

Original Article

Preservation of peritoneal morphology and function by pentoxifylline in a rat model of peritoneal dialysis: molecular studies

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Abstract

Background. High-glucose (HG) content of dialysate accelerated peritoneal fibrosis. We investigated *in vitro* mechanisms and the *in vivo* potential of pentoxifylline (PTX) to prevent this fibrogenic process.

Methods. For human peritoneal mesothelial cell (HPMC) culture, a normal-glucose (NG, 5.5 mM) or HG (138 mM) medium was established through pilot experiments. The rat peritoneal dialysis (PD) model consists of four groups ($n = 8$): group 1, intraperitoneal (IP) HG (4.25%) solution; group 2, as group 1 plus daily IP PTX (4 mg/in 1 h); group 3, IP PTX and group 4 as control.

Results. In HPMC culture, PTX significantly prevented HG-stimulated gene and protein production of collagen and transforming growth factor- β 1 (TGF- β 1) (reduction rate of 72–81%). The p38 mitogen-activated protein kinase (MAPK) pathway was activated significantly in HG-treated HPMCs. Blockade of p38 MAPK by SB203580 (25 μ M) or PTX (300 μ g/ml) resulted in an effective suppression of collagen and TGF- β 1 gene expression in HG-cultured HPMCs. In PD experimental animals, peritoneal thickness and collagen expression in the peritoneum were significantly increased in HG-treated rats, and was attenuated by PTX ($P < 0.01$). Impaired peritoneal ultrafiltration (1.9 ± 0.5 ml versus 2.4 ± 0.4 ml, $P < 0.05$) and stimulated proinflammatory IL-6, MCP-1 and TGF- β 1 activation were observed in HG-treated rats. PTX well preserved the functional characteristics of peritoneum and cytokine profiles.

Conclusions. These *in vitro* and *in vivo* data suggest that PTX may have therapeutic benefits for the prevention or retardation of peritoneal fibrosis.

Keywords: high glucose; mesothelial cell; pentoxifylline; TGF- β 1; fibrosis

Introduction

Peritoneal fibrosing syndrome (PFS) represents a wide spectrum of peritoneal alterations observed in long-term peritoneal dialysis (PD) patients [1]. PFS is characterized by extracellular matrix (ECM) accumulation such as collagen in the human peritoneum [2,3]. *In vitro* and *in vivo* studies have disclosed that the high-glucose (HG) content of PD solutions stimulate peritoneal ECM production of human peritoneal mesothelial cells (HPMCs) and fibroblasts. This aggravates the development of PFS [4–6]. Transforming growth factor- β 1 (TGF- β 1) has been regarded as the central mediator of the fibrosing process in clinical diseases. Continuous ambulatory peritoneal dialysis (CAPD) patients who have persistent TGF- β in their drained effluent were associated with an increased risk of PFS [7]. In addition, we previously reported that TGF- β 1 stimulates expression of type I and III collagen mRNA in cultured HPMCs [8]. Theoretically, HG can directly up-regulate and enhance collagen gene expression in cultured HPMCs, or indirectly through effects of TGF- β 1 [6,9,10]. Pharmacological interventions that can attenuate HG-stimulated TGF- β 1 expression and ECM accumulation in the peritoneum may have a therapeutic potential to prevent or retard PFS.

Pentoxifylline (PTX) is a clinically used anti-platelet agent. In addition to its anti-platelet effect, we previously reported that PTX exerts an anti-fibrotic effect on TGF- β 1-stimulated collagen gene expression in rat mesangial cells [11] and in HPMCs [12]. However, the *in vivo* effect of PTX in peritoneal preservation under HG stimulation has not been studied to date.

Three mitogen-activated protein kinase (MAPK) families have been identified in HPMCs. These are extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK) and p38 MAPK [8,13]. In mammalian cells, HG stimulates p38 MAPK activation and can interact with TGF- β 1 pathway [14–15]. Based on our previous findings that PTX can modulate p38 MAPK in HPMCs [12], we hypothesized that blockade of p38 MAPK activation by PTX may suppress the production of TGF- β 1 and the accumulation of ECM in HG-treated HPMCs.

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The aim of this study was to test the inhibitory effects of PTX in HG-treated HPMCs as well as TGF- β 1 production and collagen gene expressions. We also tested the ability of PTX to protect the peritoneum from HG-containing dialysate infusion *in vivo*. This study showed that p38 MAPK is involved in HG-stimulated TGF- β signalling and collagen gene expression in HPMCs and can be blocked by PTX. In addition, PTX can preserve the peritoneum from HG-stimulated morphologic and functional changes.

Materials and methods

Materials

Fetal calf serum (FCS) was obtained from Biochrome KG (Berlin, Germany). Culture flasks and plates were purchased from Corning (Corning, NY, USA) and pre-coated with 1.6 $\mu\text{g}/\text{cm}^2$ of Vitrogen 100[®] (Celtrix Lab, Palo Alto, CA, USA) before cell loading. Trypsin–EDTA, M-199 medium, glutamine and trypan blue were obtained from Gibco (Grand Island, NY, USA). Aprotinin, ATP, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), bovine serum albumin and other tissue culture reagents were purchased from Sigma (St Louis, MO, USA). Selective p38 MAPK inhibitor, SB203580, was obtained from Calbiochem (La Jolla, CA, USA). BCA reagents were obtained from Pierce (Rockford, IL, USA). Anti-phospho- and non-phospho-p38 MAPK antibodies were purchased from Cell Signaling (New England, MA, USA). Human collagen α 1 (I) cDNA was purchased from the American Type Culture Collection (Rockville, MD, USA). Agents used for isolating total RNA and northern blot analysis were obtained from Boehringer Mannheim (Mannheim, Germany) unless otherwise specified. PTX was generously provided by Aventis (Taiwan). All other chemicals used were of analytical grade.

