High glucose concentration inhibits the expression of membrane type metalloproteinase by mesangial cells: possible role in mesangium accumulation

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Abstract

Aims/hypothesis. High glucose concentration decreases the degradation of mesangium matrix, an action substantially mediated by a reduction in the activities of the matrix metalloproteinases (MMPs). Metalloproteinase-2 is unique in that it is activated on the cell surface by one of the membrane type metalloproteinases (MT1-MMP), a process involving complex interactions with tissue inhibitor of metalloproteinase-2. The aim of this study was investigate the effects of glucose concentration on mesangial cell gene expression of *MT1-MMP* and its ability to modulate the activation of metalloproteinase-2.

Methods. Gene expression was determined using competitive RT-PCR, protein expression of MMP-2 was measured by western blot and its activation by zymography. Concanavalin A, known to increase *MT1-MMP* expression was added in some experiments.

Results. High glucose concentration decreased *MT1-MMP* gene expression (11.52 ± 1.63 and 4.84 ± 0.72

amol/ μ g RNA, 5 vs 25 mmol/l glucose, respectively) and decreased activation of MMP-2 by 30 % despite a twofold increase in gene expression of *MMP-2*. Concanavalin A increased expression of *MT1-MMP* and activation of MMP-2. Irrespective of whether MMP-2 was from endogenous or exogenous source there was an excellent correlation between the *MT1-MMP* expression and degree of MMP-2 activation, whereas the gene expression of TIMP-2 was not significantly altered by high glucose concentration or concanavalin A.

Conclusions/interpretation. Our results indicate that in a high glucose milieu, suppression of *MT1-MMP* expression could explain the low MMP-2 activity in the presence of high *MMP-2* expression. This process could contribute to the mesangium matrix accumulation in diabetic nephropathy. [Diabetologia (2000) 43: 642–648]

Keywords Matrix metalloproteinase, nephropathy, extracellular matrix, degradation.

Mesangium enlargement is one of the most important factors in the pathogenesis of diabetic nephropathy and its expansion correlates well with clinical mark-

ers of this renal complication. High glucose concentration has been shown to increase the gene expression and synthesis of several extracellular matrix molecules and it is recognised that increased matrix synthesis plays a part in diabetic nephropathy [1, 2]. In contrast, the importance of impaired matrix degradation in causing mesangium accumulation has received little attention. Previous studies in our laboratory have shown that high glucose concentration decreases the ability of mesangial cells to degrade mesangium matrix, a phenomenon associated with a reduction in the activities of the matrix metalloproteinases (MMPs) [3, 4]. Other workers have shown that

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Abbreviations: MMPs, Matrix metalloproteinases; MMP-2, matrix metalloproteinase-2; MT1-MMPs, membrane type metalloproteinases; TIMP-2, tissue inhibitor of metalloproteinase; Con A, concanavalin A.

the expression of these enzymes can be altered by high glucose concentration [5–11]. Thus the effects of high glucose concentration on MMPs constitute a potential pathway of how mesangium degradation becomes impaired in diabetes.

The MMPs belong to a large family of at least 17 enzymes, which are divided according to substrate specificity into four main subgroups. These include the gelatinases, the interstitial collagenases, the stromelysins and the more recently identified membrane-type metalloproteinases (MT-MMPs) [12–14]. In mesangial cells, a diabetic milieu has been shown to decrease the expression of most of the MMPs studied [5–11]. Reports on the expression of one of the MMPs, the 72 000 M_r gelatinase or matrix metalloproteinase-2 (MMP-2) are, however, contradictory with both increased and decreased expression being reported [5–11]. This MMP is the principal MMP secreted by cultured mesangial cells and is primarily responsible for degradation of type IV collagen which is known to accumulate in the mesangium in diabetes [12, 15]. Therefore the regulation of this MMP is of particular interest in the study of diabetic nephropathy.

