gelatinase B (MMP-9) activity was decreased under the same conditions. Matrilysin (MMP-7) activity was decreased by up to 100% under hyperglycaemic conditions. Reverse transcriptase-PCR and Western-blotting analyses indicate that in all cases both the transcripts and the

protein level were correlated with enzymic activity. One tissue inhibitor of metalloproteinases, TIMP-2, was barely detectable under hyperglycaemic conditions (30 mM D-glucose). In contrast, TIMP-1 increased during the initial 2 weeks of culture in hyperglycaemic conditions and remained elevated to the end of the experiment (4 weeks). Under normoglycaemic conditions TIMP-1 decreased after 2 weeks of culture. Hyperglycaemic conditions also decreased markedly the activity of tissue plasminogen activator (t-PA). This seemed to be due to increased synthesis of its inhibitor, plasminogen activator inhibitor 1, under these conditions rather than to decreased expression of the t-PA enzyme.

INTRODUCTION

Diabetic nephropathy leading to kidney failure is a major complication for a significant number of patients with type I or type II diabetes mellitus [1]. A major characteristic of diabetic nephropathy is an enlargement of the mesangium due to the accumulation of extracellular matrix (ECM). The degree of mesangial expansion correlates with the loss of renal function [2]. The amount of ECM laid down is the result of a balance between the synthesis and degradation of the structural proteins in the mesangium. Increased synthesis of various ECM macromolecules has been demonstrated in the glomeruli of diabetic rats and in rat, calf and human mesangial cell (HMC) cultures maintained in media with elevated concentrations of glucose [3–5]. However, the effects of high concentrations of glucose, such as are found in diabetes, on the proteinase profile of mesangial cells has been less well studied.

Matrix degradation depends on a series of extracellular neutral proteinases whose properties are controlled by activating proenzymes and by specific inhibitors. The two major groups of neutral proteinases that degrade ECM are matrix metalloproteinases (MMPs) and serine proteinases. It has been suggested that the two classes are linked in an activation cascade [6,7]. The MMP family includes interstitial collagenase (MMP-1), three types of stromelysin (MMP-3, MMP-10 and MMP-11), neutrophil collagenase (MMP-8), matrilysin (PUMP-1, MMP-7) and the gelatinases A and B (MMP-2 and MMP-9). All MMPs are secreted in a latent form and are activated by cleavage of an N-terminal propeptide at a conserved sequence [8]. Although the

precise mechanism of their activation *in vivo* is unclear, two of their naturally occurring inhibitors, TIMP-1 and TIMP-2 (tissue inhibitors of metalloproteinases), have been isolated and characterized [9–11]. It has been established that TIMP-1 and TIMP-2, complexed with the latent forms of gelatinase B and gelatinase A respectively, have full inhibitory activity against active MMPs, making a tertiary 1:1 non-covalent complex with them [11].

The plasminogen activator (PA)/plasmin system is the other important protease cascade involved in regulating matrix turnover [7,12,13]. Plasmin is an active serine protease that can degrade glycoproteins, proteoglycan core proteins and type IV collagen as well as fibrin. It can also activate several proenzymes, including MMPs. Plasmin is generated from plasminogen and the process is regulated by the interaction of activators (PAs) and inhibitors (PAIs). There are two PAs, a tissue plasminogen activator (t-PA) and a urokinase-type plasminogen activator (u-PA). These are structurally and functionally different and are encoded by different genes [14,15]. There are also two plasminogen activator inhibitors, PAI-1 and PAI-2 [7].

The purpose of the present study was to investigate the possible effects of elevated concentrations of glucose on the expression of such neutral proteases and their inhibitors in primary cultured human mesangial cells (HMCs).

MATERIALS AND METHODS

Materials

Special RPMI 1640 medium, foetal calf serum and antibiotics were from Gibco (Grand Island, NY, U.S.A.). Insulin, trans-

Abbreviations used: AP-1, activator protein 1; APMA, 4-aminophenylmercuric acetate; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMCs, human mesangial cells; MMPs, matrix metalloproteinases; PAI, plasminogen activator inhibitor; RT-PCR, reverse transcriptase-PCR; TGF β , transforming growth factor β ; TIE, TGF β inhibitor element; TIMP, tissue inhibitors of metalloproteinases; t-PA tissue plasminogen activator; u-PA, urokinase-type plasminogen activator.