Establishment of the HPMC culture

Specimens of human omentum were obtained from abdominal surgical procedures for elective gastric cancer resection. The omentum was grossly inspected as normal. The HPMC culture was performed as previously reported [13,16]. Briefly, the surgically removed human omentum was washed thrice with phosphate buffered saline (PBS) and then digested with trypsin–EDTA (0.125%, Gibco) for 15 min. After centrifugation, the cell pellet was washed with a culture medium and then seeded into a gelatin-coated (1 mg/ml) flask. The medium was changed on Day 3. M-199 medium containing 100 mg/dl glucose plus 20% FCS, penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and insulin (30 $\mu\text{g}/\text{ml}$) was used. After 2–4 days, the cells became confluent and were sub-cultured with a medium containing 10% FCS. HPMCs were identified by the presence of vimentin and cytokeratin, but without desmin and factor VIII-related antigen by the immunofluorescence method. All *in vitro* experiments were performed in passage 1–3 HPMCs.

Analysis of mRNA expression by reverse transcription–polymerase chain reaction and northern blots

To determine the effect of HG on gene expressions of type I collagen and TGF- β 1, HPMCs were grown in M-199 supplemented with 10% FCS until sub-confluence. HPMCs were growth-arrested by 0.5% FCS overnight and then treated in a normal-glucose (NG) or HG medium in the absence or presence of PTX. After 24 h, the cells were harvested for isolation of total RNA, as previously described [13]. TGF- β 1 RNA probes were synthesized with primer pairs as upstream, 5'-TCCACAGAGAAGAAGTCTG-3' (corresponding to bases from 1214 to 1233) and downstream, 5'-ACTTGCAGGAGCGCACAATC-3' (corresponding to bases from 1498 to 1517) [11]. For northern blotting, a 1.5-kb EcoRI fragment of collagen 1(I) was sub-cloned into pBSII/SK (Stratagene, La Jolla, CA, USA) and used as templates for *in vitro* transcription [11]. The signal intensity recorded on x-ray film was quantified by computerized densitometry and normalized against the signal of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages.

Cell preparations and protein extraction

HPMCs were grown in 10-cm dishes until sub-confluence, growth-arrested (0.5% FCS) overnight, and then harvested after treatment at indicated time points with a 200- μl ice-cold lysis buffer. The lysis buffer contained the following: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM β -glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 200 μM Na_3VO_4 , 10 μg leupeptin/ml, 200 units of aprotinin/ml, 1 μM pepstatin A, 1 mM PMSF and 100 nM okadaic acid. The obtained cell lysate was centrifuged at 12 000 rpm (13 400 g) for 20 min. The supernatant was collected and aliquoted. The protein concentration of the supernatant was measured by BCA protein assay (Pierce).

Western blot analysis

Western blot analysis of the p38 MAPK pathway was performed by using phospho-specific antibodies according to the manufacturer's protocol. Briefly, HPMCs were low-serum (0.5% FCS) starved overnight, and then stimulated with an HG or NG medium for 24 h. For evaluating the inhibitory effects of PTX and SB203580 on glucose-stimulated activation of the p38 MAPK pathway, we incubated HPMCs with PTX (30–300 $\mu\text{g}/\text{ml}$) or SB203580 (25 μM) for 30 min before stimulation with a glucose-containing medium. Cells were harvested 24 h later for western blotting. Cell lysates (20- μg protein) were separated by SDS–PAGE (12%), and then transferred to a PVDF membrane (Millipore, Bedford, MA, USA). For immunodetection, membranes were probed with a primary antibody followed by incubation with peroxidase-conjugated secondary antibodies. Bands were visualized by ECL system (Amersham).

Enzyme-linked immunosorbent assay for the measurement of protein levels of TGF- β 1 and type 1 collagen

Cell supernatants were frozen at -20°C until assay. An enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) was used to measure the concentration of TGF- β 1 in culture supernatant of HPMCs, as reported elsewhere [17]. To assess collagen synthesis, procollagen type I C-peptide (PICP) cleaved during collagen synthesis was quantified in culture supernatant of HPMCs by using a commercial EIA kit (Takara Bio Inc., Japan) according to the manufacturer's descriptions.

Animal model

We carried out *in vivo* experiments through management of male Wistar rats (weighing 200–250 g) maintained as our previous report [18]. We adopted an animal model of short-term PD developed by Hekking *et al.* [19]. This model demonstrated changes in peritoneal tissue after 5 weeks of HG exposure. The duration was extended to 8 weeks to evaluate the *in vivo* protective effect of PTX. Experimental manipulation was carried out in accordance with the American Association for Accreditation for Laboratory Animal Care (AAALAC) regulations.

Experimental model and design. After acclimation, rats were divided into three groups with eight rats per group. Group 1 received daily intraperitoneal (IP) installation of 20 ml PD4 (4.25% glucose, Baxter, USA) solution only and group 2 received the same manipulation as group 1 plus additional infusion (IP) of PTX (4 mg/rat). Group 3 received daily IP infusion of PTX (4 mg in 20 ml 0.9% saline/rat) only. Eight additional healthy rats receiving IP infusion of 20 ml 0.9% saline were used as control. The experiments were performed daily for a total duration of 8 weeks. During experiments, rats were provided with food and water *ad libitum*.

Peritoneal function test. At the end of the study, a 1-h peritoneal equilibration test (PET) was performed on each experimental animal. The procedures were performed without peritoneal injury. Twenty millilitre of 2.5% PD solution was slowly injected intraperitoneally. After a 1-h dwell, the rats were anaesthetized with ketamine and the peritoneal cavity was opened for dialysate collection and peritoneal samplings. Individual blood samples were obtained at the same time. To establish PET profiles, we defined peritoneal ultrafiltration (UF) function by calculating volume differences between the instilled and drained dialysate. Dialysate-to-plasma ratio of creatinine (D/P creatinine) and glucose re-absorption (D_1/D_0 glucose) were measured to represent peritoneal characteristics of experimental rats in all four groups.

