Regulation of CCN2/CTGF and related cytokines in cultured peritoneal cells under conditions simulating peritoneal dialysis

Joseph C. K. Leung1, Loretta Y. Y. Chan1, Ka Ying Tam1, Sydney C. W. Tang1, Man Fai Lam1, Amy S. Cheng1, Kent Man Chu2 and Kar Neng Lai1

1Department of Medicine and 2Department of Surgery, Queen Mary Hospital, University of Hong Kong, Pokfulam, Hong Kong

Abstract

Background. Continuous ambulatory peritoneal dialysis (CAPD) is a major treatment modality for end-stage renal failure. The peritoneal membrane exhibits pathological changes that correlate with the duration of dialysis. These changes are due to the exposure of the peritoneum to non-physiologic peritoneal dialysis solution (PDS) with a high glucose content, and containing potentially toxic substances including glucose degradation products (GDP) and advanced glycation end products (AGE). Connective tissue growth factor (CTGF/CCN2) is one of the determinants of progressive fibrosis and peritoneal membrane dysfunction in CAPD. In this study, we examined the CCN2 expression and its regulation in peritoneal resident cells using a cell culture model.

Methods. The expression of transforming growth factor-β (TGF-β), CCN2 and vascular endothelial growth factor (VEGF) in human peritoneal mesothelial cells (HPMC), human peritoneal fibroblasts (HPF) or endothelial cell line EA.hy926 (EC) cultured with various PDS and their components was examined by quantitative PCR (qPCR). The modulation of CCN2 synthesis under the crosstalk between HPMC and HPF or EC was examined using a conditioned medium transfer system in which HPMC was exposed to conditioned media obtained from HPF or EC incubated with PDS and their components. The differential effects of TGF-β, CCN2 and VEGF in inducing the expression of transcriptional factors as well as interleukin-6 (IL-6), matrix metalloproteinase 9 (MMP-9) and collagen I mRNA expression.

Results. PDS and their components differentially modulated the expression of TGF-β, CCN2 and VEGF in HPMC, HPF and EC. The expression of CCN2 by HPMC was significantly increased after cultured with a HPF-conditioned medium and an EC-conditioned medium. CCN2, TGF-β and VEGF activated distinct transcriptional factors in HPMC, which resulted in divergent biological responses in terms of IL-6, MMP-9 and collagen I mRNA expression.

Conclusion. AGE and GDPs in PDS differentially regulate the synthesis of CCN2 by peritoneal resident cells. The CCN2 synthesis by HPMC can be further amplified by TGF-β released from HPF or EC. The differential activation of different transcriptional factors and diverse response of HPMC towards CCN2, TGF-β and VEGF suggest that these cytokines/growth factors have an overlapping and distinct role on HPMC.

Keywords: CAPD; CTGF/CCN2; mesothelial cell; TGF-β; VEGF

Introduction

Continuous ambulatory peritoneal dialysis (CAPD) is a major treatment modality for end-stage renal failure. The peritoneal membrane exhibits structural changes that correlate with the duration of dialysis. The pathological changes in the peritoneum are due to its exposure to non-physiologic peritoneal dialysis solution (PDS) with a low pH and high glucose content [1], and containing potentially toxic substances including glucose degradation products (GDP) and advanced glycation end products (AGE) [2,3]. These compounds cause irreversible damage to the peritoneum and may initiate the process of peritoneal fibrosis that is commonly found in patients under long-term CAPD. Transforming growth factor-β (TGF-β) is an important pro-fibrotic cytokine that has been suggested to affect the peritoneal membrane function during CAPD [4]. Connective tissue growth factor (CTGF/CCN2) is another potential candidate that may play a role in progressive fibrosis of the peritoneum during CAPD. In peritoneal mesothelial cells, CCN2 is inducible by TGF-β [5] and is up-regulated during peritonitis. In many systems, CCN2 acts both as a pro-fibrotic marker and as an effector in the downstream cascade of fibrosis induced by TGF-β [6]. CCN2 induces
fibronectin expression by renal fibroblast and is an important mediator in TGF-β-induced tubulointerstitial fibrosis [7]. In renal epithelial cells, the profibrotic effect of TGF-β is exerted through CCN2 which can be blocked by the hepatocyte growth factor (HGF) [8]. Neutralizing antibodies against CCN2 inhibits collagen synthesis in fibroblasts induced by TGF-β [9]. TGF-β is a pleiotropic growth factor that affects the normal functions of different cell types. Some of the pro-fibrogenic properties of TGF-β are associated with the induction of CCN2 by TGF-β. Blockade of CCN2, rather than TGF-β, may be a better target for selective intervention in the process involved in TGF-β-dependent tissue fibrosis. We hypothesize that residual peritoneal cells (including peritoneal mesothelial cells, fibroblasts and endothelial cells) are activated and produce CCN2 following exposure to non-physiologic PDS. The interaction between CCN2 and various angiogenic and fibrogenic factors from peritoneal cells may lead to peritoneal membrane dysfunction. Our present study aims to examine the peritoneal cell expression of CCN2 and its interaction with various peritoneal cells in the process of peritoneal fibrosis.