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ferrin, sodium selenite, and bacterial collagenase (type IV) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-Glucose was added to the RPMI medium to the required concentration. Rabbit anti-human t-PA and sheep anti-human PAI-2 and PAI-1 antibodies were from Cambio (Cambridge, U.K.). MMP-1, MMP-3, sheep anti-human TIMP-1, TIMP-2, gelatinase A and matrilysin and sheep anti-(pig gelatinase B) antibodies were a gift from Dr. Gillian Murphy (Strangeways Research Laboratory, Cambridge, 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 [16]. Primary cultures of mesangial cells were established from collagenase-treated glomeruli [17]. The growth medium consisted of RPMI 1640 supplemented with 20% (v/v) heat-inactivated foetal calf serum, 50 i.u./ml penicillin, 50 µg/ml streptomycin, 300 µg/ml glutamine and 4 mM D-glucose. Insulin, transferrin and sodium selenite (5 µg, 5 µg and 5 ng/ml final concentrations) were added routinely to all culture media. Cells were characterized as mesangial by their morphology and by their immunoreactivity [5]. Cells were stored frozen in liquid nitrogen at the fourth to sixth passage. For experiments, confluent post-exponential-phase cultures of HMCs (passage 8) were maintained in growth medium containing 10% (v/v) foetal calf serum and 4 mM (normoglycaemic), 11 mM or 30 mM (hyperglycaemic) D-glucose for up to 4 weeks. At the end of each week, cultures were washed extensively with PBS and cultured for 24 h in serum-free glucose-supplemented media. Conditioned media were collected, centrifuged at 1000 g for 10 min at 4 °C, concentrated 10-fold by freeze drying and stored at -70 °C before further analysis.

Substrate gel electrophoresis (zymography)

The SDS/PAGE procedure of Laemmli [18] was used with the following modifications. Polyacrylamide gels (10%, w/v) were polymerized in the presence of 0.1% pig skin type I gelatin (Sigma) for gelatin zymograms, or 0.1% casein (Sigma) for casein zymograms. Samples were solubilized in sample buffer without boiling. Reducing agents were not used. After electrophoresis at 4 °C, gels were washed twice in 100 ml of 2.5% (w/v) Triton X-100 for 1 h each to remove SDS and then incubated overnight at 37 °C in 100 mM Tris/HCl buffer, pH 7.5, containing 150 mM NaCl and 10 mM CaCl₂. The gels were stained with Coomassie Blue; unstained areas, corresponding to zones of digestion, were revealed after destaining with 10% (v/v) acetic acid in 20% (v/v) methanol. Prestained molecular mass standards (Amersham International) were used to monitor protein migration and were removed before development of the substrate gels. The molecular masses of the active forms of MMPs were determined by comparing their electrophoretic migrations with those of protein standards.

For plasminogen/casein zymograms, the method of Tomooka et al. [19] was followed. Briefly, plasminogen (0.03 µg) and casein (1 mg/ml) were included in the 10% polyacrylamide gel mixture prior to the casting. Samples were electrophoresed under non-reducing conditions. After electrophoresis gels were treated as for gelatin zymograms except that the incubation buffer was 100 mM glycine, pH 8.0. Samples were also run on gels with casein only, to establish that the zones of lysis were attributable to PA.

Activation of latent collagenases

Latent MMPs were activated with freshly prepared 2 mM 4-aminophenylmercuric acetate (APMA) for 1 h at 37 °C before electrophoresis.

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 7-amino-1-chloro-3-L-tosylamidoheptan-2-one/1 mM *N*-ethylmaleimide/0.1 mM 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one/2 mM PMSF; Sigma Chemical Co., Poole, Dorset, U.K.). Samples were boiled for 5 min and resolved on SDS/PAGE [15% (w/v) gel]. Proteins were transferred to poly(vinylidene difluoride) membrane filter (Immobilon-P, Millipore, Bedford, Beds., U.K.) with a Bio-Rad transfer apparatus. Immunodetection was performed essentially as described by Towbin et al. [20]. Bound antibodies were revealed by peroxidase-conjugated IgGs and 4-chloro-1-naphthol (Boehringer Mannheim Biochemicals, Lewes, East Sussex, U.K.).

RNA extraction and reverse transcriptase-PCR (RT-PCR) analysis

Total RNA was extracted from 6 × 10⁶ mesangial cells by using the RNazol B method (AMS). Samples were dissolved in 20 µl of diethyl pyrocarbonate in distilled H₂O and stored at -70 °C until used. Equal amounts of total RNA (2 µg) from each sample were first reverse-transcribed into cDNAs with SuperScript II RNase H⁺ reverse transcriptase (Gibco BRL) and random primers. Equal amounts (1 µl) of the reverse transcriptase reaction (20 µl) were in turn subjected to PCR amplification in a 100 µl volume containing 10 µl of 10 × PCR buffer, 16 µl of dNTPs (1.25 mM each), 2 mM MgCl₂, 20 µM 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 consisting 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 to confirm the use of equal amounts of the RNAs we co-amplified a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amount of reverse transcription reaction used for the amplification (1 µl) was selected as being non-saturating for the PCR product of GAPDH after 30 cycles of amplification.

The sequences of primers were all designed from the published sequence of the human genes: gelatinase B (sense), 5'-AGAC-CTGAGAACCAATCTCAC-3'; gelatinase B (antisense), 5'-GGCACTGAGGAATGATCTAA-3' (1100 bp) [21]; gelatinase A (sense), 5'-TTTTCTCGAATCCATGATGG-3'; gelatinase A (antisense), 5'-CTGGTGCAGCTCTCATATTT-3' (620 bp) [22]; matrilysin (sense), 5'-AGATGTGGAGTGCCAGATGT-3'; matrilysin (antisense), 5'-TAGACTGCTACCATCCGTCC-3' (365 bp) [23]; GAPDH (sense), 5'-ACCACAGTCCATGCC-ATCAC-3'; GAPDH (antisense), 5'-TCCACCACCCTGTTG-CTGTA-3' (450 bp) [24]. After amplification, 8 µl of each PCR reaction mix was subjected to electrophoresis through a 2% (w/v) agarose gel with ethidium bromide (0.5 µg/ml).