Histological analysis. At the time of sacrifice, peritoneal tissue specimens from the abdominal wall, the gut with the mesentery, and from the liver were taken for histological analysis as described before [18]. After formalin fixation and paraffin embedding, tissue sections were cut at a 4- μm thickness and processed by standard haematoxylin

and eosin stain and Masson's trichrome stain. The thickness of sub-mesothelial tissue (SMT) at the liver surface of individual rats was blindly evaluated (by Dr J-W.H.). Each tissue section was measured at five random locations. Three tissue sections were examined per rat. The average thickness (μm) of each rat was recorded for statistical analysis between different groups.

Northern blot analysis of collagen content. Tissue samples of omentum and the right upper quadrant of the abdominal wall obtained from sacrificed rats were immediately frozen in an ethanol/dry-ice bath and stored at -70°C . Total RNA was extracted from homogenized tissue by using TRIzol reagent (Life Tech., USA) according to the manufacturer's protocol. Total RNA (20 $\mu\text{g}/\text{lane}$) was electrophoresed on a 1% agarose/1 M formaldehyde gel and then transferred to a nylon membrane. Hybridization, washing and autoradiography for northern blot analysis were performed as described in cultured HPMCs.

Cytokine profiles of peritoneal lavage. After anaesthesia and before sacrifice, fresh 10 ml 0.9% saline was infused (IP) into each experimental rat, indwelled for 30 min, and then carefully recovered through opening of the peritoneal cavity. The appropriate time for lavage harvest is determined through serial examination of the amount and cytokine appearance at preliminary experiments (private data). The lavage fluid was spun down for removal of possible cellular debris. Supernatants were then immediately collected and frozen at -20°C until assay, as described for ELISA analysis in cultured HPMCs. We studied the levels of IL-6, MCP-1, ICAM-1 and TGF- β 1 with ELISA kits (R&D Systems).

Statistical analysis

All values were expressed as means \pm standard deviation (SD). Significance of results was determined with Instat 3.0 (GraphPad, Sacramento, CA, USA). Comparisons were performed using an unpaired Student's *t*-test, and $P < 0.05$ was considered to indicate a significant difference.

Results

Pilot study to establish HG condition in experiments of HPMCs

In Taiwan, the PD dialysate (Dianeal[®], Baxter) currently used contains 1.5%, 2.5% and 4.25% glucose, equal to glucose concentrations of 83 mM, 138 mM and 236 mM, respectively. Three types of culture media, based on M-199 medium plus additional glucose to achieve the final glucose concentrations of 83 mM, 138 mM, and 236 mM, were established before experiments. At first, HPMCs were grown in 10-cm dishes until sub-confluence, growth-arrested (0.5% FCS) overnight and then transferred to indicated culture media for experiments.

We examined the effects of glucose on collagen α 1(I) gene expression and TGF- β 1 production in HPMCs. As shown in Figure 1, media containing glucose higher than

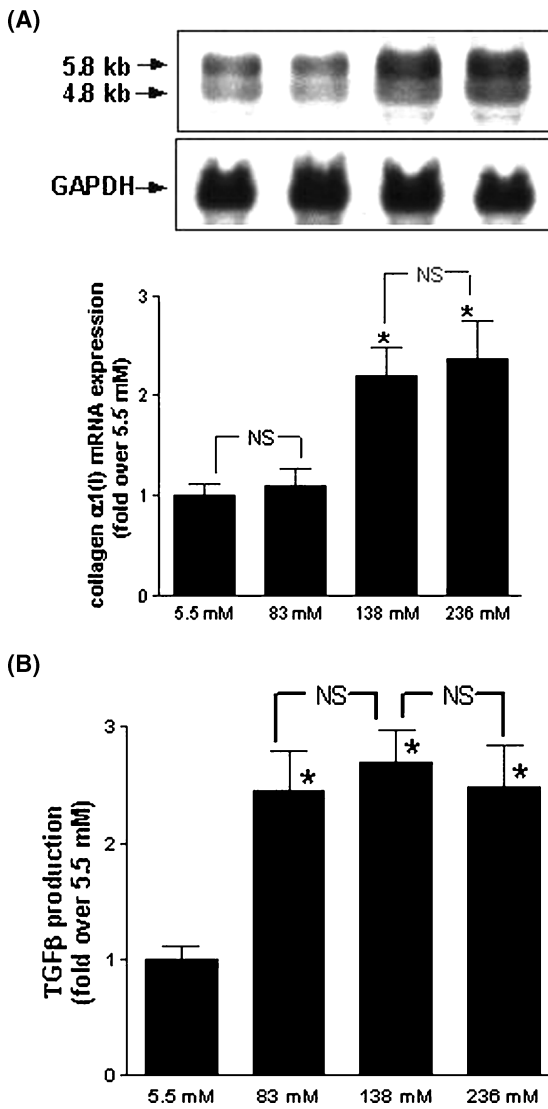


Fig. 1. (A) Type I collagen mRNA expression in HPMCs exposed to various concentrations of glucose for 24 h. A representative northern blot from experiments performed in quadruplicate is illustrated in the upper part of the figure. The mean (\pm SD) ratio of collagen $\alpha 1(I)$ mRNA over GAPDH was calculated from four experiments. Values shown in the graph are fold increase over control (NG, 5.5 mM) condition. (B) Effects of glucose concentration on the amount of TGF- $\beta 1$ production in HPMCs. Assay of TGF- $\beta 1$ production of HPMCs was performed by using an ELISA kit as described in the Methods section. Values are expressed as mean (\pm SD) from four experiments. * $P < 0.05$, compared to 5.5 mM.

138 mM significantly up-regulated the expression of collagen $\alpha 1(I)$ mRNA and the protein production of TGF- $\beta 1$ in HPMCs. The aim of this study was to test if PTX can prevent the intracellular signalling mediating the fibrogenic process of HG-stimulated HPMCs. We therefore defined the HG and NG media of these experiments as media containing glucose concentrations of 138 mM and 5.5 mM, respectively.

