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High glucose decreases collagenase expression and increases TIMP expression in cultured human peritoneal mesothelial cells

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Abstract

Background. Peritoneal fibrosis (PF), a serious problem in long-term continuous ambulatory peritoneal dialysis (CAPD) patients, is characterized by extracellular matrix (ECM) accumulation which results from an imbalance between the synthesis and the degradation of ECM components. Previous studies have demonstrated that ECM synthesis is increased in human peritoneal mesothelial cells (HPMCs) under high glucose conditions, but the effects of high glucose on degradative pathways have not been fully explored. This study was undertaken to elucidate the effects of high glucose on these proteolytic processes in cultured HMPCs.

Methods. HPMCs were isolated from human omentum and were exposed to 5.6 mM glucose (NG), 5.6 mM glucose $+34.4$ mM mannitol (NG $+$ M), or 40 mM glucose (HG) with or without PKC inhibitor (PKCi). Real-time PCR and western blot were performed to determine collagenases (MMP-1, -8 and -13) and TIMPs (TIMP-1 and -2) mRNA and protein expression, respectively. The individual activities of collagenases in culture media were determined by ELISA.

Results. Types I and III collagen protein expression were significantly increased in HG-conditioned media compared to NG media ($P < 0.05$). The MMP-1, -8 and -13/GAPDH mRNA ratios were significantly lower in HPMCs exposed to HG medium compared to NG cells by 64, 52 and 37%, respectively, and their protein expression by 76, 42 and 49%, respectively, in HG- vs NG-conditioned media. The activities of collagenases in HG-conditioned media were also significantly lower than those in NG media ($P < 0.05$). In contrast, HG significantly increased TIMPs mRNA ratios and protein expression in HPMCs. These changes in collagenase and TIMP expression induced by HG were abrogated upon pre-treatment with PKCi.

Conclusion. In conclusion, impaired matrix degradation may contribute to ECM accumulation in PF.

Keywords: collagenases; high glucose; mesothelial cells; peritoneal fibrosis; TIMPs

Introduction

Continuous ambulatory peritoneal dialysis (CAPD) has been used as a long-term renal replacement therapy in end-stage renal disease (ESRD) patients. However, after long-term treatment with CAPD, peritoneal fibrosis (PF) has been observed in some patients, resulting in membrane failure [1]. *In vitro* and *in vivo* studies have demonstrated that denudation of mesothelial cells from the peritoneum and excessive deposition of extracellular matrix (ECM) are the structural determinants of PF [1,2]. Recurrent peritonitis, bioincompatible peritoneal dialysis solution, and high glucose in dialysate *per se* have been implicated as major factors in the pathogenesis of PF, however, the precise mechanisms leading to ECM accumulation are not fully understood.

ECM accumulation is not only one of the characteristic pathological findings in various diseases such as diabetic nephropathy [3], but is also the result of an imbalance between the synthesis and degradation of ECM components, including collagen, fibronectin and laminin. It is well known that increased production of ECM [4] and decreased production of

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matrix-degrading proteinases [5] play an important role in the pathogenesis of diabetic nephropathy. Since expansion of submesothelial connective tissue layer and deposition of collagen fibres have been observed in PF, many investigators have attempted to elucidate the pathogenesis of PF in a similar manner as that of diabetic nephropathy. Peritoneal mesothelial cells, like mesangial cells, have the capacity to produce matrix proteins [6]. Previous studies on ECM biosynthesis have shown that high glucose concentrations enhance TGF-β gene expression and stimulate ECM synthesis in cultured human peritoneal mesothelial cells (HPMCs) [7,8]. In contrast, there have been only limited studies that investigated the pathogenesis of PF in terms of ECM degradation [9,10].

The degradative process of ECM is mediated by key proteolytic enzymes, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Among these, gelatinases (MMP-2 and -9) and TIMPs (TIMP-1 and -2) have been widely studied in experimental diabetic nephropathy and high glucose-stimulated mesangial cells. Decreased MMP-2 or MMP-9 gene expression has been observed in human and animal models of diabetic nephropathy [11,12]. In contrast, the expression of TIMPs was increased in mesangial cells cultured under high glucose conditions [5]. HPMCs are also known to synthesize MMP-2, -3, and -9 along with TIMP-1 and -2 and to control the accumulation of ECM by secreting these enzymes [9]. Recent studies demonstrated that activated MMP-2 was associated with chemical-induced peritoneal injury [13] and that MMP-2 levels in peritoneal effluents were significantly higher in patients with peritoneal injury compared to control patients [14], while another study revealed that MMP-9 and TIMP-1 activities were higher at the onset of peritonitis, suggesting the involvement of MMP-9 and TIMP-1 in peritoneal remodelling [15]. MMPs and TIMPs have distinct substrate specificities [16]. Therefore, it is reasonable to investigate corresponding enzymes involved in the degradation of ECM accumulated in various diseases. In contrast to diabetic nephropathy in which type IV collagen and fibronectin are the main components of ECM deposited [3], type I and III collagens are the major ECM accumulated in the submesothelial layer in PF [17]. Since the degradation of type I and III collagens is mediated by interstitial collagenases (MMP-1, -8 and -13) [16], studies on the degradative process of ECM in PF should focus on collagenases. Most of the previous studies on PF, however, have investigated the changes in gelatinases rather than collagenases [9,10].