Quantitative analysis

Zymograms were air-dried between sheets of clear cellulose film (Promega, Southampton, Hants., U.K.). Dried zymograms, Western blots and photographs of DNA gels were scanned with an Epson GT-8000 scanner into Adobe PhotoShop software; the pixel intensity for each band (average pixel intensity multiplied by area) was analysed with the Image software (NIH Shareware). Peak areas were measured in arbitrary units. To minimize error

either in the scanning procedure or due to variation in band width and thickness, the procedure was repeated three times and the mean value was used.

RESULTS

Confluent cultures of HMCs at passage 8 were maintained in media containing 4 mM (normoglycaemic), 11 mM or 30 mM (hyperglycaemic) D-glucose for up to 28 days. Aliquots of 24 h-conditioned media equivalent to 2.5×10^4 cells were run on SDS/PAGE [10% (w/v) gel]/0.1% gelatin substrate gel under non-reducing conditions. Gelatinolytic activity was revealed by zones of clearing. The major zone of lysis corresponded to gelatinase A (66–72 kDa) and a less prominent zone to gelatinase B (88–92 kDa) (Figure 1). Because the zymogram was run in the presence of SDS, the proenzyme forms of metalloproteinases would be activated and, in the zone lysed by gelatinase B, two bands were separated corresponding to progelatinase B (92 kDa) and active gelatinase B (88 kDa). Prior treatment with or without APMA, a non-protease activator of pro-MMPs, did not result in any significant shift in band mobilities (results not shown). In contrast, the intense lytic zone corresponding to gelatinase A was present as a single broad band on the zymogram. The difference in molecular mass between the proenzyme (72 kDa) and the proteolytically activated enzyme (68 kDa) is small [25] and the lytic zone probably contained both forms. Prior treatment with or without APMA did not affect the appearance of this band (results not shown).

Trace amounts of lytic activity were detected in the molecular mass range 44–55 kDa and might have been due to interstitial collagenase and/or stromelysin. These trace activities do not show up on photographs of the original gel. All the above lytic activities were blocked by prior treatment with 5 mM EDTA or

Figure 1 Gelatin zymogram of serum-free conditioned media derived from HMCs

Cells were grown to confluence and subsequently maintained in the presence of different glucose concentrations (4, 11 or 30 mM; top scale) for 1, 2 or 4 weeks (bottom scale). Aliquots of conditioned media equivalent to 2.5×10^4 cells/24 h were run on SDS/PAGE [10% (w/v) gel]/0.1% gelatin substrate gel under non-reducing conditions. Zones of clearing indicate gelatinolytic activity.

Figure 2 Western-blot analysis of gelatinase A secreted by HMCs

Cells were grown to confluence and subsequently maintained in the presence of different glucose concentrations (4, 11 or 30 mM) for 1, 2 or 4 weeks. At the end of each period cells were incubated in fresh media without FCS for 24 h. Volumes equivalent to 3.0×10^4 cells/24 h were subjected to SDS/PAGE [15% (w/v) gel] under reducing conditions. Proteins were electroblotted on nitrocellulose membrane and probed with anti-(human gelatinase A) antibody.

Table 2 Scanning analysis of gelatinase A secreted by HMCs and cross-reacted with anti-(human gelatinase A) antibody

Results represent the means $\pm$ S.E.M. for six analyses for two independent experiments and do not represent the analysis in Figure 2 alone.

[D-Glucose] (mM) ...	Gelatinase A (pixel intensity units/ 2.5×10^4 cells per 24 h)		
	4	11	30
7 days	1203 $\pm$ 27	1199 $\pm$ 38	1332 $\pm$ 24
14 days	770 $\pm$ 15	1117 $\pm$ 17	1402 $\pm$ 25
28 days	390 $\pm$ 6	964 $\pm$ 39	1292 $\pm$ 12

1 mM 1,10-phenanthroline, but not by PMSF (results not shown).

The gelatinase A activity seemed to increase in cultures exposed to high glucose compared with those maintained in 4 mM glucose (Table 1). Western blots were performed to confirm that the lytic activity in the 66–72 kDa range was due to gelatinase A, and to investigate whether the amount of enzyme present varied with the culture conditions (Figure 2). The blots showed a single band of 68 kDa that cross-reacted with anti-(human gelatinase A). The amount of enzyme decreased over the 28-day culture period under normoglycaemic conditions (Table 2). Hyperglycaemic culture conditions stimulated the production of gelatinase A (Table 2). RT-PCR indicated that the increased synthesis of this enzyme is correlated with increased transcription (Figure 3 and

Table 1 Scanning analysis of zones of gelatinolytic activity related to gelatinase A and gelatinase B

Results represent the means $\pm$ S.E.M. for four independent experiments and do not represent the analysis in Figure 1 alone. * $P < 0.001$, ** $P < 0.005$ compared with 4 mM D-glucose.