In previous dose-response experiments [12], we demonstrated that PTX at a concentration of 30–300 μ g/ml dose dependently reduced serum-stimulated collagen synthesis in HPMCs. Through cell viability tests, the trypan blue

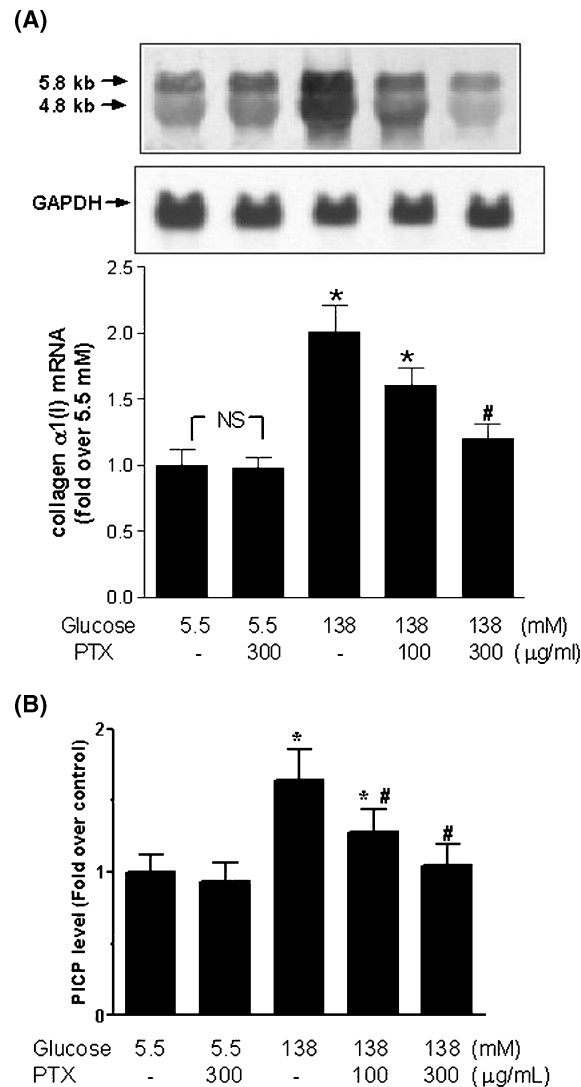


Fig. 2. (A) Type I collagen mRNA expression and (B) protein levels of procollagen type I C-propeptide (PICP) in HPMCs cultured in the NG (5.5 mM) or HG (138 mM) medium for 24 h. (A) PTX dose dependently suppressed gene expression of collagen $\alpha 1(I)$ in HPMCs exposed to HG media. Data from one representative experiment performed in quadruplicate are illustrated in the top part of the figure. The mean (\pm SD) ratio of collagen $\alpha 1(I)$ mRNA over GAPDH was calculated from each experiment ($n = 4$). Values shown in the lower panel are fold increases over that of the NG (5.5 mM) condition. (B) Protein synthesis of PICP in HPMCs. Values (mean \pm SD) in the graph are shown as fold increase over control ($n = 4$). * $P < 0.05$, compared to NG (5.5 mM) control; # $P < 0.05$, significantly lower than the HG culture (lane 3).

exclusion method and by measuring the lactate dehydrogenase (LDH) activity of the supernatant [12,17], we also excluded the potentially toxic effects of PTX and such concentrations of glucose media under these experiments.

In vitro studies

Effect of PTX on HG-induced type I collagen gene expression and TGF- $\beta 1$ production in HPMCs. We evaluated the ability of PTX to reduce HG-induced type I collagen and TGF- $\beta 1$ gene expression as well as individual protein productions in HPMCs. As shown in Figure 2, $\alpha 1(I)$

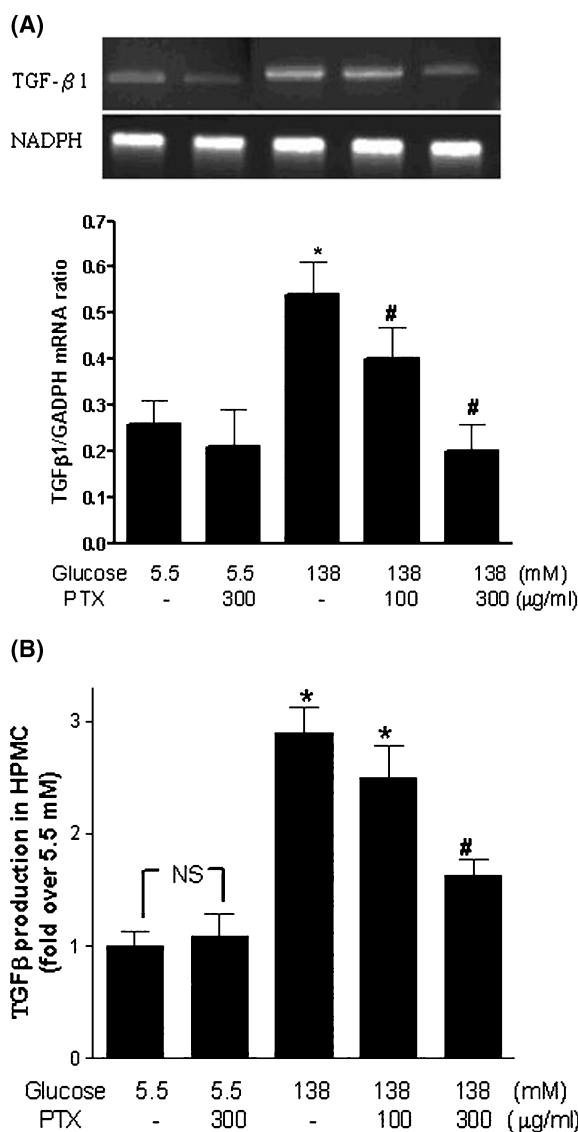


Fig. 3. PTX reduced the HG-induced increase in (A) TGF-β1 gene expression, and (B) protein production in cultured HPMCs. (A) Representative results of RT-PCR from four isolated experiments with similar results shown in the upper panel. Results of densitometric analysis ($n = 4$) shown in the lower panel. (B) Protein level of TGF-β1 production in HPMCs. Values (mean \pm SD) in the graph expressed as fold increase over control ($n = 4$). * $P < 0.05$, compared to 5.5 mM; # $P < 0.05$, significantly lower than the HG culture (lane 3).