In the present study, we examined the degradative pathways involved in the turnover of types I and III collagens in HPMCs isolated from human omentum. To clarify whether the degradative process is involved in the pathogenesis of PF, the enzymatic activities of collagenase (MMP-1, -8 and -13) as well as the expression of collagenases and TIMPs (TIMP-1 and -2) were determined in HPMCs cultured under high glucose conditions.

Methods

HPMC culture

HPMCs were isolated according to the method described by Stylianou et al. [6]. Briefly, a piece of human omentum, obtained from consenting patients undergoing elective abdominal surgery, was washed three times with sterile phosphate-buffered saline (PBS) and incubated in 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution for 20 min at 37°C with continuous shaking. After incubation, the suspension containing free mesothelial cells was centrifuged at $100\,g$ for 10 min at 4°C. The cell pellet was then washed once and re-suspended in M199 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 26 mM $NaHCO_3$, and seeded onto culture dishes. The cells were grown in the same medium at 37°C in humidified 5% CO_2 in air, and the medium was changed 24 h after seeding, and then every 3 days.

To investigate the effect of high glucose on the degradative pathway of ECM, subconfluent HPMCs were incubated with medium containing 0.5% FBS for 24 h to arrest and synchronize cell growth. After this time period, the cells were treated with 0.5% FBS media containing 5.6 mM D-glucose (NG), 5.6 mM D-glucose and 34.4 mM mannitol (NG + M), or 40 mM D-glucose (HG) with or without a PKC inhibitor, 10^{-7} M calphostin C, and were harvested after 24 h (for mRNA analysis) or 72 h (for all other experiments). The concentrations of glucose and incubation time for the present study were determined based on the results of preliminary experiments with different glucose concentrations [40 mM, 83.3 mM (1.5%) and 222.2 mM (4.0%)] and with different treatment duration.

Measurement of cytotoxicity

The cytotoxicity was measured by the alamar blue assay according to the manufacture's instruction (AbD Serotec Ltd, Oxford, UK). Briefly, HPMCs were seeded in a 96-well plate and were cultured under 0.5% FBS media containing various glucose concentrations (40, 83.3 and 222.2 mM) in triplicate for 72 h. After washing three times with fresh media, the cells were incubated in alamar blue (1:10 dilution with culture media) at 37°C for 3 h and the fluorescence was measured using a microtitre reader (Molecular Devices Corp., Sunnyvale, CA, USA) with excitation at 570 nm and emission at 600 nm. The percent viability was expressed as fluorescence emitted by treated cells compared to NG cells.

Total RNA extraction and reverse transcription

HPMC RNAs from each plate were extracted and first strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany) as previously described [8]. Reverse transcription of 2 μg of HPMC RNA from each plate was performed using 10 μM random hexanucleotide primer, 1 mM dNTP, 8 mM $MgCl_2$, 30 mM KCl, 50 mM Tris–HCl, pH 8.5, 0.2 mM dithiothreitol, 25 U RNAse inhibitor and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 min and 42°C for 1 h followed by enzyme inactivation at 99°C for 5 min.

Table 1. Primer sequences and PCR conditions

	Sequence (5′ → 3′)	Size of PCR product (bp)
GAPDH		
Sense	GATTCCACCCATGGCAAATT	65
Antisense	AGATGGTGATGGGATTTCCATT	
MMP-1		
Sense	GGG AGA TCA TCG GGA CAA CTC	72
Antisense	GGG CCT GGT TCA AAA GCA T	
MMP-8		
Sense	GCC ATC CCT TCC AAC TGG TA	88
Antisense	AGC CCA GTA TTG GTT GCC TTT	
MMP-13		
Sense	TGT TGC TGC GCA TGA GTT C	61
Antisense	TGC TCC AGG GTC CTT GGA	
TIMP-1		
Sense	CTG TGG CTC CCT GGA ACA	67
Antisense	CCA ACA GTG TAG GTC TTG GTG AAG	
TIMP-2		
Sense	AAG AGT TGT TGA AAG TTG ACA AGC A	63
Antisense	CGG ACC GAC CGA TTG C	

Real-time polymerase chain reaction (RT–PCR)

The primers used for the experiments are summarized in Table 1.

Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), PCR was performed with a total volume of 20 µl in each well, containing 10 µl of SYBR Green® PCR Master Mix (Applied Biosystems), 5 µl of cDNA, and 5 pmol sense and antisense primers. Primer concentrations were determined by preliminary experiments that analysed the optimal concentrations of each primer. Each sample was run in triplicate in separate tubes to permit quantification of the gene normalized to GAPDH. The PCR conditions used were as follows: for GAPDH, 36 cycles of denaturation at 94.5°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min; and for all other genes, 42 cycles of denaturation at 94.5°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min. Initial heating at 95°C for 9 min and final extension at 72°C for 7 min were performed for all PCR reactions.

After RT–PCR, the temperature was increased from 60°C to 95°C at a rate of 2°C/min to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative C_T method with $2^{-\Delta\Delta CT}$. The results are given as relative expression of certain genes normalized to the GAPDH housekeeping gene. Signals from NG cells were assigned a relative value of 1.0. In pilot experiments, PCR products run on agarose gels revealed a single band.

Western blot analysis

For western blot, conditioned media were collected, lyophilized and reconstituted in 100 µl sodium dodecyl sulphate (SDS) sample buffer [2% sodium dodecyl sulphate, 10 mM Tris–HCl, pH 6.8, 10% (vol/vol) glycerol]. Protein

concentrations were determined using a Bio-Rad kit (Bio-Rad Laboratories, Inc.). Aliquots of 50 µg of protein were treated with Laemmli sample buffer, heated at 100°C for 5 min and electrophoresed (50 µg/lane) in an 8% SDS–polyacrylamide gel. Proteins were transferred to Hybond-ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA) using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA). The membrane was incubated in blocking buffer A (1 × PBS, 0.1% Tween-20 and 8% non-fat milk) for 1 h at room temperature, and then incubated overnight at 4°C with a 1:200 ~ 1:1000 dilution of polyclonal antibody to type I collagen, type III collagen, MMP-1, MMP-8, MMP-13, TIMP-1 or TIMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was then washed once for 15 min and twice for 5 min in 1 × PBS with 0.1% Tween-20, and incubated in buffer A with horseradish peroxidase-linked goat anti-mouse IgG or goat anti-rabbit IgG (Amersham Life Science, Inc.) at a 1:1000 dilution. The washes were repeated, and the membrane developed with chemiluminescent reagent (ECL; Amersham Life Science, Inc.). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany), and the percent changes in the optical densities of bands from the treated cells relative to NG cells were used for analysis.

ELISA assay

The total activities of MMP-1, -8 and -13 in culture-conditioned media were determined by commercial ELISA kits (MMP-1 and MMP-8, Biotrak, Amersham Biosciences, Buckinghamshire, UK; MMP-13, Fluorokine, R&D systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol. Briefly, for MMP-1 and MMP-8, 100 µl each of pro-MMP-1 (0.1–12.5 ng/ml) or pro-MMP-8 standards (0.05–6 ng/ml), concentrated culture conditioned media, and 100 µl assay buffer as a blank were incubated at 4°C overnight in microtitre wells pre-coated with anti-MMP-1 or anti-MMP-8 antibody. Any MMP-1 or MMP-8 present in samples bound to the wells prior to removal of other components by washing four times with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.05% Tween-20. To measure the total activity of MMP-1 or MMP-8, the bound pro-MMP-1 or pro-MMP-8 was activated with 50 µl of *p*-aminophenylmercuric acetate (APMA) in assay buffer at 37°C for 1 h. Detection reagent (50 µl) was then added to each well and incubated at 37°C for 2 h. Active MMP-1 or MMP-8 was detected though activation of a modified prodetection enzyme and the subsequent cleavage of its chromogenic peptide substrate. The resultant colour was read at 405 nm in a microtitre plate reader. For MMP-13, 200 µl each of pro-MMP-13 (0.06–6 ng/mL), concentrated culture conditioned media, and 200 µl Calibrator Diluent as a blank were incubated at room temperature for 3 h in microtitre wells pre-coated with anti-MMP-13. Any MMP-13 present in samples bound to the wells prior to removal of other components by washing four times with 300 µl of Wash Buffer. To measure the total activity of MMP-13, bound pro-MMP-13 was activated with 200 µl of APMA in assay buffer at 37°C for 1 h. After washing four times with 300 µl of Wash Buffer, quenched fluorogenic substrate (200 µl) was added to each well and incubated at 37°C for 18 h in the dark room. Active MMP-13 was detected though the elimination of

distance-dependent resonance energy transfers between fluorophore and quencher molecules, by cleavage of the peptide linker. The relative fluorescence units were determined using a fluorescence plate reader set with the following wavelength parameters; excitation at 320 nm and emission at 405 nm. The activities of MMP-1, -8 and -13 in each sample were determined by interpolation of the standard curve.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical analysis was performed using the statistical package SPSS for Windows Ver. 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analysed using the Kruskal–Wallis non-parametric test for multiple comparisons. Significant differences by the Kruskal–Wallis test were further confirmed by the Mann–Whitney U-test. P-values <0.05 were considered to be statistically significant.