Methods

Materials

All reagents for tissue culture were obtained from Invitrogen Co. (Carlsbad, CA, USA). The GDP preparations of methylglyoxal (M-Glx), 3-deoxyglucose (3-DG) and 3,4-dideoxyglucose-3-ene (3,4-DGE) were obtained from Gambro AB (Lund, Sweden). Advanced glycation endproduct (AGE)-BSA was obtained from MBL International Corporation (Woburn, MA, USA). Conventional 1.5% dextrose-based PDS (Dianeal), amino-acid-based PDS (Nutrineal) and polyglucose-based PDS (Icodextrin) were obtained from Baxter Healthcare Corporation (McGraw Park, IL, USA). Recombinant human TGF-β and VEGF proteins, ELISA kits for human TGF-β and VEGF, and neutralizing anti-human TGF-β and anti-human VEGF antibodies were obtained from R&D System (Minneapolis, MN, USA). Recombinant human CCN2 was obtained from Peprotech Inc. (Rocky Hill, NJ, USA). CCN2 ELISA kit was obtained from Antigenix America Inc. (Huntington Station, NY, USA). All other chemicals were obtained from Sigma (St Louis, MO, USA).

Cell culture

The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee for human studies. Omental tissue was obtained from five non-uremic, non-diabetic patients (male, age 43–52) under elective abdominal surgery for early gastric cancer with full informed consent. These patients with abdominal inflammation had not been exposed to peritoneal dialysis treatment previously. Histological examination revealed that these omental samples were free of other pathological conditions including inflammation, metastasis and endometriosis. Cells were isolated and characterized as previously described [10]. Mesothelial cells showed typical cobblestone appearance at confluence and stained positively for vimentin and cytokeratin 18 but negatively with anti-factor VIII antibodies. The cells were maintained in medium 199 (Life Technologies, Paisley, United Kingdom) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), l-glutamine (2 mM), transferrin (5 µg/ml), insulin (5 µg/ml) and 10% vol/vol fetal bovine serum (FBS). Human peritoneal mesothelial cells (HPMC) of second or third passage from two or three donors (cells were isolated from a total of five donors) were used in experiments. Once the monolayer of HPMC reached confluence, the culture medium was replaced with medium 199 containing 0.1% vol/vol FBS and growth-arrested for 48 h prior to further experiments. Under these conditions, HPMC remained in a non-proliferative viable condition. Human peritoneal fibroblasts (HPF) were isolated by collagenase digestion [10]. In brief, omental tissue was dissociated in DMEM containing antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin), 2 mg/ml collagenase and 20 mg/ml bovine serum albumin. The fibroblast fraction was pelleted by centrifugation (200 g, 10 min) before seeding onto culture plates in low glucose DMEM supplemented with 10% heat-inactivated FBS and antibiotics. Adherent cells were first expanded before disociation (0.25% trypsin EDTA) and replating at 1000–3000 cells/cm² after reaching 70% confluence. HPF were growth-arrested in M199 containing 0.1% vol/vol FBS for 48 h prior to experiment. HPF was bipolar or multipolar shaped in culture and stained positively for vimentin and negatively for cytokeratin 18, factor VIII. The HPF stained negatively for alpha-smooth muscle actin suggesting non-myofibroblast nature. The purity of HPMC and HPF used for experiments was >99%. The endothelial cell line EA.hy926 (EC) was used as a source of endothelial cells. These cells were maintained and arrested using identical protocol for culturing HPMC as described above.

Quantification of cell viability and cell proliferation

Growth-arrested HPMC, HPF and EC were cultured with different PDS and their components for 48 h. The final concentration of PDS components and the preparation of PDS used in culture experiments were selected from preliminary experiments on HPMC (Table 1). The cytotoxic effects of different PDS and its components were measured by LDH assay kit (Roche Diagnostic, Indianapolis, IN, USA). Results were expressed as the percentage changes of LDH release (absorbance ratio between LDH release and the total intracellular LDH) relative to that of the medium control. Cell proliferation was measured using a BrdU incorporation assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) and results were expressed as percentage changes in absorbance compared with that of the medium control.