collagen mRNA expression and procollagen type I C-peptide (PICP) synthesis were significantly higher under the HG condition (1.8-fold and 1.6-fold, respectively). PTX dose dependently reduced the increment by 81% and 72%, respectively. Compared with the NG condition, the HG culture also increased TGF-β1 mRNA levels and protein production in HPMCs (Figure 3). Lower doses of PTX (100 μg/ml) partially (21.1%) reduced the HG-increased production of TGF-β1 in HPMCs. This suppressive effect was more prominent (67.4%, $P < 0.05$) when 300 μg/ml PTX was added to the HG medium. PTX had no significant inhibition on protein levels of TGF-β1 and α1(I) collagen production of HPMCs cultured in NG media (Figures 2 and 3).

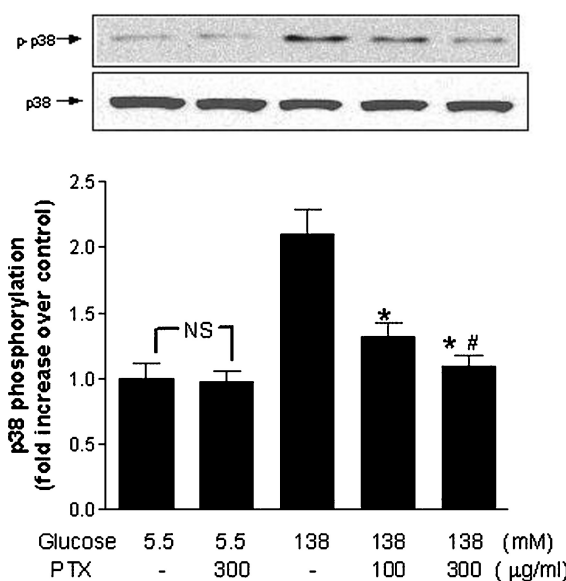


Fig. 4. Inhibitory effect of PTX on p38 phosphorylation in HPMCs exposed to HG (138 mM) for 24 h. A representative blot from one experiment out of four with similar results is shown at the top. The mean results of densitometric analyses from four separate experiments are shown at the bottom. Values in the graph are expressed as fold increases over control. PTX did not influence p38 activity in NG-treated HPMCs. * $P < 0.05$, relative to the HG culture (lane 3); # $P = NS$, compared to NG control (lane 1).

HG-stimulated p38 MAPK activation in HPMCs suppressed by PTX. We tested our hypothesis that the increased TGF-β1 production and α1(I) mRNA expression in HG-treated HPMCs is mainly through activation of the p38 MAPK pathway. The p38 MAPK activity was assayed using immunoblotted anti-phospho-p38 and anti-p38 antibody, respectively, as previously described [8,13]. HPMCs were exposed to either NG (5.5 mM) or HG (138 mM) for 24 h. As shown in Figure 4, PTX dose dependently prevented the activation of p38 MAPK by HG in HPMCs. PTX at doses of 100 μg/ml and 300 μg/ml suppressed HG-stimulated p38 activity by 71% and 91.8%, respectively. In contrast, there was no significant difference in p38 phosphorylation when PTX (300 μg/ml) was added to the NG medium.

Blockade of p38 MAPK activity suppressed HG-induced TGF-β1 production and collagen α1(I) gene expression in HPMCs

As PTX inhibited the enhanced fibrogenic gene expression and the p38 MAPK activation by HG in HPMCs (Figures 2 and 4), we assumed that the former result of PTX was originated from the latter effect by PTX. To test this hypothesis, we cultured HPMCs in HG with SB203580, a specific inhibitor of p38 MAPK activity, for 24 h. Addition of SB203580 (25 μM) completely inhibited the activity of p38 in HG-treated HPMCs (data not shown). We then demonstrated that SB203580 (25 μM) significantly suppressed gene expression of collagen α1(I) and protein production of TGF-β1 of HPMCs in response to HG (Figure 5). The data indicated that PTX can inhibit TGF-β1