Results

Types I and III collagen are accumulated in HG-conditioned media

We first examined whether types I and III collagen were accumulated in HG-conditioned culture media. To determine the concentrations of glucose and incubation time for the present study, HPMCs were exposed to various glucose concentrations (40, 83.3 and 222.2 mM) with different treatment duration. As seen in Figure 1A, types I and III collagen protein expression were significantly increased in 40 and 83.3 mM glucose-conditioned media ($P < 0.05$), whereas their expression was significantly decreased in 222.2 mM glucose-conditioned media at 72 h ($P < 0.01$). The alamar blue assay revealed that the viability of HPMCs exposed to 222.2 mM glucose was significantly decreased compared to NG and cells cultured under media containing 40 mM glucose ($P < 0.01$). The glucose concentration of 83.3 mM also reduced the cell survival but did not reach statistical significance (Figure 1B). The glucose concentrations of 40 mM significantly induced types I and III collagen protein expression in conditioned media at 24 h and further increased their expression at 72 h (Figure 1C). Equal protein loading was confirmed by Ponceau S staining of the membrane (data not shown). Based on these results, we used the glucose concentration of 40 mM (HG) and treated the cells for 72 h.

Type I collagen protein expression, assessed by western blot, was increased in HG-conditioned media at 72 h. Densitometric quantitation revealed a 72% increase in type I collagen protein expression in HG- *vs* NG-conditioned culture media ($P < 0.05$). There was also a 63% increase in type III collagen protein expression in HG- compared to NG-conditioned culture media ($P < 0.05$) (Figure 1A and C).
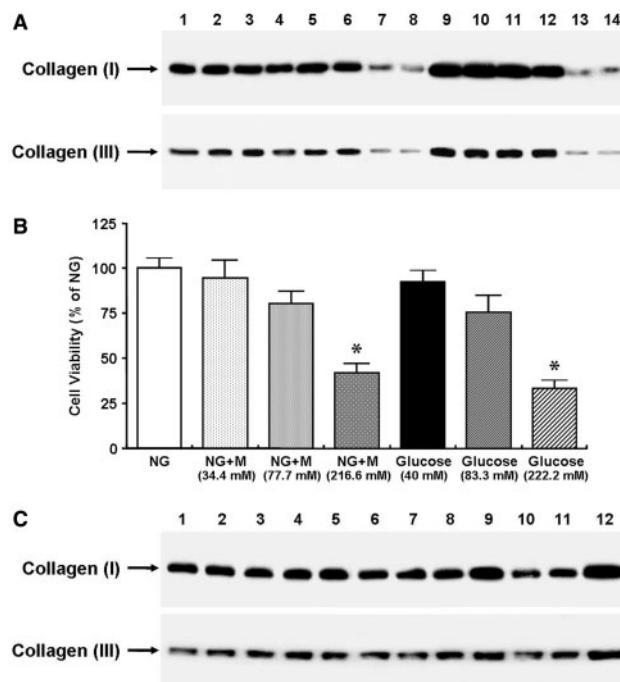

Fig. 1. (**A**) Western blot of types I and III collagen in HPMCs exposed to 5.6 mM glucose (NG) (lanes 1 and 2), NG + 34.4 mM mannitol (M) (lanes 3 and 4), NG + 77.7 mM M (lanes 5 and 6), NG + 216.6 mM M (lanes 7 and 8), 40 mM glucose (lanes 9 and 10), 83.3 mM (1.5%) glucose (lanes 11 and 12) and 222.2 mM (4.0%) glucose (lanes 13 and 14) (media) (representative of four blots). Types I and III collagen protein expression was significantly increased in 40 mM and 83.3 mM glucose-conditioned media compared to NG media ($P < 0.05$), whereas their expression was significantly decreased in NG + 216.6 mM M- and 222.2 mM glucose-conditioned media at 72 h ($P < 0.01$). (**B**) The cell viability, assessed by alamar blue assay, was significantly decreased in HPMCs exposed to NG + 216.6 mM M and 222.2 mM glucose compared to the other groups. The glucose concentration of 83.3 mM also reduced the cell survival but did not reach statistical significance ($n = 5$). *$P < 0.01$ *vs* NG, NG + 34.4 mM M and 40 mM glucose. (**C**) Western blot of type I and III collagen in HPMCs exposed to NG (lanes 1, 4, 7 and 10), NG + 34.4 mM M (lanes 2, 5, 8 and 11) and 40 mM glucose (HG) (lanes 3, 6, 9 and 12) at 2 (lanes 1–3), 6 (lanes 4–6), 24 (lanes 7–9), and 72 h (lanes 10–12) (media) (representative of four blots). Types I and III collagen were significantly accumulated in HG-conditioned media compared to NG media at 72 h. Densitometric quantitation revealed 72 and 63% increases in types I and III collagen protein expression in HG- *vs* NG-conditioned culture media, respectively ($P < 0.05$). On the other hand, mannitol had no effect on types I and III collagen protein expression.