Treatment of HPMC, HPF and EC with PDS (and their components) or recombinant CCN2, TGF-β or VEGF

HPMC, HPF or EC were grown to confluence. The culture medium was removed and replaced by M199 containing...
0.1% vol/vol FBS for 48 h prior to further culture experiments. Cells were exposed to various PDS and their components for 4 and 48 h at 37°C. In a parallel experiment, cells were treated with CCN2 (50 ng/ml), TGF-β (25 ng/ml) or VEGF (25 ng/ml), for 4 h at 37°C. The concentration of recombinant cytokines was determined from preliminary dose-response experiments on the mesothelial expression of collagen I (for CCN2 and TGF-β) or cell proliferation (VEGF) for EC. Cells were harvested at 4 h for total RNA isolation and supernatants were collected at 48 h and stored at −70°C for protein assay.

**Treatment of HPMC with conditioned media prepared from HPF or EC activated with different PDS and their components**

HPF or EC were grown to confluence before growth-arrested with M199 containing 0.1% vol/vol FBS for 48 h prior to culture with different PDS and their components for further 48 h. Supernatants were collected as HPF- or EC-conditioned media and stored at −70°C until experiment. Similarly, confluent HPMC were arrested with M199 containing 0.1% vol/vol FBS for 48 h prior to culture with different HPF- or EC-conditioned media (twofold diluted with M199 containing 0.1% vol/vol FBS) for 4 and 48 h. In parallel experiments, growth-arrested HPMC were incubated with neutralizing antibodies to TGF-β or/and VEGF (10 µg/ml each) for 1 h before cultured with conditioned media. Preliminary experiment had shown that the neutralizing antibody at this concentration was able to block 100 ng/ml recombinant TGF-β or VEGF. Total RNA isolation and supernatant collection were conducted as described above.

**Real-time PCR for determination of the CCN2, TGF-β or VEGF mRNA expression**

Total RNA was extracted using a Qiagen RNaseasy kit (Qiagen GmbH, Hilden, Germany). Five micrograms of total RNA was reverse transcribed to cDNA with Superscript II reverse transcriptase (Life Technologies) in a 20 µl reaction mixture containing 160 ng oligo (dT)12–18, 500 µM of each dNTP and 40 U RNase inhibitor for 10 min at 37°C, 60 min at 42°C and 5 min at 99°C. cDNA was stored at −20°C until PCR. Real-time PCR amplification was performed in an ABI Prism 7500 sequence detection system using the SYBR-Green kit (Applied Biosystems, Foster City, CA, USA). Primer sequences and gene bank accession numbers are listed in Table 2. Data obtained were analysed using the comparative CT (cycle threshold) method. To determine the quantity of the target transcripts (CCN2, TGF-β, VEGF, IL-6, MMP-9 or collagen I) in cultured cells, the CT value of target transcripts was obtained by subtracting the CT value of the GAPDH control (ΔCT = CTarget - C GAPDH).

### Table 1. Information on PDS and their components used in culture experiments

<table>
<thead>
<tr>
<th>PDS &amp; their components</th>
<th>Concentration used in cell culture experiments</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-glucose (Glucose)</td>
<td>30 mM</td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>30 mM</td>
<td></td>
</tr>
<tr>
<td>Methyglyoxal (m-Glx)</td>
<td>1 µg/ml (13.9 µM)</td>
<td>2-23 µM² in PDS</td>
</tr>
<tr>
<td>3-Dideoxynucleoside-3-ene (3-DGE)</td>
<td>20 µg/ml (122 µM)</td>
<td>118-154 µM² in PDS</td>
</tr>
<tr>
<td>Advanced glycation endproduct (AGE-BSA)</td>
<td>4 µg/ml (17 µM)</td>
<td>9-22 µM² in PDS</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>10 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Fetal bovine serum (FBS)</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>Conventional PDS dextrose 1.5% (Dianeal)</td>
<td>4 x diluted; with 5% FBS</td>
<td>Low pH, high GDPs</td>
</tr>
<tr>
<td>Amino acids based PDS (Nutrineal)</td>
<td>4 x diluted; with 5% FBS</td>
<td>pH 6.7, no GDPs</td>
</tr>
<tr>
<td>Polyglucose based PDS (Icodextrin)</td>
<td>4 x diluted; with 5% FBS</td>
<td>Low pH, low GDPs</td>
</tr>
</tbody>
</table>

*Data adopted from [2,3].