[D-Glucose] (mM) ...	Gelatinase A (pixel intensity units/ 2.5×10^4 cells per 24 h)			Gelatinase B (pixel intensity units/ 2.5×10^4 cells per 24 h)		
	4	11	30	4	11	30
7 days	3253 $\pm$ 44	3337 $\pm$ 41	3396 $\pm$ 107	3222 $\pm$ 18	2321 $\pm$ 84*	1173 $\pm$ 45*
14 days	2893 $\pm$ 145	3280 $\pm$ 42	3605 $\pm$ 189	960 $\pm$ 33	2124 $\pm$ 66	171 $\pm$ 46*
28 days	1978 $\pm$ 45	2471 $\pm$ 57**	3069 $\pm$ 32*	677 $\pm$ 95	240 $\pm$ 59	438 $\pm$ 59

Figure 3 The effect of D-glucose concentration on mRNA expression of gelatinase A, gelatinase B, matrilysin and GAPDH in HMCs

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

Table 3). Transcription of the housekeeping gene, GAPDH, was unchanged at different glucose concentrations. The gelatinase B activity seemed to decrease after 7 days of culture under hyperglycaemic conditions, especially in 30 mM D-glucose (Table 1). Thereafter gelatinase B activity was decreased under both normoglycaemic and hyperglycaemic culture conditions compared with 7-day cultures. We were unable to detect gelatinase B by Western blotting, suggesting that this enzyme is produced in very small amounts. However, transcripts for gelatinase B were detected by RT-PCR (Figure 3 and Table 3). These decreased in hyperglycaemic cultures.

Figure 4 Western-blot analysis of matrilysin secreted by HMCs

Cells were grown under the conditions and for the periods described in the Materials and methods section. Volumes equivalent to 2.5×10^4 cells/24 h were subjected to SDS/PAGE [15% (w/v) gel] under reducing conditions. Proteins were electroblotted on nitrocellulose membrane and probed with anti-(human matrilysin) antibody.

When equivalent samples were applied to a casein substrate gel, the zymogram revealed one zone of caseinolytic activity of about 30 kDa. This was inhibited by 5 mM EDTA or 1 mM 1,10-phenanthroline, but not by PMSF (results not shown). This zone of caseinolytic activity is consistent with the molecular mass of matrilysin (26–31 kDa), identified as a product of mesangial cells by Marti et al. [26]. The enzymic activity of matrilysin was decreased markedly under hyperglycaemic conditions and could not be detected at all at 30 mM glucose (Table 4). The loss of matrilysin activity correlated with decreased gene transcription in 11 mM D-glucose, and undetectable levels of transcription in 30 mM D-glucose, as shown by RT-PCR (Figure 3 and Table 3). Matrilysin protein could be detected only in cultures maintained in 4 mM D-glucose (Figure 4 and Table 4).

Table 3 Quantification of cDNA species 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 7, 14 or 28 days before analysis as shown in Figure 3. The RT-PCR products corresponding to gelatinase A, gelatinase B, matrilysin and GAPDH were separated on an agarose gel. Gels were photographed, scanned and bands were quantified by analysis with Image software. The pixel intensity of each band is shown in arbitrary units. The results shown are the means $\pm$ S.E.M. for three separate RT-PCR analyses and do not represent the analysis in Figure 3 alone. ** $P < 0.005$, † $P < 0.05$ compared with 4 mM D-glucose.

[D-Glucose] (mM) ...	Gelatinase A (pixel intensity units)			Gelatinase B (pixel intensity units)			Matrilysin (pixel intensity units)			GAPDH (pixel intensity units)		
	4	11	30	4	11	30	4	11	30	4	11	30
7 days	866 $\pm$ 102	1144 $\pm$ 63	1172 $\pm$ 87	500 $\pm$ 32	396 $\pm$ 24	360 $\pm$ 35†	175 $\pm$ 25	92 $\pm$ 8†	0	709 $\pm$ 47	769 $\pm$ 54	761 $\pm$ 43
14 days	750 $\pm$ 79	1122 $\pm$ 68†	1131 $\pm$ 166†	449 $\pm$ 29	236 $\pm$ 45†	280 $\pm$ 57	163 $\pm$ 18	57 $\pm$ 23†	0	719 $\pm$ 47	753 $\pm$ 118	710 $\pm$ 13
28 days	630 $\pm$ 62	1036 $\pm$ 104	967 $\pm$ 88†	444 $\pm$ 26	312 $\pm$ 34†	220 $\pm$ 25**	155 $\pm$ 27	0	0	718 $\pm$ 41	725 $\pm$ 59	649 $\pm$ 31

Table 4 Scanning analysis of the caseinolytic activity and protein level of matrilysin secreted by HMCs maintained in the presence of different D-glucose concentrations (4, 11 or 30 mM) for 1, 2 or 4 weeks

Aliquots of conditioned media equivalent to 2.5×10^4 cells/24 h were run on SDS/PAGE [10% (w/v) gel] with casein substrate under non-reducing conditions for zymography or run on SDS/PAGE [15% (w/v) gel] under reducing conditions for Western blotting. Gels were processed as in the Materials and methods section and bands were quantified by analysis with Image software. The results represent means $\pm$ S.E.M. for three independent experiments. * $P < 0.001$, *** $P < 0.01$ compared with 4 mM D-glucose.