HG inhibits MMP-1, -8 and -13 mRNA and protein expression

We secondly evaluated whether the increases in type I and III collagen protein expression in HG-conditioned culture media were attributed to the decreases in collagenases expression. MMP-1, -8 and -13 mRNA expression were significantly decreased in HPMCs exposed to HG medium compared to cells cultured under NG conditions ($P < 0.05$), whereas there was no difference in GAPDH mRNA expression between NG and HG cells (data not shown). The MMP-1, -8 and -13/GAPDH mRNA ratios were significantly lower in HG-stimulated HPMCs relative to NG cells
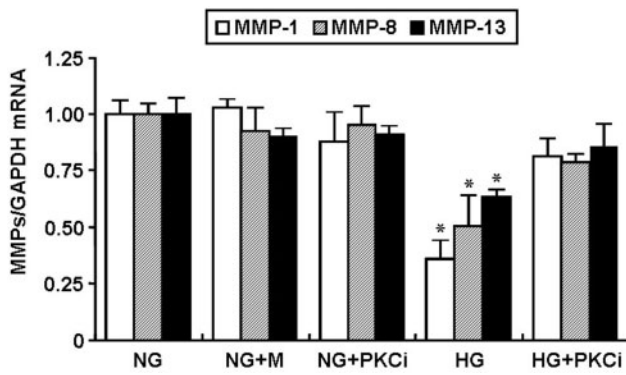
Fig. 2. Collagenase/GAPDH mRNA ratios in HPMCs exposed to 5.6 mM glucose (NG), NG + 34.4 mM mannitol (M) and 40 mM glucose (HG) with or without a PKC inhibitor, 10^{-7} M calphostin C (PKCi) ($n = 5$). There were significant decreases in MMP-1, -8 and -13/GAPDH mRNA ratios in HPMCs cultured under HG conditions by 64, 52, and 37%, respectively, compared to NG cells. Pre-treatment with calphostin C for 6 h nearly abolished the decreases in MMP-1, -8 and -13/GAPDH mRNA ratios in HPMCs exposed to HG medium. $^*P < 0.05$ *vs* other groups.

by 64, 52 and 37%, respectively ($P < 0.05$) (Figure 2). MMP-1, -8 and -13 protein expression were also decreased by 76, 42 and 49%, respectively, in HG- *vs* NG-conditioned culture media (Figure 3)

HG inhibits MMP-1, -8 and -13 activities

We next explored the activities of collagenases to ascertain whether the changes in their mRNA and protein expression led to changes in enzymatic activities. The collagenase activities showed a similar pattern to the observed changes in mRNA and protein expression. MMP-1 and MMP-8 activities in HG-conditioned culture media, assessed by ELISA, were 0.13 ± 0.03 ng/ml and 0.15 ± 0.09 pg/ml, respectively, which were significantly lower compared to NG media (MMP-1, 0.36 ± 0.05 ng/ml; MMP-8, 0.31 ± 0.07 ng/ml, $P < 0.05$). In addition, there was also a significant decrease in the activity of MMP-13 in HG- compared to NG-conditioned culture media (HG, 0.24 ± 0.05 ng/ml; NG, 0.38 ± 0.04 ng/ml, $P < 0.05$) (Figure 4).

HG increases TIMP-1 and -2 mRNA and protein expression

Since the net activities of MMPs are determined by the interaction between MMPs and TIMPs, we examined mRNA and protein expression of TIMP-1 and -2. TIMP-1 and -2 mRNA expression were significantly increased in HPMCs exposed to HG medium compared to cells cultured under NG conditions ($P < 0.05$) (data not shown). The TIMP-1 and -2/GAPDH mRNA ratios were significantly higher in HG-stimulated HPMCs relative to NG cells by 148 and 79%, respectively ($P < 0.05$) (Figure 5). TIMP-1 and -2 protein expression, assessed by western blot, were also increased in HG-conditioned culture media compared to NG media. Densitometric quantitation
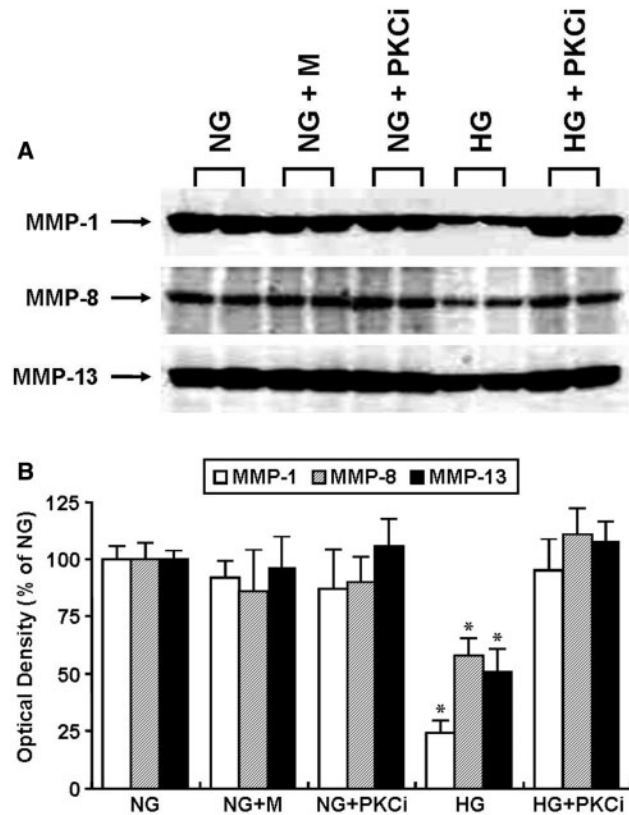