### Table 2. Primer sequence for quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5'-ACCACAGTTGATCCATGCCATCAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCCACACCCCTGTTGCTGTA-3'</td>
</tr>
<tr>
<td>CCN2</td>
<td>Forward</td>
<td>5'-GAGGAAACATTTAAGAAGGGCAA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGGCCACAGTCTTGAAGTC-3'</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Forward</td>
<td>5'-GACTTCCGAAGCCTTCACGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGCCACAGTATCTGGGACAG-3'</td>
</tr>
<tr>
<td>VEGF</td>
<td>Forward</td>
<td>5'-TCCTCACACACTTAAAAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TTGAGGAAAGTGTTCAACCACT-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>5'-ATGAACTCTCTTACAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TGTCATATTGTTCTTAAAGAG-3'</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Forward</td>
<td>5'-CAGGAAATTGGGCTTCTAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGTTGTAGTTGGGTCTTGG-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Forward</td>
<td>5'-GCGGGGCGGTTAGTGAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GTGGTGGCAGGGGAGTGATTG-3'</td>
</tr>
</tbody>
</table>
GAPDH). Relative mRNA fold changes of target transcripts were calculated by subtracting the normalized CT values obtained from various experimental groups (exp) relative to the medium control (ctl) [(ΔΔCT = ΔCT exp - ΔCT ctl)] and the relative mRNA fold changes of target transcripts were determined (2^−ΔΔCT).

ELISA of CCN2, TGF-β or VEGF in culture supernatants

Immuno-reactive TGF-β or VEGF in supernatant was quantified by a commercial ELISA kit. The detection sensitivities for TGF-β or VEGF were 32 pg/ml or 50 pg/ml with the coefficient of variation of 6.5% or 7.3% respectively. CCN2 was determined with the ELISA construction kit from Antigenix America Inc. Briefly, wells of Immulon 2 microtiter plates (Dyntachew, Marnes la Coquette, France) were coated with 100 µl capture antibody at 1 µg/ml in a 0.05 M carbonate–bicarbonate buffer (pH = 9.6). The plates were incubated overnight. All subsequent incubation steps were done at room temperature. The plates were then washed three times with a wash buffer (PBS with 0.05% Tween-20). Wells were aspirated to remove all liquid and were washed four times using 300 µl of wash buffer per well. After the last wash, 200 µl of coating stabilizer and blocking mixture was added (Antigenix America Cat # EA150) and incubated for 60 min. After further washing three times with the wash buffer, 100 µl of samples or standard CTGF (from 20 to 320 pg/ml diluted 0.05% Tween-20, 0.1% BSA in PBS) were introduced. After incubation for 2 h, the plates were washed three with the wash buffer and 100 µl of biotin labelled tracer antibody (0.25 µg/ml) was added. After incubation for 2 h, the plates were washed three times with the wash buffer and 100 µl of streptavidin-horseradish peroxidase (HRP) conjugate (1:500) was added with further incubation for 30 min. After incubation, the plates were washed three times with the wash buffer and 100 µl of freshly prepared tetramethyl benzidine (TMB) substrate were added. The plates were incubated at room temperature for a further 20 min and the reaction was stopped with 100 µl 2 M sulphuric acid. The absorbances were measured at 450 nm with background correction at 630 nm using a microplate reader (Labsystem, Helsinki, Finland). No significant cross-reactivity or interference was observed when 500 ng/ml of recombining TGF, VEGF, PDGF, FGF, TNF or IL-6 was assayed. The detection sensitivity for CCN2 was 86 pg/ml with the coefficient of variation of 8.9%.

Electrophoretic mobility shift assay (EMSA)

Standard EMSA was used to further examine the activity of the transcription factors activator protein-1 (AP-1), nuclear factor kappa-B (NF-κB) and Sp-1 by CCN2, TGF-β and VEGF. Briefly, HPMC were cultured in a T75 tissue culture flask and were growth-arrested upon confluence. Cells were then treated with CCN2 (50 ng/ml), TGF-β (25 ng/ml) or VEGF (25 ng/ml) for 1 h. At the end of the incubation, nuclear extract was prepared using NE-PER nuclear extraction reagent (Pierce Biotechnology Inc., Rockford, IL, USA) and was stored at −70°C until assay. Gel shift oligonucleotide for NF-κB (5’-AGTTGAGGGACTT-CGCCAGGC) or AP-1 (5’-CCGTTTGAATCTAGCCCGAA) or Sp-1 (5’-ATTGATCGG-GCCGGGGCGAG) was biotinylated using a Biotin 3’-end labelling kit (Pierce) and the EMSA was carried out with the LightShift chemiluminescent EMSA kit (Pierce). To confirm whether the DNA binding activity is specific, 100-fold molar excess of unlabelled probes were added to the mixture (labelled probe and nuclear extract) before EMSA. For supershift assay, anti-Sp-1, anti-NF-κB p65 and anti-c-Fos (2 µg, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was incubated with the corresponding nuclear extract for 15 min at 4°C before EMSA to further confirm the specificity.