[D-Glucose] (mM) ...	Matrilysin caseinolytic activity (pixel intensity units/ 2.5×10^4 cells per 24 h)			Matrilysin protein level (pixel intensity units/ 2.5×10^4 cells per 24 h)		
	4	11	30	4	11	30
7 days	388 $\pm$ 21	284 $\pm$ 16***	0	1322 $\pm$ 27	0	0
14 days	243 $\pm$ 25	106 $\pm$ 9*	0	1091 $\pm$ 30	0	0
28 days	212 $\pm$ 10	74 $\pm$ 5*	0	322 $\pm$ 13	0	0

Figure 5 Western-blot analysis of TIMP-1 and TIMP-2 secreted by HMCs

Cells were grown under the conditions and for the periods described in the Materials and methods section. Volumes equivalent to 3.0×10^4 cells/24 h were subjected to SDS/PAGE [15% (w/v) gel]. Proteins were electroblotted on nitrocellulose membrane and probed with anti-(human TIMP-1) (a) or anti-(human TIMP-2) (b) antibodies.

We examined by Western blotting the potential role of TIMPs in lowering the amount of metalloproteinase enzyme activity. Aliquots of conditioned media (equivalent to 3.0×10^4 cells/24 h) were subjected to SDS/15% (w/v) gel electrophoresis. Proteins were electroblotted on nitrocellulose membranes and probed with anti-(human TIMP-1) and anti-(human TIMP-2) antibodies. Figure 5(a) shows a 28 kDa polypeptide that reacted with TIMP-1 antibody. The amount of this protein increased after 2 weeks of all treatments. It then decreased to a very low level under normoglycaemic conditions, but stayed at a high level under hyperglycaemic conditions (Table 5). Figure 5(b) shows a 21 kDa polypeptide that reacted with TIMP-2 antibody. The amount of TIMP-2 protein seemed to be decreased in cultures exposed to 30 mM D-glucose but not in those maintained in 11 mM D-glucose (Table 5).

When plasminogen was included in the casein substrate gel, the zymogram showed a 70 kDa plasminogen-dependent zone of lysis in the analyses of conditioned media of cells maintained under normoglycaemic conditions. This was not present in hyperglycaemic cultures (Figure 6a and Table 6). Western-blotting analysis confirmed that the PA activity in normoglycaemic cultures was due to t-PA. Moreover, similar or even slightly increased amounts of t-PA (especially after 7 days) were present in hyperglycaemic cultures (Figure 6b, and Table 6). Western blotting analysis also confirmed the presence of the 45 kDa PAI-1 (Figure 6c), whereas PAI-2 protein was not detected. Figure 6(c) and Table 6 show that the protein level of PAI-1 was barely detectable under normoglycaemic conditions but was markedly increased after 2 weeks of hyperglycaemic culture.

Figure 6 Analysis of t-PA and PAI-1 secreted by HMCs

Cells were grown under the conditions and for the periods described in the Materials and methods section. Volumes equivalent to 3.0×10^4 cells/24 h were subjected to (a) SDS/PAGE/casein-plasminogen zymography or (b, c) SDS/PAGE/Western blotting with anti-(human t-PA) antibodies (b) or PAI-1 antibodies (c).

DISCUSSION

The results indicate that hyperglycaemic culture conditions modulate the potential proteolytic activity of the neutral enzymes secreted by HMCs. During long-term exposure to high glucose concentrations, potential gelatinase A activity was always greater than in cultures maintained under normoglycaemic conditions. Increased transcription and protein synthesis for gelatinase A occurred in hyperglycaemic cultures. This suggests a possible increase in gelatinase A activity in hyperglycaemia. Similar results have been reported by Wang et al. [27] with murine mesangial cells. These authors also showed that the increase in gelatinase A activity was due specifically to 30 mM D-glucose because no significant increase was found in cultures maintained in either 30 mM L-glucose or in 30 mM mannitol.

The effect of hyperglycaemic conditions on gelatinase B was also marked after 7 days of culture in 30 mM D-glucose but, in contrast with gelatinase A, potential gelatinase B activity fell to approx. 40% of that found in normoglycaemic cultures. Over longer periods it was difficult to distinguish any effects of the glycaemic conditions on enzyme activity because the level of gelatinase B in 4 mM cultures also decreased very markedly with increasing time. Although protein for gelatinase B could not be detected by Western blotting there was a clear decrease in mRNA in hyperglycaemic cultures as shown by RT-PCR.