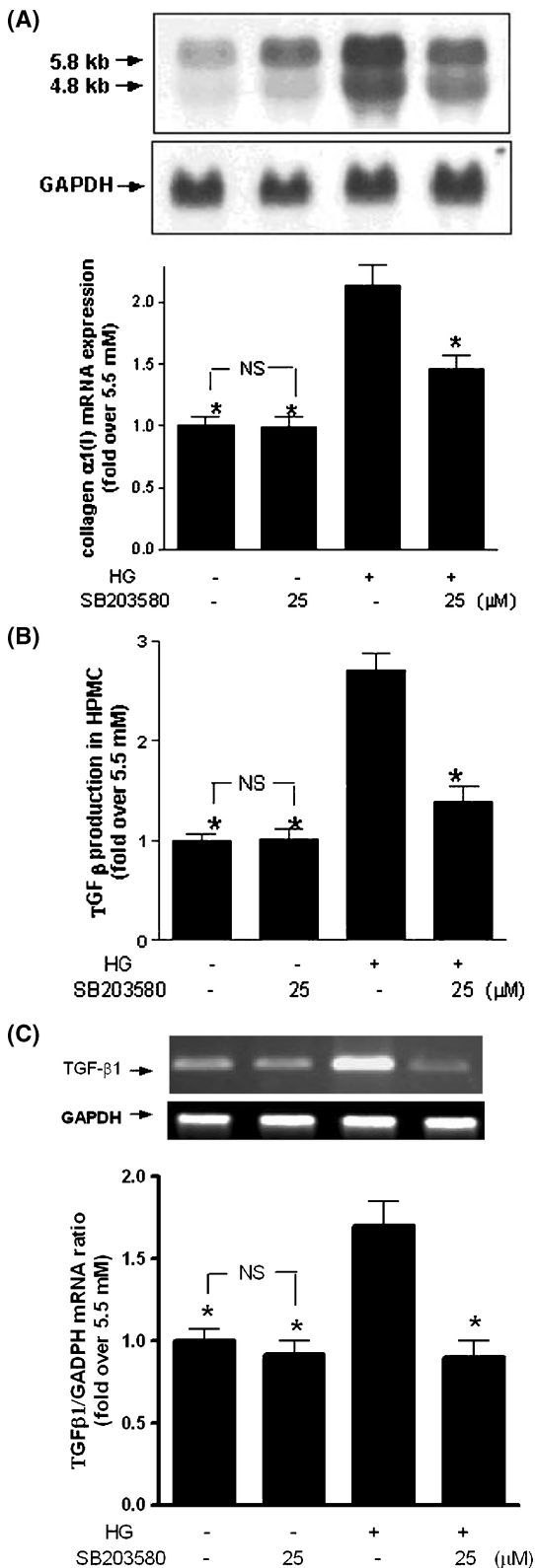


Fig. 5. Effect of SB203580 on (A) $\alpha 1(I)$ collagen mRNA expression and (B) TGF- $\beta 1$ protein production of HPMCs. Cells were incubated with HG (138 mM) media in the presence of various concentrations of SB203580 (25 μ M) for 24 h. Results of densitometric analyses from four isolated experiments with similar results are shown. Values shown in the graph are fold increases over control. * $P < 0.05$, compared to lane 3 (HG alone).

and collagen production of HG-treated HPMCs through suppression of the p38 MAPK pathway.

In vivo studies. PTX is an anti-platelet agent. In this work, PTX was administered via IP route; therefore, the risk of bleeding is expected to be low. During experiments, we did not find significant changes in the blood platelet count and bleeding time in rats receiving IP injection of PTX. The haemoglobin level was 18.1 ± 0.2 g/dl and 17.4 ± 0.2 g/dl before and after PTX, respectively ($P = \text{n.s.}$). The platelet counts were 874 ± 110 ($10^3/\text{mm}^3$) and 942 ± 120 ($10^3/\text{mm}^3$) before and after PTX, respectively ($P = \text{n.s.}$), and the bleeding times were 132 ± 10 (s) and 143 ± 14 (s) before and after PTX, respectively ($P = \text{n.s.}$).

Histological analysis. At the time of sacrifice and by gross inspection of the opened abdominal cavity, we found no gross adhesion between abdominal tissues of rats in four experimental groups (data not shown). We then checked tissue fibrosis and the thickness of SMT between experimental groups. Figure 6 shows photomicrographs of representative sections obtained from experimental animals. In control rats treated with saline alone, no fibrosis was observed in the histology specimen. In comparison, an increased connective tissue deposit and thickened SMT at the liver surface was noted in rats (group 1) that received daily HG indwell for 8 weeks. In rats treated with PTX, in addition to HG infusion (group 2), there was no prominent collagen deposit or increment of the SMT connective tissue thickness, compared with that of group 1 ($P < 0.05$). Figure 6(E) shows results of the quantitative comparison of the mean SMT thicknesses between treatment groups. Group 1 rats had the highest mean value (0.88 ± 0.1 μ m) of SMT thickness, and rats treated with PTX (group 2) had a significantly reduced SMT thickness (0.42 ± 0.05 μ m, $P < 0.05$). Control rats and rats that received IP PTX only did not show a statistical difference between individual thicknesses of SMT layers (Figure 6E).

Northern blot analysis. Northern blot analysis was applied to detect collagen $\alpha 1(I)$ mRNA transcripts in peritoneal samples of omentum tissues obtained from four groups. The collagen gene expression was significantly increased in samples from group 1 rats that received HG IP infusion. We found that PTX treatment can effectively reduce the collagen gene expression within peritoneal tissue ($P < 0.05$, Figure 7).

Cytokine profiles of peritoneal lavage. Table 1 shows the results of the PET analysis and the cytokine profiles obtained from peritoneal lavage of experimental rats. The lavage values of IL-6 and MCP-1 were significantly higher in HG-treated rats (group 1), compared with those of PTX-treated (group 2) and control rats. TGF- $\beta 1$ levels of peritoneal lavage from group 1 (HG-treated) rats were significantly higher than those treated with PTX or control rats. In addition, ICAM-1 levels of lavage in group 1 rats were higher than those in the PTX-treated (group 2) or control rats, but the results were not statistically significant.