Like other MMPs, MMP-2 is secreted as a 72 000 M_r proenzyme which requires activation by cleavage of the N-terminal domain to yield the active 57 000 M_r enzyme. Unlike the other MMPs which are activated after secretion by plasmin, MMP-2 is unique in that it is activated on the cell surface by MT-MMP [12–15]. The predominant MT-MMP in the kidney is MT1-MMP which has been shown to be present in mesangial cells of rat or human origin [15, 16]. Adding to the complexity of its regulation, the activation and activity of MMP-2 are also affected by the somewhat opposing actions of tissue inhibitor of metalloproteinase-2 (TIMP-2). This inhibitor binds to MMP-2 to facilitate its activation by MT1-MMP and also inhibits the activity of the activated MMP-2 [15–20]. The importance of the MT1-MMP cascade is well documented in tumours where increased expression of MT1-MMP is associated with invasiveness [13]. Its role in renal disease which is characterised by matrix accumulation has not, however, been evaluated. Taking these factors into consideration, our study was undertaken to examine the effects of high glucose concentration on the interaction of MT1-MMP, TIMP-2 and MMP-2 expression and activation.

Materials and methods

Cell culture

Human mesangial cells were isolated from fetal kidneys using a sieving technique as described previously [3]. Briefly, these cells are cultured from glomeruli isolated from kidneys ob-

tained from terminations of pregnancies in the second trimester. Their identity and purity have been established by criteria outlined in previous publications [3, 4]. Cells were cultured at 37°C in an environment of 95% air and 5% CO₂ in RPMI media (Sigma, St Louis, Mo., USA) containing 5 mmol/l glucose, 15 mmol/l HEPES and 10% fetal calf serum (Trace, Sydney, Australia). Cells from the third and fourth passage were used in experiments.

Experimental design

To test the effects of varying glucose concentration, human fetal mesangial cells cultured as described above were trypsinised and seeded into 90 mm² tissue culture dishes (Iwaki, Sydney, Australia). When the cells reached confluence the media was removed, the cells were washed twice with phosphate buffered saline (PBS) and the media replaced with FCS free RPMI containing 0.1% albumin and either 5 mmol/l or 25 mmol/l glucose. After 72 h the media was collected for determination of MMP-2 activation by zymography and MMP-2 protein concentration by western analysis. The cell layer was then washed with PBS before extraction of RNA for measurement of gene expression of *MMP-2*, *TIMP-2* and *MT1-MMP* or the preparation of plasma membranes for studying the effects of manipulating *MT1-MMP* concentration on the activation of exogenous MMP-2.

To test the effects of increasing *MT1-MMP* expression on MMP-2 activities, concanavalin A (Con A, 20 µg/ml) which is known to increase the expression of *MT-MMPs* was added to mesangial cells in the presence of either 5 or 25 mmol/l glucose for the final 24 h [21]. The media and cells were collected and studied as above.

In some experiments mesangial cells were also cultured in the presence of 20 mmol/l mannitol and 5 mmol/l glucose as an osmotic control.

SDS/PAGE zymography

For all studies the protein concentration of the media was determined using BioRad DC (Sigma) and aliquots containing 10 µg protein were analysed by zymography (10% SDS/PAGE containing 1 mg/ml gelatin) as reported previously [4]. As in previous reports, the predominant band was the 72 000 M_r proenzyme form of MMP-2, together with a less intense band at 57 000 M_r being the activated form of MMP-2. The degree of MMP-2 activation was determined by densitometry and calculated as follows:

$$\% \text{ activation of MMP-2} = 57\,000\,M_r \div [72\,000\,M_r + 57\,000\,M_r] \times 100$$

Control gels were incubated in TRIS-HCl buffer containing 10 mmol/l EDTA. No activity was seen on these gels verifying that the degradation of gelatin was due to MMPs.