The observation that hyperglycaemic culture has opposite effects on the expression of gelatinase A and gelatinase B is of interest. The gene for gelatinase A, which shows elevated expression, does not have a transforming growth factor β (TGF β)

Table 5 Scanning analysis of TIMP-1 and TIMP-2 secreted by HMCs and cross-reacted with anti-(human TIMP-1) and anti-(human TIMP-2) antibodies

Results represent means $\pm$ S.E.M. for six analyses for two independent experiments and do not represent the analysis in Figure 5 alone. * $P < 0.001$ compared with 4 mM D-glucose.

	[D-Glucose] (mM) ...	TIMP-1 (pixel intensity units/ 3.0×10^4 cells per 24 h)			TIMP-2 (pixel intensity units/ 3.0×10^4 cells per 24 h)		
		4	11	30	4	11	30
7 days		0	0	0	318 ± 18	138 ± 5*	0
14 days		56 ± 13	299 ± 27	340 ± 26	320 ± 12	167 ± 4*	0
28 days		0	182 ± 13	384 ± 27	172 ± 15	133 ± 10	0

Table 6 Scanning analysis of t-PA and PAI-1 secreted by HMC

The plasminogen-dependent caseinolytic activity zones in zymograms as well as bands cross-reacted with anti-(human t-PA) and anti-(human PAI-1) antibodies by Western blotting were quantified by analysis with Image software. Data are means $\pm$ S.E.M. for three independent experiments and do not represent the analysis in Figure 6 alone.

[D-Glucose] (mM) ...	t-PA activity (pixel intensity units/ 2.5×10^4 cells per 24 h)			t-PA protein level (pixel intensity units/ 2.5×10^4 cells per 24 h)			PAI-1 protein level (pixel intensity units/ 2.5×10^4 cells per 24 h)		
	4	11	30	4	11	30	4	11	30
7 days	207 $\pm$ 11	0	0	1374 $\pm$ 104	1747 $\pm$ 62	1525 $\pm$ 30	0	993 $\pm$ 128	855 $\pm$ 107
14 days	378 $\pm$ 40	0	0	1813 $\pm$ 53	1717 $\pm$ 57	1428 $\pm$ 61	0	1016 $\pm$ 213	1766 $\pm$ 53
28 days	384 $\pm$ 36	0	0	1759 $\pm$ 48	2516 $\pm$ 49	1873 $\pm$ 60	0	1284 $\pm$ 122	787 $\pm$ 60

inhibitor element (TIE) in its promoter sequence [28]. In contrast, gelatinase B, the expression of which is decreased under hyperglycaemic conditions, does contain a putative TIE in its promoter sequence. Moreover, TGF β induces high expression levels of Jun-B, which is a negative regulator of *c-jun* in the AP-1 (activator protein 1) transcription factor [29,30]. Jun-B-containing AP-1 complexes bind to the AP-1 promoter sites and repress gene expression [31,32]. The gelatinase A promoter does not contain an AP-1 site, whereas the promoter for the gelatinase B gene has this site [28] and would therefore be down-regulated in the presence of increased levels of TGF β . In experiments reported elsewhere [5] we have shown that HMC cultures increase their production of TGF β 1 under hyperglycaemic conditions. The increased levels of TGF β 1 are maintained over the 28-day period under which cells were cultured in the present experiments. Although the difference in the promoter structures of the two gelatinase genes might provide a possible explanation for the different responses to hyperglycaemia, this proposal could be an oversimplification. Although other investigators have found that TGF β induces gelatinase A in rat and HMCs [33], the growth factor can also induce both gelatinases A and B in some other cell types [33–36].

Hyperglycaemic culture had a very clear effect on matrilysin (MMP-7) expression and activity. In 30 mM glucose the activity of this enzyme was completely suppressed, and even in 11 mM glucose after 2 weeks the activity of matrilysin was greatly decreased. The matrilysin promoter has three putative TIE boxes and one AP-1 element within its sequence [28]. Apart from a marked reduction in the expression of matrilysin in hyperglycaemic mesangial cells, it is also likely that the activity of this and other metalloproteinases would be decreased by TIMP-1. The inhibitor was barely detected in normoglycaemic cultures but its synthesis was markedly increased under hyperglycaemic conditions. TIMP-1 inhibits collagenase, gelatinases and stromelysin-type enzymes, of which matrilysin is one [37].

If similar changes occurred in mesangial metalloproteinase activity in the hyperglycaemic mesangium *in vivo* they would lead to a decrease in the turnover of a number of matrix components. For example, matrilysin digests type IV collagen well [38]. Although we found that hyperglycaemic culture conditions do not stimulate type IV collagen synthesis in cultures of HMCs [5], an observation that contrasts with their effect on rat mesangial cells [3,4], this basement-membrane-type collagen nevertheless accumulates in the diabetic mesangium *in vivo* [39]. The decrease in matrilysin expression and increase in TIMP-1 in hyperglycaemic cultures suggest that this accumulation could be due to reduced matrilysin expression and increased TIMP-1 in the mesangium of hyperglycaemic diabetics. Decreased expression of gelatinase B with increased levels of TIMP-1 could also contribute

to the accumulation of type IV collagen, although gelatinases are not particularly good at degrading the latter [40].