Statistics

All data were expressed as means ± standard error of the mean (SEM). Inter-group differences between two variables were assessed by the unpaired t-test. The data from more than two study groups were analysed using multivariate ANOVA followed by the Bonferroni correction. All *P* values quoted are two-tailed and the significance is defined as *P* < 0.05.

Results

Effects of PDS and their components on cell proliferation and cell viability

HPMC, HPF and EC were cultured with different PDS and their components for 48 h. There was no observable change in cell viability as measured by LDH release (data not shown). Only HPF cultured with 5% FBS induced significant cell proliferation by BrdU incorporation assay (*P* < 0.001), but similar findings were not observed in HPMC or EC (data not shown).

Effects of PDS and their components on the CCN2, TGF-β or VEGF expression

Following culture with different PDS and their components, a significant up-regulation of the CCN2 mRNA expression was detected in HFP or HPMC by AGE-BSA, all GDPs and PDS preparations when compared with medium control (HPMC cultured with medium only) (Figure 1A). The mRNA expression of CCN2 was up-regulated by glucose only in HPF (*P* < 0.05). There was no change in the CCN2 expression in EC cultured with different PDS and their components. The mRNA expression of TGF-β by HPF and HPMC was significantly up-regulated by glucose, AGE-BSA, m-Glx, 3,4-DGE, 3-DG and all tested PDS (Figure 2A). As observed in CCN2, no change in the TGF-β expression was detected in EC cultured with PDS or their components. In contrast, the mRNA expression of VEGF by HPF, HPMC and EC was significantly up-regulated by AGE-BSA, all tested GDPs and PDS preparations (Figure 3A). The VEGF mRNA expression was selectively up-regulated in HPMC and HPF by glucose, but not in EC. Data of the protein expression of CCN2, TGF-β and VEGF in HPMC, HPF and EC, as determined by ELISA, mirror imaged the mRNA expression...
and the pattern of protein expression under various stimuli (Figures 1B, 2B and 3B). Table 3 summarizes data on the CCN2, TGF-β and VEGF mRNA expression and protein synthesis by HPMC, HPF and EC when cultured with PDS or their components.

Effect of HPF- or EC-conditioned media on the CCN2 expression by HPMC

To explore the possible role of HPF or EC in regulating the CCN2 expression by HPMC, growth-arrested HPMC were cultured with an HPF- or EC-conditioned medium prepared by incubating HPF or EC with various PDS and their components. The CCN2 mRNA expression and protein release by HPMC were significantly up-regulated by HPF-conditioned media prepared from incubation with glucose, AGE-BSA, all tested GDPs and PDS preparations ($P < 0.001$ except $P < 0.05$ for glucose) (Figure 4). The CCN2 mRNA expression and protein release by HPMC were significantly up-regulated by an EC-conditioned medium prepared from incubation with AGE-BSA alone ($P < 0.001$). Although EC-conditioned media prepared from incubation with glucose, GDPs and PDS preparations all up-regulated CCN2 expression by HPMC ($P < 0.05$), but the magnitude of increase is less than that in HPF.

Effect of TGF-β or/and VEGF blockade on the CCN2 expression by HPMC activated with HPF- or EC-conditioned media

To further explore the role of TGF-β and VEGF in the mesothelial synthesis of CCN2 induced by HPF- or EC-conditioned media, growth-arrested HPMC were cultured
CTGF/CCN2 synthesis by peritoneal cells

Fig. 2. Effects of PDS and their components on the TGF-β expression. Growth-arrested HPMC, HPF and EC were cultured with various PDS and their components. (A) TGF-β mRNA expression by HPF or HPMC was up-regulated by glucose, AGE-BSA, all tested GDPs and PDS. There was no change in the TGF-β expression when EC were cultured with PDS or their components as compared with medium control. (B) Results of the TGF-β protein expression mirror imaged the mRNA expression as determined by ELISA. The results represent the mean ± SEM of five separate experiments using HPMC or HPF from three donors.

with HPF-conditioned media prepared from HPF incubated with glucose, 3,4-DGE, AGE-BSA, glucose-based PDS (Dianeal) or 5% FBS (control for the FBS concentration in diluted Dianeal) in the presence or absence of neutralizing antibodies to TGF-β or/and VEGF. TGF-β blockade, but not VEGF blockade, reduced the up-regulatory CCN2 expression by HPMC activated with HPF-conditioned media prepared following incubation with glucose, 3,4-DGE, AGE-BSA or Dianeal (Figure 5). Neutralizing antibodies against TGF-β, VEGF, or in combination failed to alter the up-regulatory CCN2 expression by HPMC activated with EC-conditioned media prepared following incubation with AGE-BSA or Dianeal (Figure 6). There were no changes in the mRNA or protein expression in HPMC cultured with HPF- or EC-conditioned media prepared from incubation with 5% FBS in the presence or absence of neutralizing antibodies.