Western blot analysis

The amount of MMP-2 protein in the medium was determined by western blot. For these studies the conditioned medium was concentrated (five to tenfold) and the protein concentration determined as above. Samples containing 30 µg protein were loaded onto a SDS-PAGE gel (10%) and transferred (13 mA

Table 1. Primer sequences and expected PCR product sizes for both the wild-type mRNA and its RNA_{comp}

	Oligonucleotide sequence		Wild type	RNA _{comp}
MT1-MMP	sense	5' ccc tat gcc tac atc ctg ga	550 bp	466 bp
	antisense	5' tcc atc cat cac ttg gtt at		
MMP-2	sense	5' cgc cgt cgc cca tca tca agt	400 bp	347 bp
	antisense	5' tgg att cga gaa aac cgc agt gg		
TIMP-2	sense	5' ctc tgg aaa cga cat tta tgg c	332 bp	365 bp
	antisense	5' aga tgt agc acg gga tca tgg g		
β -actin	sense	5' gaa ttc tgg cca cgg ctg ctt cca gct	164 bp	254 bp
	antisense	5' aag ctt ttt cgt gga tgc cac agg act		

for 1 h) to PVDF membrane (Immobilon, Millipore, Bedford, Mass., USA). Membranes were blocked (PBS containing 0.1% Tween 20, and 5% skim milk powder for 1 h) and washed with PBS (three changes, 5 min) before overnight incubation with mouse monoclonal MMP-2 antibody (1:1000, ICN Biomedicals, Seven Hills, Australia). After extensive washing (PBS, three changes, 15 min) the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG second antibody (1:10000 Santa Cruz, Santa Cruz, Calif., USA) for 2 h. Bound second antibody was detected after washing (PBS, three changes, 15 min) by chemiluminescence (Amersham Pharmacia Biotech, Sydney, Australia) and quantitated by densitometry.

Competitive RT-PCR

Preparation of competitor molecules. To enable quantification of the effect of high glucose concentration on the expression of *MT1-MMP*, *MMP-2* and *TIMP-2* internal RNA competitor molecules (RNA_{comp}) were designed and constructed for each MMP by modification of *v-erb* (PCR mimic construction kit) (Clontech, Palo Alto, Calif., USA). Briefly, two rounds of PCR were used to incorporate firstly primers specific for the gene of interest and secondly a T₇ transcription site at the 5' end and a poly A tail at the 3' end. The products from these PCR reactions were then transcribed to RNA using the Maxiscript T₇ In Vitro Transcription kit (Ambion, Austin, Texas, USA). RNA_{comps} were prepared in this manner for *MT1-MMP*, *MMP-2* and *TIMP-2*. An additional RNA_{comp} for the housekeeping gene β -actin was also prepared and used to confirm PCR efficiency and to act as a loading control. The oligonucleotide sequences and PCR product sizes for wild type and RNA_{comps} for each gene (i.e. *MT1-MMP*, *MMP-2*, *TIMP-2* and β -actin) are shown in Table 1.

RNA extraction and RT-PCR. Mesangial cells were washed in PBS and RNA was extracted using TRIzol (1 ml per 90 mm² dish) (Gibco BRL/Life Technologies, Mulgrave, Australia). The RNA concentration was determined using SYBR green (Molecular Probes, Eugene, Ore., USA). First strand cDNA was prepared by concurrent reverse transcription of increasing dilutions of RNA_{comp} and wild-type RNA (250 ng/ μ l) using Superscript (Gibco BRL/Life Technologies), random hexamers (50 ng/ μ l), PCR buffer, 25 mmol/l MgCl₂, 10 mmol/l deoxyribonucleoside triphosphate (dNTP), 0.1 mol/l dithiothreitol and RNase free H₂O, according to the manufacturers' instructions. First strand cDNA (2 μ l) was amplified using *AmpliTa*q DNA polymerase (2 U) (Perkin Elmer, Scoresby, Australia), 10 mmol/l dNTPs and 20 mmol/l of the gene specific primers as follows: *MT1-MMP*, 94°C for 3 min, followed by 35 cycles

of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, *MMP-2* and *TIMP-2*, 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, β -actin 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min. For each gene product a final extension of 72°C for 7 min was used. The resulting PCR products were separated on a 2.0% agarose gel containing ethidium bromide. Results were recorded using the Gel Doc system (UVP, Uppland, Calif., USA) and the intensity of the ethidium bromide stained bands analysed by densitometry using Phoretix (Phoretix, Newcastle-upon-Tyne, UK). The concentration of wild-type mRNA was determined from the equivalence point i.e. where the ratio of wild-type mRNA:RNA_{comp} equals one and expressed as attomoles of gene per μ g RNA.

