Connective tissue growth factor in renal development and injury
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Citation for published version (APA):

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Connective tissue growth factor (CTGF/CCN2) is increased in peritoneal dialysis patients with high peritoneal solute transport rate

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doi: 10.1152/ajprenal.00368.2009
http://ajprenal.physiology.org/content/298/3/F721.full.pdf+html

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CTGF is associated with high peritoneal transport

ABSTRACT

Peritoneal fibrosis (PF) is an important complication of peritoneal dialysis (PD) therapy that often occurs in association with peritoneal high transport rate and ultrafiltration failure (UFF). To study the possible pathogenic role of Connective Tissue Growth Factor (CTGF) in the relationship of PF and UFF, dialysate CTGF contents (n=178) and tissue CTGF expression (n=61) were investigated by ELISA, real-time PCR, immunohistochemistry and in-situ hybridization. CTGF production with and without TGF-β1 stimulation in human peritoneal mesothelial cells (HPMC) from the spent patients’ peritoneal dialysate (n=32) was studied in vitro. The dialysate-to-plasma ratio for creatinine (D/P Cr) was positively correlated to dialysate CTGF concentration and estimated local peritoneal production of CTGF. CTGF mRNA expression was 11.4-fold higher in peritoneal membranes with UFF than in pre-PD renal failure peritoneum and was correlated with thickness of the peritoneum. CTGF protein and mRNA were detected in mesothelium and in fibroblast-like cells. In cultured HPMC, TGF-β1-induced expression of CTGF mRNA was increased at 12 and 24 hours and was correlated with D/P Cr. In contrast, bone morphogenic protein-4 mRNA expression was inversely correlated with D/P Cr. Our results suggest that high peritoneal transport state is associated with fibrosis and increased peritoneal CTGF expression and production by mesothelial cells which can be stimulated by TGF-β1. Dialysate CTGF concentration could be a biomarker for both peritoneal fibrosis and membrane function. Functional alteration of mesothelial cells may be involved in progression of peritoneal fibrosis in high transport state.
INTRODUCTION

Long-term peritoneal dialysis (PD) treatment is accompanied by functional and histopathological alterations in the peritoneum (10, 14, 28). The characteristic feature of chronic peritoneal damage in PD treatment is decreased ultrafiltration capacity associated with submesothelial fibrosis, accumulation of extra-cellular matrix and neoangiogenesis leading to a large vessel peritoneal surface area (49). The decrease in ultrafiltration capacity seen after prolonged PD is one of the important reasons for its discontinuation (22). The pathogenesis of peritoneal fibrosis is attributed to a combination of bioincompatible factors in dialysate, including high osmolality, high glucose (10), advanced glycation products (47) and glucose degradation products (48), uremic inflammation (49) and acute peritonitis with inflammation (11, 12, 40). Importantly, peritoneal fibrosis/sclerosis often occurs in association with high transport rate and ultrafiltration failure (UFF). The mechanism of these interactions between peritoneal fibrosis and UFF, which may become a target to prevent the peritoneal damage, is still not clear. In addition, there is no biomarker which reflects both conditions.

Connective tissue growth factor (CTGF; CCN-2) is a 349 amino acid cysteine-rich polypeptide belonging to the CCN (CTGF/Cyr61/Nov) family. CTGF was first identified in conditioned media of endothelial cells as a 36-38-kDa polypeptide containing chemotactic activity toward fibroblasts. The CCN family consists of six regulatory proteins, which participate in diverse biological processes like angiogenesis and wound healing, and are involved in control of migration, cell proliferation and differentiation, and epithelial-to-mesenchymal transition (EMT) (7, 39). CTGF is highly expressed during development of various fibrotic disorders and has been acknowledged as one of the key growth factors in extracellular matrix production and other profibrotic activities mediated by transforming growth factor (TGF)-β (25, 52, 53). In patients with type 1 diabetic nephropathy, we observed that plasma CTGF is an independent predictor of end-stage renal disease and mortality (34) and that urinary CTGF excretion is correlated with clinical markers of renal disease (33). CTGF was also detected in peritoneal fluid of patients undergoing peritoneal dialysis (57). However, since these latter studies used small numbers of patients, they were not sufficiently powered to draw conclusions on the potential use of CTGF as biomarker in PD patients and the possible pathophysiological role of CTGF in peritoneal transport. Therefore, we investigated CTGF expression in human peritoneal fibrosis and peritoneal transport dysfunction using human peritoneal tissue, dialysate and cultured mesothelial cells from CAPD patients with varying rates of peritoneal transport.
SUBJECTS AND METHODS

Patients and Experimental Design
All studies were approved by the Ethics Committee for Human Research of the Faculty of Medicine, Nagoya University (Approval #298 - peritoneal fluid experiment; #299 - peritoneal tissues experiment), and all patients provided informed consent prior to participation in the study.