Differential regulation of transcriptional factors by TGF-β, CCN2 or VEGF in HPMC

HPMC were treated with recombinant TGF-β, CCN2 or VEGF, and the activation of transcriptional factors including AP-1, NF-κB and Sp-1 was examined by EMSA. AP-1 was activated by CCN2 or VEGF, but not TGF-β, whereas only VEGF activated NF-κB (Figure 7A). TGF-β activated Sp-1 more potently than CCN2 or VEGF.

Divergent biological response of HPMC to TGF-β, CCN2 or VEGF

Finally, HPMC were treated with recombinant TGF-β, CCN2 and VEGF, and the expression of IL-6, MMP-9 and collagen I was determined by quantitative PCR. Interleukin-6 expression by HPMC was significantly up-regulated by
Fig. 3. Effects of PDS and their components on the VEGF expression. Growth-arrested HPMC, HPF and EC were cultured with various PDS and their components. (A) VEGF mRNA expression by HPF, HPMC or EC was up-regulated by AGE-BSA, all tested GDPs and PDS. VEGF mRNA expression by HPF and HPMC was also up-regulated by glucose. (B) Results of the VEGF protein expression mirror imaged the mRNA expression as determined by ELISA. The results represent the mean ± SEM of five separate experiments using HPMC or HPF from three donors.

Table 3. A summary of the effects of PDS and their components on the synthesis of TGF-β, CCN2 and VEGF by HPMC, HPF and EC

<table>
<thead>
<tr>
<th></th>
<th>HPMC</th>
<th></th>
<th></th>
<th>HPF</th>
<th></th>
<th></th>
<th>EC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TGF-β</td>
<td>CCN2</td>
<td>VEGF</td>
<td>TGF-β</td>
<td>CCN2</td>
<td>VEGF</td>
<td>TGF-β</td>
<td>CCN2</td>
<td>VEGF</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Mannitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-Glx</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>3,4-DGE</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>3-DG</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>AGE-BSA</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dianeal</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Nutrineal</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Icodextrin</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

+ : up-regulated expression ($P < 0.05$). ++: up-regulated expression ($P < 0.001$). −: no alteration in expression.
Fig. 4. Effect of HPF- or EC-conditioned media on the CCN2 expression by HPMC. CCN2 mRNA expression by HPMC was up-regulated by HPF- or EC-conditioned media prepared from incubation with glucose, AGE-BSA, all tested GDPs and PDS. (A) CCN2 mRNA expression by HPMC was up-regulated by EC-conditioned media prepared from incubation with AGE-BSA only (\( P < 0.05 \)). (B) Results of the CCN2 protein expression mirror imaged the mRNA expression as determined by ELISA. The results represent the mean ± SEM of five separate experiments using HPMC or HPF from three donors.

CCN2 and VEGF but not TGF-\( \beta \) (Figure 7B). Compared with CCN2, induction of the IL-6 expression was more potent with VEGF. The MMP-9 mRNA expression was only inducible by VEGF. TGF-\( \beta \) provided a stronger stimulation for the collagen I mRNA expression than CCN2, whereas VEGF did not induce the collagen I mRNA expression.