Preparation of Plasma membrane for activation of exogenously added MMP-2

For preparation of plasma membranes, mesangial cells were grown to confluence in 75 cm² tissue culture flasks (Iwaki). We used 15 flasks of cells for each of the conditions tested. Cells were washed with PBS before the addition of TRIS-HCl buffer (1 ml, 50 mmol/l, pH 7.5). The cells were then scraped from the flask using a rubber policeman and lysed by homogenisation on ice in cold TRIS-HCl buffer containing 20 mmol/l glucose and 0.02% NaN₃. The whole lysate was centrifuged at 16 000 g for 20 min. The supernatant was removed and centrifuged (100 000 g, 1 h at 4°C) and the resulting pellet was washed in TRIS-HCl buffer and centrifuged as before. Plasma membranes were then resuspended in TRIS buffer and stored at -20°C until use.

For each experiment, 500 μ g of plasma membrane protein was incubated for 24 h with MMP-2 obtained from one ml of a pooled FCS-free mesangial cell conditioned medium. The degree of MMP-2 activation was determined by zymography and quantified as described above.

Statistical analysis

To analyse the effects of glucose and Con A on the expression and activities of *MT1-MMP* and *MMP-2*, each experiment was done at least three times. Results are expressed as means $\pm$ SD. Statistical analysis was done using post hoc analysis of variance and significance determined using Duncans *T* test. Coefficient of correlation was calculated using the least squares method.

Fig. 1 **A** A zymogram of media obtained from cells grown in 5 mmol/l glucose or 25 mmol/l glucose which showed a decrease in activated MMP-2 (57 000 M_r) in the high glucose environment. **B** Western blot showing an increase in the protein expression of MMP-2 in the media from cells cultured in 25 mmol/l glucose

Results

The effects of glucose concentration on activation and expression of MMP-2. Zymography showed that growth of mesangial cells in high glucose (25 mmol/l) concentration for 72 h caused a 30% decrease in the activated MMP-2 (57 000 M_r form) in the culture medium (Fig. 1). By contrast, western analysis of the same sample showed an increase in the MMP-2 protein (Fig. 1). Similarly the zone of equivalence is shifted to the left in the 25 mmol/l glucose sample, indicating that the gene expression of MMP-2 by mesangial cells was also increased by high glucose concentration (Fig. 2). The results from three experiments confirming this pattern of response are shown in Table 2. High glucose concentration did not affect expression of the housekeeping gene β -actin and its effects on MMP-2 activation and expression were not observed in the mannitol control experiments.

The roles of MT1-MMP and TIMP-2 in MMP-2 activation. Using the primers described in Table 1, a 550 bp product was amplified by PCR and confirmed to be *MT1-MMP* by restriction enzyme digestion. Its DNA sequencing showed 100% homology with the reported sequence (Genebank Accession No. D26512). As shown by the shift in equivalence point to the right in the 25 mmol/l sample, the expression of *MT1-MMP* in mesangial cells was decreased by high glucose concentration (Fig. 3, Table 3). The expression of *MT1-MMP* was increased when Con A

Fig. 2 **A, B.** Typical competitive RT-PCR profiles comparing the effects of **(A)** 5 mmol/l or **(B)** 25 mmol/l glucose on the gene expression of MMP-2. The concentration in attomoles of competitor added to each RT reaction are: lane 1 competitor alone 27306 amol, lane 2 109226 amol, lane 3: 54613 amol, lane 4: 27306.5 amol, lane 5: 13663.25 amol, lane 6 6826.6 amol, lane 7: 3413.3 amol. WT, wild-type

was added to 5 mmol/l glucose but not 25 mmol/l glucose (Table 3). In all cases, the amount of activated MMP-2 in the culture medium changed in the same direction as *MT1-MMP* expression but not that of *MMP-2* gene expression (Table 3). The degree of MMP-2 activation correlated well with the *MT1-MMP* expression (Fig. 4).