Fig. 3. (**A**) Western blot of collagenase protein in HPMCs exposed to 5.6 mM glucose (NG), NG + 34.4 mM mannitol (M) and 40 mM glucose (HG) with or without a PKC inhibitor, 10^{-7} M calphostin C (PKCi) (media) (representative of four blots). MMP-1, -8 and -13 protein expression were significantly decreased in HG-conditioned media compared to NG media. (**B**) Densitometric quantitation revealed 76, 42 and 49% decreases in MMP-1, -8 and -13 protein expression in HG- *vs* NG-conditioned culture media, respectively. Pre-treatment with calphostin C for 6 h ameliorated the decreases in MMP-1, -8 and -13 protein expression in HG-conditioned media. $^*P < 0.05$ *vs* other groups.
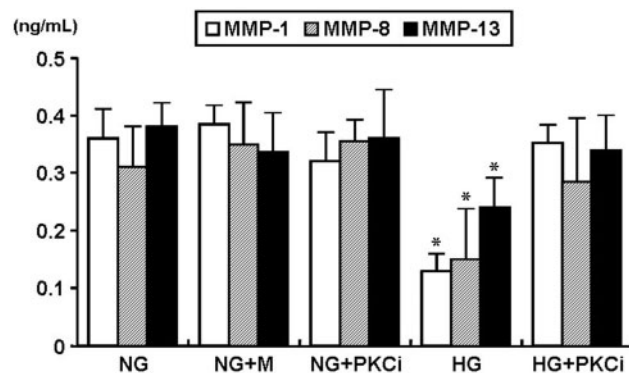

Fig. 4. The collagenase activities, assessed by ELISA, in HPMCs exposed to 5.6 mM glucose (NG), NG + 34.4 mM mannitol (M) and 40 mM glucose (HG) with or without a PKC inhibitor, 10^{-7} M calphostin C (PKCi) (media) ($n = 4$). MMP-1, -8 and -13 activities in HG-conditioned culture media were significantly lower compared to NG media. The HG-induced changes in their activities were ameliorated by calphostin C pre-treatment. $^*P < 0.05$ *vs* other groups.
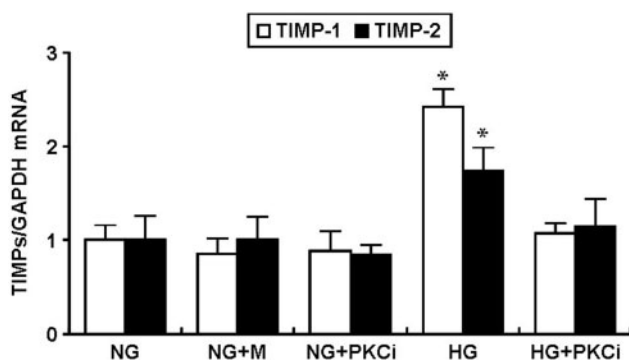
Fig. 5. TIMP-1 and -2/GAPDH mRNA ratios in HPMCs exposed to 5.6 mM glucose (NG), NG + 34.4 mM mannitol (M) and 40 mM glucose (HG) with or without a PKC inhibitor, 10^{-7} M calphostin C (PKCi) ($n = 5$). There were significant increases in TIMP-1 and -2/ GAPDH mRNA ratios in HPMCs cultured under HG conditions by 148 and 79%, respectively, compared to NG cells. Pre-treatment with calphostin C for 6 h nearly abolished the increases in TIMP-1 and -2/GAPDH mRNA ratios in HPMCs exposed to HG medium ($P < 0.05$). $^*P < 0.05$ vs other groups.

revealed a 69% increase in TIMP-1 protein and a 93% increase in TIMP-2 protein expression in HG- vs NG-conditioned culture media ($P < 0.05$) (Figure 6).