Discussion

The majority of previous in vitro studies focused at examining the pathogenic effects of PDS and their components on peritoneal mesothelial cells in the clinical setting of CAPD [3,11]. Other peritoneal resident cells including fibroblasts and endothelial cells that play equally important roles in membrane dysfunction have been less studied. In fact, these cells are also the source of pro-inflammatory and pro-fibrogenic mediators and communicate with each other in affecting the overall peritoneal integrity. Understanding their interactions will provide valuable information about peritoneal membrane dysfunction in CAPD. Our in vitro data suggest that peritoneal fibroblasts may be the most active cell type in terms of the TGF-\( \beta \), CCN2 and VEGF expression compared to HPMC or EC, under identical stimulation by PDS and their components. CCN2 is detected in the peritoneal dialysate from patients on CAPD [5], suggesting that abundant CCN2 in the peritoneal cavity during CAPD may modulate the function of other cell types in the peritoneum. Our data suggest that HPF and HPMC are the producers of CCN2 in vitro; it remains to be confirmed whether our findings reflect the in vivo situation in the peritoneal cavity. AGE-BSA and 3,4-DGE are the most potent inducers for CCN2 synthesis by HPF, followed by m-Glx and 3-DG. CCN2 release from HPMC was strongly induced by AGE-BSA, and less by GDPs and PDS. Most interestingly, while glucose is a potent activator for TGF-\( \beta \)
Fig. 5. Effect of TGF-β or and VEGF blockade on the CCN2 expression by HPMC induced by HPF-conditioned media. (A) Blockade of TGF-β, but not VEGF, reduced the up-regulated CCN2 mRNA expression by HPMC cultured with HPF-conditioned media prepared from HPF incubated with glucose, 3,4-DGE, AGE-BSA or glucose-based PDS (Dianeal) (*P < 0.05). (B) Results of the CCN2 protein expression mirror imaged the mRNA expression as determined by ELISA. The results represent the mean ± SEM of five separate experiments using HPMC or HPF from two donors.

production by HPF and HPMC, it did not directly induce CCN2 synthesis in HPMC and EC. It has been reported that in renal fibroblast, high glucose increased CCN2 expression in the absence of TGF-β signalling [12]. The mechanism of TGF-β-independent CCN2 synthesis by AGE needs further study. AGE-BSA activated TGF-β production in HPF and HPMC. All GDPs activated TGF-β synthesis by HPF while the same GDPs induced comparatively less amount of TGF-β synthesis by HPMC. In contrast, AGE-BSA and all tested GDPs induced VEGF synthesis by HPF, HPMC and EC. These findings in peritoneal resident cells cultured with different PDS largely resembled that of the individual AGE or GDP. Dianeal, a glucose-based PDS with a high level of glucose and GDPs, induced a significant release of TGF-β, CCN2 and VEGF by HPF or HPMC, and VEGF release by EC. Icodextrin, a polyglucose-based PDS with low GDPs, induced less growth factors or cytokines in HPF, HPMC or EC. Nutrineal, an amino-acid-based PDS with no GDP, induced the least release of these cytokines by HPF, HPMC or EC. Taken together, our data suggest that there is differential regulation of CCN2, TGF-β and VEGF by peritoneal cells. The regulation of CCN2, TGF-β and VEGF synthesis by peritoneal cells depends on the unique biochemical properties of the PDS components and their interaction with individual cell type of the peritoneum.

The peritoneal concentration of VEGF and TGF-β correlates with the peritoneal membrane function [13]. During CAPD, glucose, GDP or AGE increases the expression of VEGF in the peritoneum and the peritoneal concentration of VEGF and TGF-β correlates with the peritoneal
membrane function. TGF-β exerts adverse fibrogenic and functional effects on the peritoneal membrane [4]. The promoter of the CCN2 gene contains the NF-κB and TGF-β response elements that could participate in cellular events mediated by TGF-β [14]. VEGF induces neoangiogenesis in the peritoneal microcirculation [15]. Animal and human data suggest that neoangiogenesis plays an important role in the development of ultrafiltration failure [16]. Our present study suggests that, in the context of peritoneal dialysis, CCN2 is produced mainly by HPF and partially by HPMC upon AGE-BSA activation. Synthesis of CCN2 by HPMC was further amplified by conditioned media prepared from HPF or EC cultured with GDPs and AGE-BSA. Nevertheless, neither TGF-β blockade or/and VEGF blockade could completely abolish the increased CCN2 production by HPMC induced by in HPF- or EC-conditioned media, suggesting the involvement of other unidentified mediator(s). AGE-BSA induced the gene and protein expression of CCN2 and epithelial-to-mesenchymal transition (EMT) of rat tubular kidney epithelial cell line NRK-52E. However, the CCN2-induced EMT was not blocked by neutralizing anti-TGF-β antibodies [17]. Furthermore, exposure of rat mesangial cells to AGEs in vitro significantly increases both TGF-β and CCN2 mRNA expression; blockade of TGF-β also did not significantly prevent the AGE-increased expression of CTGF mRNA and protein [18]. We speculate that AGE-induced CTGF expression in HPMC may be regulated through a TGF-β-independent pathway.

We have also examined the crosstalk between HPMC and HPF or EC by exposing HPMC to conditioned media prepared from HPF or EC conditioned media prepared from HPF or EC cultured with PDS and their components. Increased CCN2 synthesis by HPMC following incubation with HPF-conditioned media prepared from HPF cultured with AGE-BSA, GDP and AGE-BSA suggested that soluble mediators released from HPF induced or amplified CCN2 synthesis by HPMC. Similar amplification was observed in EC-conditioned media. These findings implicate that AGE-BSA is a potent universal activator for CCN2 synthesis by HPMC or HPF and the release of CCN2 by HPMC could be further amplified following the crosstalk.
from HPF or EC. This crosstalk network initiated by CCN2, TGF-β, VEGF or other soluble factors will orchestrate interactions between different peritoneal resident cells, forming a major driving force of peritoneal dysfunction under the setting of long-term CAPD.