As predicted from their actions on *MT1-MMP*, high glucose decreased and Con A increased the ability of plasma membrane to activate exogenously added MMP-2. There was a good correlation between MMP-2 activation and *MT1-MMP* gene expression (Fig. 5). These effects of glucose were not observed in the mannitol control experiments.

The gene expression of *TIMP-2* was not altered by either 25 mmol/l glucose or the addition of Con A (Table 3).

Table 2. The effect of glucose concentration on MMP-2 activation and expression

Condition	Activation of media MMP-2 ^a (% of 5 mmol/l) ^b	MMP-2 protein ^c (% of 5 mmol/l) ^b	MMP-2 gene expression (amol/ μ g RNA)
5 mmol/l glucose	100	100	44.2 $\pm$ 0.75
25 mmol/l glucose	70.05 $\pm$ 11.42 ^d	167 $\pm$ 6.25 ^d	85.67 $\pm$ 0.22 ^d
5 mmol/l glucose + 20 mmol/l mannitol	105 $\pm$ 5.62	110 $\pm$ 8.68	54.34 $\pm$ 0.17

^a Activation of MMP-2 measured as the area of 57 000 M_r /[area 72 000 M_r + 57 000 M_r]

^b Expressed as a percentage of values obtained in the 5 mmol/l glucose control samples

^c Arbitrary densitometry units

^d $p < 0.05$ significantly different from 5 mmol/l glucose control values

Fig. 3 A, B. Typical competitive RT-PCR profiles comparing the effects of (A) 5 mmol/l or (B) 25 mmol/l glucose on the gene expression of MT1-MMP. The concentration in attomoles of competitor added to each RT reaction are: lane 1: 8530 amol, lane 2: 2560 amol, lane 3: 853 amol, lane 4: 256 amol, lane 5: 85.3 amol, lane 6: 25.6 amol, lane 7: 8.53 amol. C Example of the analysis required to determine MT1-MMP mRNA expression in 5 mmol/l glucose and 25 mmol/l (-▲-) glucose. Band densities of the competitor (comp) and target molecules (WT) were determined as described. The log ratio of competitor:target band intensities are plotted against the log of the concentration of the competitor molecule in each RT-PCR reaction. At $Y = 0$ (equivalence point) the corresponding X-axis value can be determined and the concentration of the target mRNA calculated

Discussion

Previous studies by ourselves and other workers have shown that high glucose concentration alters the expression and activation of MMPs [3–8], phenomena which could have important roles in explaining the

Fig. 4. The correlation between MT1-MMP expression by mesangial cells and activated of MMP-2 in the culture medium obtained from these cells. $r = 0.744$

Fig. 5. The correlation between MT1-MMP expression by mesangial cells and the activation of exogenous MMP-2 by plasma membranes prepared from these mesangial cells. $r = 0.742$

mesangium accumulation of diabetic nephropathy. Metalloproteinases are regulated at several levels and understanding how high glucose exerts its effects is of potential importance in devising strategies to

Table 3. The effect of concanavalin A on MT1-MMP, MMP-2, TIMP-2 gene expression and MMP-2 activation

	MT1-MMP gene expression (amol/μg RNA)	MMP-2 gene expression (amol/μg RNA)	TIMP-2 gene expression (amol/μg RNA)	Activation of media MMP-2 (% of 5 mmol/l) ^a
5 mmol/l glucose	11.5 ± 1.6	41.4 ± 17.3	93.8 ± 26.1	100
25 mmol/l glucose	4.8 ± 0.7 ^b	94.3 ± 16.4 ^b	73.5 ± 19.2	78.7 ± 7.7 ^b
5 mmol/l glucose + Con A	14.0 ± 0.5 ^b	56.4 ± 17.8	114.5 ± 20.3	168.5 ± 9.6 ^b
25 mmol/l glucose + Con A	10.2 ± 1.2	36.2 ± 15.6	105.5 ± 13.2	93.5 ± 5.8