Several other matrix proteins that accumulate in the diabetic mesangium, for example fibronectin and laminin [39], are also substrates for matrilysin [37]. In their case accumulation is likely to be due to both decreased turnover and increased synthesis because the expression of these proteins is increased in hyperglycaemic mesangial cells [5].

It is of interest that Fadly et al. [41] found increased type IV collagenase activity in the urine of streptozotocin-diabetic rats. The enzyme responsible was not further defined but one possibility is that it could have been gelatinase A, whose expression and activity were increased in our long-term hyperglycaemic experiments. Gelatinase A was recently shown to have a collagenolytic-type activity against type I collagen [42]. Type I collagen is not generally detected in the diabetic mesangium [39], although its expression and synthesis are up-regulated in hyperglycaemic HMC cultures [5]. One speculation might be that if its synthesis is turned on *in vivo*, deposition of type I collagen might be prevented through the action of gelatinase A. Against this stand the observations that type V collagen is present in the diabetic mesangium [39] and that it too is a good substrate for gelatinase A [40]. Thus it seems more likely that gelatinase A has only a low level of activity in the diabetic mesangium, possibly as a result of inhibition by TIMP-1.

No t-PA activity was detected in hyperglycaemic cultures by zymography, although the enzyme was detected by Western blotting. The lack of activity in hyperglycaemic cultures seems to be due to an increased synthesis of PAI-1 under these conditions. Enzyme-inhibitor complexes would be preserved under the conditions used for zymography but would be dissociated after boiling under reducing conditions before SDS/PAGE and Western blotting. In the absence of plasminogen, t-PA activity was not detected on the casein zymogram.

Elevated levels of PAI-1 have been reported generally in diabetic patients [43,44], so the results *in vitro* are in accord with observations *in vivo*. TGF β 1 induces both PA and PAI-1 in other cell systems but the production of inhibitor exceeds that of the proteinase [45]. In the mesangial cell cultures there was marginal evidence for increased t-PA protein levels in hyperglycaemic cultures, but PAI-1 levels were clearly increased. A decline in t-PA activity would lead to a decrease in plasmin production *in vivo* with a possible decrease in the conversion of some proMMPs to active enzymes [37] and decreased proteolytic attack on mesangial matrix protein substrates. However, this effect of decreased t-PA activity *in vivo* could be compensated for by the fact that, although mesangial cells in culture do not synthesize u-PA, they express its cellular receptor u-PAR [46] by which they can bind u-PA produced by other cells *in vivo*.

The results of the present experiments *in vitro* with mesangial cell cultures suggest that hyperglycaemic conditions might reduce the turnover of the mesangial matrix macromolecules and therefore contribute to their accumulation in diabetic nephropathy. A recent report indicates that high glucose concentrations do indeed reduce the degradation of the mesangial matrix *in vitro* [47].

Hyperglycaemic culture of HMCs induces an increase in mRNA and protein for a number of extracellular macromolecules including type I collagen, fibronectin, laminin and decorin. These increases are only apparent after 14–21 days of culture under hyperglycaemic conditions, whereas TGF β 1 levels are greatly elevated after 7 days in response to high concentrations of glucose. This led us to question whether elevated TGF β 1 is the only signal stimulating the transcription of these matrix proteins in hyperglycaemia [5]. In contrast the decrease in gelatinase B and matrilysin (PUMP-1) and increase in PAI-1 is marked after 7 days of exposure to hyperglycaemic conditions. Thus, taking account of their promoter structures, an elevation in TGF β 1 level is a likely candidate for stimulating these changes. TIMP-1 is also induced by TGF β [48]. As discussed previously, the acceptability of this hypothesis will depend on demonstrating the presence of bioactive TGF β 1 and its receptors in hyperglycaemic mesangial cell cultures [5].