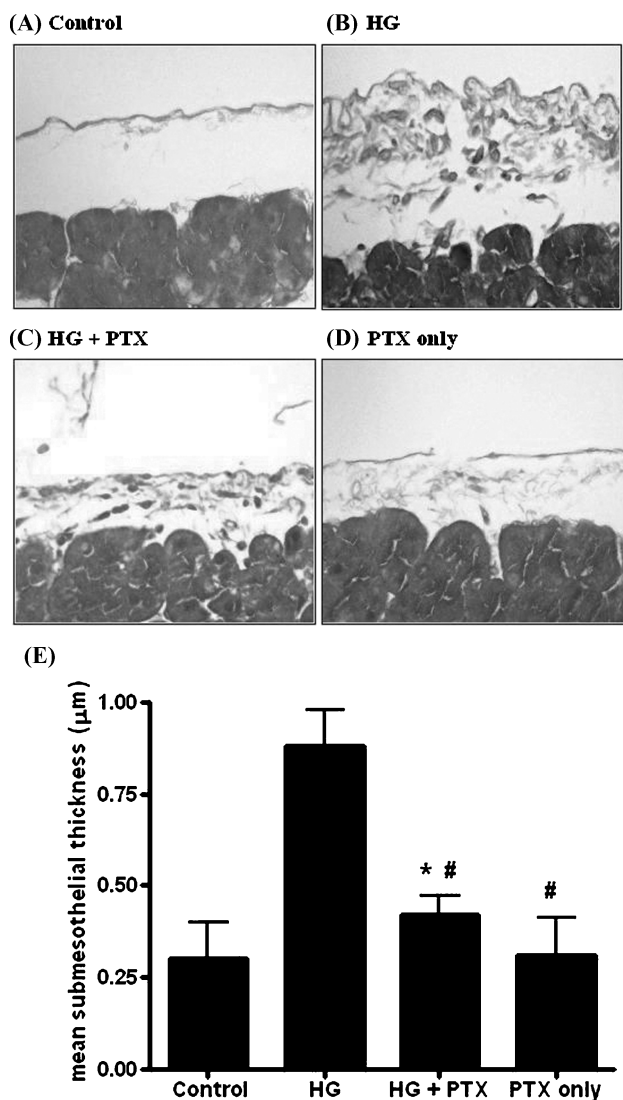


Fig. 6. Histological assessment of fibrotic reaction in peritoneal tissues. (A) Control; (B) HG treated; (C) HG + PTX group and (D) PTX only. Representative sections of Masson's trichrome-stained peritoneum showing fibrotic thickening between mesothelial layers and the liver surface (original magnification 200 \times). (E) Quantitative comparisons of connective tissue thickness between different groups. Thickness, in μm , was assessed using Masson trichrome's stain. * $P < 0.05$ compared to group 2.

Cytokine profiles of rats treated with PTX only (group 3) were not different from controls.

PET study. Peritoneal functional changes were evaluated as for PET analysis performed in clinical practice. As shown in Table 1, no statistical differences of D/P creatinine and D_1/D_0 glucose were found between all four groups. However, HG-treated rats (group 1) had a relatively higher mean value of D/P creatinine than the other three groups ($P = 0.72$). HG-treated rats (group 1) had a trend of lower values of D_1/D_0 glucose and lower UF volume compared with the other three groups ($P = 0.79$ and $P = 0.29$, respectively). PTX therapy alone did not result in altered PET profiles from those of controls.

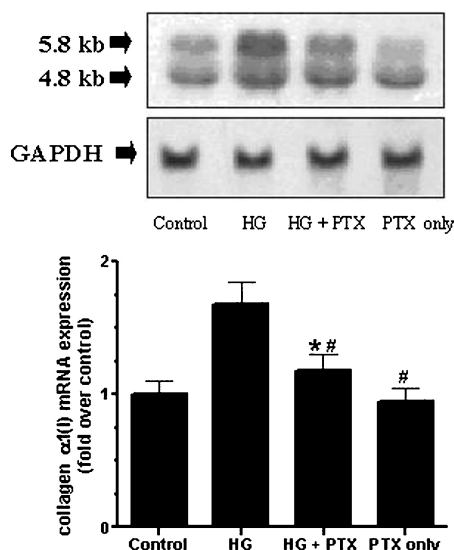


Fig. 7. Gene expression of collagen $\alpha 1(I)$ in peritoneal tissue of omentum tissue obtained from experimental rats was shown. Expression of collagen $\alpha 1(I)$ mRNA was significantly increased in group 1 (rats treated with hypertonic PD solution), and was significantly reduced by intraperitoneal infusion of pentoxifylline (group 2). PTX only did not result in significant change in collagen $\alpha 1(I)$ mRNA in rats without exposure to a hypertonic PD solution. Values (mean \pm SD) are shown as fold increases over control, analysed from four separate experiments. * $P < 0.05$, compared to control; # $P < 0.05$, significantly lower than the HG group.

To summarize, we demonstrated in these experimental rats that HG exposure of the peritoneal cavity can stimulate early peritoneal fibrotic change (SMT thickness) as well as enhanced collagen gene expression in peritoneal tissue. Both histological changes of the peritoneum can be effectively prevented by PTX. These anatomical alterations in the peritoneum were accompanied by early, but relatively minor changes in peritoneal function. In addition, the pro-inflammatory status of the peritoneal cavity under HG stimulation can be largely preserved with PTX therapy.

Discussion

Through *in vitro* experiments, we showed that the p38 MAPK pathway is activated in HG-treated HPMCs (Figure 4), and is required for the increased production of TGF- $\beta 1$ and type I collagen in HG-treated HPMCs (Figure 5). Our *in vivo* experiments demonstrated the therapeutic potential of PTX for PFS with a direct inhibitory effect on matrix accumulation of peritoneum, and also the safety concern at long-term clinical application. Since TGF- $\beta 1$ is a multiplicative growth factor participating in numerous functions of various cell types in the body [20], chronic over-suppression of TGF- $\beta 1$ may have a safety concern. In this study, PTX reduced TGF- $\beta 1$ production only in the HG-treated HPMCs, but results were non-significant in the NG media. IP infusion of PTX in healthy or saline-treated rats did not result in alterations of peritoneal morphology or peritoneal function. These safety and therapeutic advantages of PTX support its clinical application to preserve the peritoneum during PD therapy.

Table 1. Results of peritoneal equilibrium test (PET) analysis and cytokine profiles in different groups of experimental rats

	Experimental groups			
	Control <i>n</i> = 8	1 (HG indwell) <i>n</i> = 8	2 (HG indwell + PTX) <i>n</i> = 8	3 (PTX only) <i>n</i> = 8
PET analysis				
D/P creatinine	0.52 ± 0.14	0.60 ± 0.14	0.54 ± 0.16	0.55 ± 0.13
D ₁ /D ₀ glucose	0.58 ± 0.12	0.51 ± 0.12	0.55 ± 0.16	0.56 ± 0.18
Ultrafiltration (ml)	2.4 ± 0.4	2.0 ± 0.5	2.3 ± 0.5	2.1 ± 0.4
Peritoneal lavage				
IL-6 (pg/ml)	80.1 ± 36.2	212.1 ± 38.6 [#]	118.1 ± 40.0	122.1 ± 44.3
MCP-1 (pg/ml)	88.2 ± 12.6	146.5 ± 38.4 [#]	110.5 ± 26.7	104.3 ± 24.8
ICAM-1 (ng/ml)	0.23 ± 0.15	0.38 ± 0.21	0.27 ± 0.25	0.24 ± 0.18
TGF-β1 (ng/ml)	2.1 ± 0.5	4.8 ± 1.0 [#]	3.5 ± 0.9 [#]	3.0 ± 1.1

HG = high glucose; PTX = pentoxifylline; D/P creatinine = dialysate-to-plasma ratio of creatinine; D₁/D₀ glucose = glucose concentration in drained dialysate at 1 h (D₁) and at original fresh dialysate before indwell (D₀); peritoneal ultrafiltration (UF): the volume difference between the instilled and drained dialysate.