We next examined the activation of transcriptional factors, namely Sp-1 AP-1 and NF-κB, by HPMC activated with CCN2, TGF-β or VEGF. The three selected transcriptional factors control major events involved in connective tissue formation during fibrinogenesis. Sp-1, a zinc-finger family transcription factor that binds to GC-rich consensus sequence of the collagen I gene promoter and induces the COL1A2 promoter activity [19], is implicated in controlling collagen I synthesis in skin fibroblasts. AP-1 belongs to a transcription factor family that contains Jun D, c-Jun, and c-Fos, and modulates the target gene expression as homo- or heterodimer. The intron of collagen I gene contains an AP-1 binding site that involved in transcriptional regulation of the collagen gene [20]. NF-κB is present in many genes that are important in immune and inflammatory reaction [21]. Our data suggest differential activation of these transcriptional factors by HPMC cultured with different cytokines/growth factors. Sp-1 and NF-κB were only activated by TGF-β and VEGF respectively whereas AP-1 was activated by both CCN2 and VEGF. It should be pointed out that the signalling pathways involved in vivo in the peritoneum under CAPD are much more complicated. Whether data from our in vitro model apply to in vivo environment needs to be explored. We further studied the expression of three important genes that control the extracellular matrix (ECM) accumulation or turnover. The collagen I mRNA expression in HPMC was significantly increased by TGF-β followed by CCN2, supporting the pathological role of these two cytokines in fibrogenesis. In contrast, the MMP-9 mRNA expression in HPMC was only up-regulated by VEGF, but not by TGF-β or CCN2. Matrix metalloproteinases (MMPs) are key enzymes involved in the turnover of the ECM including collagen. MMP-9 is involved in metastasis due to its role in degradation of the ECM of the basement membrane [22]. VEGF may participate in the epithelial to mesenchymal transition (EMT) process through inducing the release of MMP-9 and promoting the degradation of basement membrane collagen. The lack of up-regulation of the collagen I expression by HPMC culturing with VEGF may suggest its major role in ECM remodelling, rather than participating in up-regulation of collagen production during the EMT process. We found NF-κB activation by VEGF in HPMC. Down-regulation of NF-κB activity has been reported to associate with increased MMP-9 level [23]. MMP-9 promoter contains a cis-acting regulatory element for NF-κB (located at −600 bp), which is involved in the induction of the MMP-9 gene [24]. This piece of evidence leads us to speculate that VEGF may induce MMP-9 synthesis through activation of NF-κB. IL-6 mRNA in HPMC was also induced by CCN2 and VEGF. IL-6 is implicated in the progression of peritoneal fibrosis through its stimulatory role in cell proliferation and extracellular matrix synthesis. Different signal transduction pathways are involved in the regulation of the IL-6 gene. Various transcription factors can interact with corresponding binding sites on the IL-6 promoter to initiate IL-6 mRNA transcription. We had previously shown that the increased synthesis of IL-6 in glomerular mesangial cells was dependent on NF-κB and AP-1 [25].

Our current data suggest that the up-regulation of the IL-6 mRNA expression by VEGF or CCN2 may be attributed to a similar mechanism. Our EMSA data show that VEGF activated both AP-1 and NF-κB whereas CCN2 only activated AP-1. This may explain the observation that VEGF, compared with CCN2, induced more IL-6 expression in HPMC.

In conclusion, AGE and GDPs in PDS differentially regulate the synthesis of CCN2 by cultured peritoneal resident cells. The differential activation of different transcriptional factors and diverse response of HPMC towards CCN2, TGF-β and VEGF suggest that these cytokines/growth factors have an overlapping and distinct role on HPMC.
Acknowledgements. The study was partly supported by the Research Grant Council, Hong Kong (HKU 7415/04M). JCL was supported by the L & T Charitable Fund and the House of INDOCAFE.

Conflict of interest statement. None declared.

References

3. Welten AG, Schalkwijk CG, ter Wee PM. Single exposure of mesothelial cells to glucose degradation products (GDPs) yields early advanced glycation end-products (AGEs) and a proinflammatory response. Perit Dial Int 2003; 23: 213–221

Received for publication: 27.11.07
Accepted in revised form: 25.8.08