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REFERENCES

- Pirart, J. (1978) *Diabetes Care* **1**, 168–188
- 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
- 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
- Danne, T., Spiro, M. J. and Spiro, R. G. (1993) *Diabetes* **42**, 170–177
- Abdel Wahab, N., Harper, K. and Mason, R. M. (1996) *Biochem. J.* **316**, 985–992
- Werb, Z., Mainardi, C. L., Vater, C. A. and Harris, Jr., E. D. (1977) *New Engl. J. Med.* **296**, 1017–1023
- Saksela, O. and Rifkin, D. B. (1988) *Annu. Rev. Cell Biol.* **4**, 93–126
- Sanchez-Lopez, R., Nicholson, R., Gesnel, M.-C., Matrisian, L. M. and Breathnach, R. (1988) *J. Biol. Chem.* **263**, 11892–11899
- Docherty, A. J. P., Lyons, A., Smith, B. J., Wright, E. M., Stephens, P. E. and Harris, T. J. R. (1985) *Nature (London)* **318**, 66–69
- Stetler-Stevenson, W. G., Brown, P. D., Onista, M., Levy, A. T. and Liotta, L. A. (1990) *J. Biol. Chem.* **265**, 13933–13938
- Ward, R. V., Hembray, R. M., Reynolds, J. J. and Murphy, G. (1991) *Biochem. J.* **278**, 179–187
- Anderasen, P. A., George, B., Lund, L. R., Riccio, A. and Stacey, S. N. (1990) *Mol. Cell. Endocrinol.* **68**, 1–19
- Vassalli, J. D., Sappino, A. P. and Belin, D. (1991) *J. Clin. Invest.* **88**, 1067–1072
- Triputti, P., Blasi, F., Verde, P., Cannizano, L. A., Emmanuel, B. S. and Croce, C. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4448–4452
- Verheijen, J. H., Visse, R., Wijnen, J. T. H., Chang, G. T. G., Kluft, C. and Meera Kahn, P. (1986) *Hum. Genet.* **72**, 153–156
- Striker, G. E., Killen, P. D. and Farin, F. M. (1980) *Transplant. Proc.* **12**(3) (suppl. 1), 88–99
- Bernik, M. B. (1969) *Nephron* **6**, 1–10
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Tomooka, S., Border, W. A., Marshall, B. C. and Noble, N. A. (1992) *Kidney Int.* **42**, 1462–1469
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A. and Goldberg, G. I. (1989) *J. Biol. Chem.* **264**, 17213–17221
- Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C. S., Bauer, E. A. and Goldberg, G. I. (1988) *J. Biol. Chem.* **263**, 6579–6587
- Muller, D., Quantin, B., Gesnel, M. C., Millon-Collard, R., Abecassis, J. and Breathnach, R. (1988) *Biochem. J.* **253**, 187–192
- Ercolan, I. L., Florence, B., Denaro, M. and Alexander, M. (1988) *J. Biol. Chem.* **263**, 15335–15341
- Nagase, H. (1994) *Contrib. Nephrol.* **107**, 85–93
- Marti, H. P., McNeil, L., Thomas, G., Davies, M. and Lovett, D. H. (1992) *Biochem. J.* **285**, 899–905
- Wang, Z. J., Bacchicchio, J., Fadly, M., Sorbi, D. and Arbeit, L. A. (1994) *J. Am. Soc. Nephrol.* **5**, 974
- Gaire, M., Magbanua, Z., McDonnell, S., McNeil, L., Lovett, D. H. and Matrisian, L. M. (1994) *J. Biol. Chem.* **269**, 2032–2040
- Chiu, R., Angel, P. and Karin, M. (1989) *Cell* **59**, 979–986
- Schutte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J. and Minna, J. (1989) *Cell* **59**, 987–999
- Deng, T. and Karin, M. (1993) *Genes Dev.* **7**, 479–490
- Mauviel, A., Qiu Chen, Y., Dong, W., Evans, C. H. and Uitto, J. (1993) *Curr. Biol.* **3**, 822–831
- Pullig, O., Marx, M., Netzer, K. and Weber, M. (1993) *J. Am. Soc. Nephrol.* **4**, 663
- Salo, T., Lyons, J. G., Rahemtulla, F., Birkedal-Hansen, H. and Larjava, H. (1991) *J. Biol. Chem.* **266**, 11436–11441
- Overall, C. M., Wrana, J. L. and Sodek, J. (1989) *Connect. Tiss. Res.* **20**, 289–294
- Overall, C. M., Wrana, J. L. and Sodek, J. (1991) *J. Biol. Chem.* **266**, 14064–14071
- Murphy, G. and Reynolds, J. (1993) in *Connective Tissue and its Heritable Disorders* (Royce, P. M. and Steinman, B., eds.), pp. 287–316, Wiley-Liss, New York
- Murphy, G., Cockett, M. I., Ward, R. V. and Docherty, A. J. P. (1991) *Biochem. J.* **277**, 277–279
- Nerlich, A. and Schleicher, E. (1991) *Am. J. Pathol.* **139**, 889–899
- Nagase, H., Ogata, Y., Suzuki, K. O., Enghild, J. J. and Salvesen, G. (1991) *Biochem. Soc. Trans.* **19**, 715–718
- Fadly, M., Sorbi, D., Hicks, R., Alexander, S. and Arbeit, L. (1993) *J. Am. Soc. Nephrol.* **4**, 794
- Aimes, R. T. and Quigley, J. P. (1995) *J. Biol. Chem.* **270**, 5872–5876
- Auwerx, J., Bouillon, R., Collen, D. and Geboers, J. (1988) *Arteriosclerosis* **8**, 68–72
- Mall, M., Kluft, C., MacCuish, A. C. and Lowe, G. D. O. (1989) *Diabetes Care* **12**, 655–658
- Laiho, M., Saksela, O., Andreason, P. A. and Keski-Oja, J. (1989) *J. Cell Biol.* **103**, 2403–2410
- Nguyen, G., Li, X.-M., Peraldi, M. N., Rondeau, E. and Sraer, J. D. (1993) *J. Am. Soc. Nephrol.* **4**, 661
- McLennan, S. V., Fisher, E. J., Yue, D. K. and Turtle, J. R. (1994) *Diabetes* **43**, 1041–1045
- Edwards, D. R., Murphy, G., Reynolds, J. J., Whitham, S. E., Docherty, A. J., Angel, P. and Heath, J. K. (1987) *EMBO J.* **6**, 1899–1904