[#]*P* < 0.05 versus control rats.

Based on our observations in this work, three clinically therapeutic implications of PTX were established. Firstly, PTX may prevent the development of PFS by effectively suppressing the production of TGF-β1 in HG-stimulated HPMCs (Figure 3). HG [9–10] or pro-inflammatory cytokines [16,21] can induce TGF-β1 in HPMCs. In CAPD patients, an increased gene expression of the TGF-β1 is associated with an increased risk of PFS [7,22]. The reduction of TGF-β1 production in HPMCs by PTX may implicate the potential of PTX for the prevention of PFS. We also provided *in vivo* experiments demonstrating that IP PTX effectively reduced HG induction of increased dialysate production of TGF-β1 and pro-inflammatory cytokines, including the IL-6, MCP-1 and TGF-β1 (Table 1). However, the mechanisms through which PTX attenuates TGF-β1 production remain undetermined. PTX can act directly by blocking HG-stimulated TGF-β1 production, or indirectly through manipulation against pro-inflammatory cytokines (IL-6, MCP-1) (Table 1) provoked during IP HG infusion. Experiments on a special species of knock-out rats with similar design may clarify this hypothesis.

The second clinical implication of PTX originated from its effectiveness to suppress collagen gene expression and reduced SMT thickness, as demonstrated in our *in vitro* and *in vivo* experiments (Figures 2, 6 and 7). In addition, those beneficial effects of PTX may be potentiated further by the reduced local production of TGF-β1. In the *in vitro* model, we demonstrated that p38 MAPK is involved in HG-activated collagen α1(I) gene expression, which can be suppressed by PTX (Figures 2 and 4). This p38 MAPK cascade theoretically might not be the only signalling pathway responsible for HG-induced collagen gene expression in HPMCs. The near complete blockade of the p38 MAPK activity by SB203580 (25 μM) resulted in only partial (60%) suppression of increased collagen α1(I) gene expression in HG-treated HPMCs (Figure 5). A recent study by Chen *et al.* demonstrated that protein kinase C (PKC) pathways can synergistically interact with MAPK to induce mesangial fibrosis [23]. Effects of PTX on other signalling pathways,

such as the PKC-mediated fibrogenic response in HPMCs, require further research.

The third clinical implication and also a novel aspect of PTX is its anti-inflammatory effects in HG-treated rats. Mesothelial cells can synthesise IL-6 and MCP-1 that then mediate the development of PFS [24–26]. The immunomodulatory effect of PTX on MCP-1 production through increased intracellular cAMP levels was previously reported [11]. The reduction of IL-6 and MCP-1 in lavage from IP HG-treated rats is compatible with the morphological change observed in PTX-treated experimental rats. In contrast to the significant increase of IL-6 and MCP-1 in HG-treated rats, ICAM-1 level in peritoneal lavage tends to increase in HG-treated rats. This result was not statistically significant (Table 1). However, as the ICAM-1 is dominant under conditions with significant peritoneal adhesion [27–28], a reasonable explanation for this observation could be that our PFS model is too mild to induce a gross adhesion between peritoneal tissues. We tested the induction of these pro-inflammatory cytokines in more advanced peritoneal fibrosis models that were reported elsewhere [18]. ICAM-1 could be significantly induced together with large production of IL-6 and MCP-1 (unpublished data). Longitudinal clinical studies examining the role of peritoneal ICAM-1 levels in predicting peritoneal adhesion under medical and surgical conditions may be of value.

In this work, we did not measure the formation of GDP within the peritoneal cavity of experimental rats. As we found during experiments that almost all of IP infusion fluids were well absorbed by these experimental rats, the chance of GDP formation was expected to be low due to limited time to formation. Fluorescent evaluation of AGE formation in the peritoneal tissue with a spectrofluorimeter could be helpful in further studies.

Compared with our previous peritonitis rat model of PFS [18], this HG rat model is more appropriate for clinical PD situations. Hypertonic PD solutions still comprise nearly 90% of PD fluid prescribed worldwide. In this chronic PD model, although we failed to produce a gross fibrotic

change in the peritoneum after 8 weeks of IP HG treatment, PTX can still effectively preserve peritoneal function as revealed in the PET profile of experimental rats (Table 1). It has been known that in the early stage of PFS, only sub-clinical anatomical and minimal functional alterations can be identified [28–30]. Our animal model therefore can be regarded as the early stages of PFS. The observed peritoneal fibrotic changes (Figures 6 and 7) and cytokine profiles in peritoneal lavage (Table 1), together with alterations of PET profiles towards higher transporter and less UF characteristics in HG-treated rats are compatible with what we learned from asymptomatic long-term PD patients [31–32]. A correlation study between SMT thickness and individual PET data among a larger sample of experimental rats could establish a relationship between anatomical and functional changes after PD therapy. The early application of PTX to preserve the peritoneum with PD therapy is of value, since chronic exposure of the peritoneum to hypertonic PD solution may change the peritoneal character and may predispose to worse patient and technique outcomes [31–33].

In conclusion, we demonstrated that PTX, by multidisciplinary inhibition of HG-induced fibrogenic effects in HPMCs accompanied by structural and functional preservation, can provide therapeutic potential for the prevention of PFS.

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Conflict of interest statement. None declared.

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