HG decreases collagenase expression and increases TIMPs expression via PKC-dependent pathway

Finally, we examined the effects of a PKC inhibitor, calphostin C, on the observed changes in MMP-1, MMP-8, MMP-13, TIMP-1 and TIMP-2 expression induced by HG. Pre-treatment with calphostin C for 6 h nearly abolished the decreases in MMP-1, -8 and -13/GAPDH mRNA ratios in HPMCs cultured under HG conditions, and their protein expression and activities in HG-conditioned culture media (Figures 2–4). The HG-induced increases in TIMP-1 and -2 expression were also abrogated by the PKC inhibitor (Figures 5 and 6).

Discussion

PF is one of the most serious complications of long-term CAPD, and leads to membrane failure [1]. It is pathologically characterized by a denudation of mesothelial cells from the peritoneum and the deposition of ECM [1,2]. It is well known that ECM accumulation is the result of an imbalance between the synthesis and degradation of ECM components. During biosynthesis, mesothelial cells in vitro have the capacity to produce a variety of matrix proteins, including collagen α1(I) and α1(III), laminin and fibronectin [6]. Several studies have shown that high glucose concentrations enhance their gene expression in HPMCs, resulting in ECM deposition [7,8]. In contrast, few studies have investigated the degradative processes of ECM in the pathogenesis of PF.
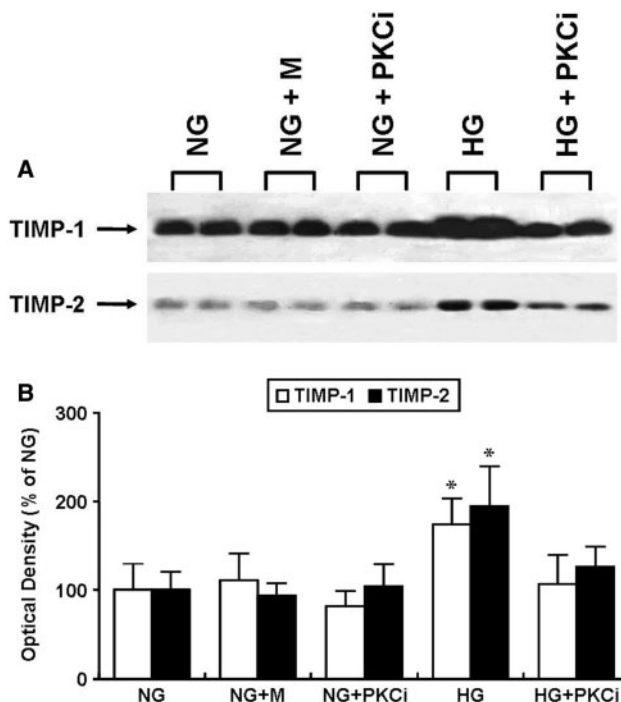

Fig. 6. (**A**) Western blot of TIMP-1 and -2 protein in HPMCs exposed to 5.6 mM glucose (NG), NG + 34.4 mM mannitol (M) and 40 mM glucose (HG) with or without a PKC inhibitor, 10^{-7} M calphostin C (PKCi) (media) (representative of four blots). TIMP-1 and -2 protein expression was significantly increased in HG-conditioned media compared to NG media. (**B**) Densitometric quantitation revealed a 69% increase in TIMP-1 protein and a 93% increase in TIMP-2 protein expression in HG- vs NG-conditioned culture media. Pre-treatment with calphostin C for 6 h ameliorated the increases in TIMP-1 and -2 protein expression in HG-conditioned media. $^*P < 0.05$ vs other groups.

The current study demonstrates for the first time that decreased collagenase expression along with increased TIMP-1 and -2 expression may contribute to ECM accumulation in PF.

The degradative process of ECM is mediated by key proteolytic enzymes, MMPs and TIMPs. The role of these enzymes in the pathogenesis of various diseases characterized by the deposition of ECM has been extensively explored. In particular, decreased production of matrix-degrading proteinases in conjunction with increased production of ECM is considered one of the pathogenetic mechanisms in the development of diabetic nephropathy [4,5,11,12]. Peritoneal mesothelial cells, like mesangial cells, have the capacity to produce matrix proteins [6]. Prior studies have shown that high glucose concentrations activate PKC and p38 mitogen-activated protein kinase, one of PKC's downstream signal pathways, thus leading to increased expression of type I collagen and fibronectin in cultured HPMCs [7,8]. Besides high glucose-induced synthesis of matrix proteins in HPMCs, some investigators have explored the degradative pathway of ECM to clarify the pathophysiology of ECM accumulation in PF in a similar manner as in diabetic nephropathy [9,10]. HPMCs are known to constitutively secrete MMP-2 and MMP-3, while MMP-9 mRNA and

protein expression in HPMCs are induced in response to the proinflammatory cytokine, IL-1β [9]. In addition, TGF-β increases TIMP-1, TIMP-2 and TIMP-3 mRNA expression in cultured HPMCs [9]. These findings suggest that these enzymes are important in the normal maintenance of peritoneal membrane integrity and in the changes that occur following prolonged peritoneal dialysis.