^a Activation of MMP-2 measured as the area of 57 000 M_r [area 72 000 M_r + 57 000 M_r] expressed as a % of control values obtained in the 5 mmol/l glucose samples

^b $p < 0.05$ significantly different from 5 mmol/l glucose control values

prevent this diabetic complication. In this study we have focussed on MMP-2 as it is the major MMP expressed by human mesangial cells and is involved in the degradation of type IV collagen known to accumulate in extracellular matrix in diabetes [12]. Moreover, as distinct from other MMPs, the activation of MMP-2 is mediated by MT1-MMP on the cell surface and the effects of high glucose concentration on this process have not been studied previously.

One of the intriguing findings of our results was that high glucose concentration for 72 h increases the gene and protein expression of MMP-2 but its activation to the 57 000 M_r active moiety is decreased. This dichotomy of *MMP-2* expression and activation can now be explained by our findings that *MT1-MMP* expression is reduced by high glucose, thus reducing the capacity of mesangial cells to activate MMP-2. The good correlation between *MT1-MMP* expression and activation of MMP-2 irrespective of whether it is from an endogenous or exogenous source lend support to this notion.

A previous study using human mesangial cells exposed to high glucose concentration for four weeks has shown that the expression of MMP-2 is increased at both the gene and protein level [7]. There are, however, some contradictory reports in the literature [9–11]. Down-regulation of *MMP-2* expression in glomeruli of patients with Type II (non-insulin-dependent) diabetes mellitus was shown by RT-PCR [11]. Studies using either human or rat mesangial cells have also reported down-regulation of *MMP-2* gene expression after 7 days exposure to high glucose concentration although no data is available on MMP-2 protein concentrations [9–10]. The basis for these divergent trends of MMP-2 response to high glucose reported in the literature is not clear. The findings of increased gene expression of *MMP-2* both by us and others were supported by the increased MMP-2 protein from the same specimens whereas the contradictory reports were on gene expression alone. The discrepancy in *MMP-2* gene expression also cannot be explained solely on the basis of difference in time as the above experiments overlapped in duration of exposure to high glucose concentration. Moreover, in our further experiments *MMP-2* expression is also increased after a longer exposure of 7 days to high glucose and in glomeruli of rats after 12 weeks of streptozocin induced diabetes (results not shown). The finding of a simultaneous increase in *MMP-2* gene expression but decreased MMP-2 activation is relevant to the understanding of this important pathway of mesangium degradation.

The mechanism of MMP-2 activation by MT1-MMP is complex and also involves the actions of TIMP-2. In addition to the traditional concept of TIMP-2 binding to MMP-2 to inhibit its action, studies have suggested that TIMP-2 also competitively in-

hibits the binding of proMMP-2 to the MT1-MMP to inhibit the activation process [19, 20]. Other workers have also suggested that in a somewhat contradictory manner the proMMP-2/TIMP-2 complex can also facilitate MMP-2 activation on the cell surface [16]. Thus decreased activation of MMP-2 observed in a high glucose environment could also be caused by changes in TIMP-2 concentration. Because we have, however, been unable to observe any glucose-induced change in *TIMP-2* expression, the decrease in expression of *MT1-MMP* is more likely to have the important role in this context. Adding to the complexity is the role of TGF- β in the activation of MMP-2. It has been reported that increased TGF- β can decrease the activation of MMP-2 [22]. As high glucose concentration can increase TGF- β this provides another possible mechanism affecting MMP-2 activation.

One of the difficulties in studying the roles of metalloproteinases in diabetic nephropathy is that there are multiple enzymes of overlapping specificity, each with many potential levels of regulation. It is therefore difficult to ascertain the causal effects of any changes observed. Our results indicate, however, that in a high glucose milieu, suppression of *MT1-MMP* expression could explain the low MMP-2 activity in the presence of high *MMP-2* expression. This process could contribute to the mesangium matrix accumulation in diabetic nephropathy.

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