MMPs are Zn^{2+}-dependent endopeptidases which participate in ECM degradation. To date, at least 24 different vertebrate MMPs have been identified and they can be divided into six subgroups on the basis of substrate specificity, sequence similarity, and domain organization [16]: collagenases (MMP-1, -8, -13, -18); gelatinases (MMP-2, -9); stromelysins (MMP-3, -10, -11); matrilysins (MMP-7, -26); membrane-type MMPs (MT-1, -2, -3, -4, -5, MT6-MMPs); and other MMPs (MMP-12, -19, -20, -21, -23, -27, -28). It is well known that collagenases are primarily responsible for the degradation of type I, II and III collagen, whereas gelatinases are involved in the degradation of type IV, V and VI collagen, fibronectin and gelatin. Since type IV collagen and fibronectin are mainly accumulated in diabetic nephropathy [3], decreased expression and/or activities of gelatinases may be involved in the pathogenesis of diabetic nephropathy. In contrast, peritoneal mesothelial cells synthesize laminin, fibronectin, and type I and III collagen, but not type IV collagen [6]. Moreover, in a rat model of CAPD with 4.0% glucose, thickening of the peritoneum was observed along with a corresponding increase in the deposition of types I and III collagen by 92 and 32%, respectively, in the submesothelial interstitium [10]. Similar pathological findings with types I and III collagen accumulation were demonstrated in chemical peritonitis induced by intraperitoneal colloidal silica in rats [17]. In this study, we observed a significant increase in types I and III collagen protein expression in high glucose-conditioned culture media. Considering these findings, we thought that it was reasonable to investigate collagenases rather than gelatinases in the pathophysiology of ECM accumulation in PF. However, only one study by Higuchi *et al.* [10] investigated the changes in MMP-13 (but not MMP-1 or MMP-8) in mesothelial cells exposed to 4.0% glucose medium. They demonstrated not only an increase in MMP-13 mRNA expression but also significant decreases in type I and III collagen mRNA expression. However, they did not examine the effect of mannitol with same osmolality nor the cytotoxic effect of 4.0% glucose medium. In the preliminary experiments, we also observed decreases in type I and III collagen expression in 222.2 mM glucose and NG + 216.6 mM mannitol-conditioned media, but based on the results of the alamar blue assay we submitted that these changes in collagen expression were attributed to the cytotoxicity of hyperosmolality rather than the exclusive effect of high glucose. This study demonstrates for the first time that MMP-1, -8 and -13 expression and enzymatic activities were decreased by high glucose, which may

also contribute to type I and III collagen accumulation in PF.

MMPs are secreted in latent forms which are incapable of effecting proteolysis. Their activation is achieved by cleavage of the linkage between the cysteine in the proenzyme domain and Zn^{2+} in the catalytic site in which plasmin or stromelysins are involved [16]. Once activated, the ability of MMPs to degrade ECM is further modulated by TIMPs, specific inhibitors of MMPs which bind in a 1:1 stoichiometry [16]. Under pathological conditions associated with unbalanced MMPs activities, changes in TIMP levels are considered to be important because TIMPs directly affect the levels of MMP activities. A recent study demonstrated that TIMP-1 mRNA expression and secreted TIMP-2 protein were increased in mesothelial cells following TGF-β1 treatment [18]. Another study revealed that TIMP-1 activities were higher at the onset of peritonitis rather than either during the recovery phase of peritonitis or in control individuals, suggesting the involvement of TIMP-1 in peritoneal remodelling [15]. The present study demonstrates that TIMP-1 and -2 mRNA and protein expression are increased in HPMCs exposed to high glucose and in high glucose-conditioned culture media, respectively, resulting in reduced potency of MMPs to degrade ECM.

MMP and TIMP expression can be regulated by several factors including PKC [19]. Because the activity of PKC was increased by high glucose in mesangial cells [20], it was thought to be potential mechanisms capable of modifying MMP and TIMP expression in diabetes. In cultured HPMCs, high glucose also activated PKC, which largely mediated high glucose-induced TGF-β1 and fibronectin synthesis [7]. Therefore, we speculated as to whether the expression of MMPs and TIMPs in HPMCs were under the control of PKC as previously determined in other cell lines. The current study demonstrates for the first time that changes in the expression patterns of MMP-1, -8 and -13 as well as TIMP-1 and -2 by high glucose were ameliorated with the pre-treatment of a PKC inhibitor in HPMCs. These data suggest that the PKC pathway is responsible for increased synthesis and reduced degradation of ECM, leading to progressive accumulation of ECM and eventual PF.

In summary, high glucose decreased the activities and expression of collagenases and increased the expression of TIMPs in HPMCs or in conditioned media via the PKC pathway, resulting in accumulation of type I and III collagen. These findings suggest that reduced degradation of ECM may play an important role in the pathogenesis of PF.

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