Peritoneal transport of CTGF in PD patients.
CTGF concentration in peritoneal effluent was measured in over-night dwelled (8.79±2.10 hours) samples collected from 155 PD patients (63 females, 92 males) treated between January 2005 to December 2007 at the Department of Nephrology and Renal Replacement Therapy of Nagoya University Hospital (Nagoya, Japan) and at affiliated hospitals including Handa Municipal Hospital, Nagoya Kyoritsu Hospital, Kounan-Kousei Hospital and Anjo-Kosei Hospital. The mean age of all patients was 58.4±13.7 (range, 27 to 89) years and the mean duration of CAPD treatment was 41.2±36.1 (range, 1 to 180) months. Diabetic nephropathy was the cause of end-stage renal disease in 52 PD patients (33.5%). All patients were free from peritonitis for at least one month prior to the study, and patients with other diseases, such as liver or lung diseases and malignancy, were excluded. Patients undergoing combination therapy (HD + PD) were not included in this study. Peritoneal transport was assessed in 144 PD patients by ratios of creatinine concentrations in dialysate and plasma (D/P Cr) and the average value was 0.65±0.15 (range, 0.20 to 0.96). Correlation between CTGF concentration in PD effluent and D/P Cr was analyzed in 144 PD patients. A separate study using 23 stable PD patients of Nagoya University hospital (14 males and 9 females, mean age 52.8±11.5 years, mean PD treatment duration 30.2±25.3 months) was designed to estimate the proportion of PD effluent CTGF that is derived from local production in the peritoneal cavity. CTGF, β2-microglobulin, IgG and α2-macroglobulin content were measured in blood (Na₂EDTA plasma or serum) and dialysate samples at 4 hr of Peritoneal Equilibration Tests (PET). The fast PET was performed using 2.27% glucose-based dialysis solutions (Dianeal-N PD-4, Baxter) as described by Twardowski et al (45). Serum creatinine levels were measured enzymatically on an automated analyzer (JCA-BM6050, JEOL, Tokyo, Japan). β2-Microglobulin was determined using microparticle enzyme immuno-assay β2-microglobulin kit (Denka, Niigata, Japan). Albumin, immunoglobulin G and α2-macroglobulin concentration were measured by turbidimetric immunoassay using Albumin kit (Shino-test, Sagamihara, Japan), IgG kit (Nittobo, Tokyo, Japan) and N antiserum to human α2-macroglobulin (Dade Behring Marburg GmbH, Marburg, Germany), respectively. Na₂EDTA plasma of fifteen healthy controls (6 males and 9 females, mean age 40.9±7.7 years) were used to measure levels of CTGF in healthy individuals.
CTGF mRNA expression and correlation with histology of peritoneum.

Sixty-one peritoneal tissue samples were obtained from 35 PD patients and 26 pre-PD controls (chronic renal failure patients who needed PD catheter insertion because of advanced renal failure). Among the 35 PD patients: 7 were regarded as having impaired ultra-filtration capacity (UFF), which was defined by use of more than four hypertonic bags (2.27% glucose, 3.86% glucose or icodextrin) in each 24h to maintain fluid balance (17); 5 patients were peritonitis-positive; and, 23 patients (incidental) had their catheters removed because of transplantation, mental disorders, severe exit site infection or difficulty to do the bag exchanges (Table 1). Correlation of CTGF mRNA expression with peritoneal membrane thickness and number of vessels was evaluated.

Table 1. Peritoneal biopsy cases evaluated for CTGF mRNA expression.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>UFF</th>
<th>Peritonitis</th>
<th>Incidental</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>26</td>
<td>7</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>male</td>
<td>17</td>
<td>3</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>female</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>age (year)</td>
<td>62.0±12.8</td>
<td>55.9±11.6</td>
<td>72.5±12.8</td>
<td>60.8±13.2</td>
</tr>
<tr>
<td>duration of treatment (years)</td>
<td>0</td>
<td>9.4±6.6</td>
<td>2.4±1.8</td>
<td>3.7±3.0</td>
</tr>
<tr>
<td>average thickness of peritoneum (μm)</td>
<td>157.9±62.1</td>
<td>308.6±129.2</td>
<td>432.1±322.8</td>
<td>155.7±93.1</td>
</tr>
</tbody>
</table>

Values are means ± SD. CTGF, connective tissue growth factor; Control, peritoneal tissues were taken at time when a peritoneal dialysis (PD) catheter was inserted because of renal failure; UFF, cases of Ultra-Filtration Failure; Incidental, peritoneal tissues were taken when the catheter was removed because of reasons other than UFF.

Table 2. Patients profiles of the culture studies.

<table>
<thead>
<tr>
<th></th>
<th>PD duration (&lt; 2 yr)</th>
<th>All patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>male</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>female</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>total</td>
<td>25</td>
<td>32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>D/P Cr</th>
<th>PD duration (&lt; 2 yr)</th>
<th>All patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>low</td>
<td>~0.49</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>low average</td>
<td>~0.5 - 0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>high average</td>
<td>~0.65 - 0.81</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>high</td>
<td>~0.82</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D/P Cr; ratio of creatinine concentrations in dialysate and plasma, an index of the peritoneal transport.
**CTGF production in human mesothelial cells.**

Human peritoneal mesothelial cells (HPMC) were isolated from spent glucose-based peritoneal dialysis fluid (Dianeal-N PD-4, pH 6.5-7.5, Baxter, Tokyo, Japan) taken from 32 clinically stable patients (Table 2) and cultured using a modified method described previously (5, 26). Basal and TGF-β1 induced CTGF mRNA expression in both HPMC and human mesothelial cell line, Met-5A, were studied as detailed below.

**Processing of Biopsy Samples and Morphological Analysis**

Samples of parietal peritoneum were biopsied in the standard manner and processed as reported previously (17, 35, 49). The tissue samples were fixed with 10% buffered formalin overnight, routinely processed for light microscopy and embedded in paraffin. Four-μm-thick sections were cut and stained with hematoxylin and eosin (HE) and Masson’s-trichrome. Before analysis of peritoneal thickness, each specimen was assessed for size, site and direction of the peritoneum, then judged adequate as described by Honda et al (17). In 40 of 61 samples, thickness and the number of the vessels could be measured. In order to assess the extent of peritoneal thickening, the submesothelial compact zone was identified as the peritoneal fibrosis between basal border of the surface mesothelial cells and upper border of the peritoneal adipose tissues (17, 49). We measured peritoneal thickness at 5 random points using a Zeiss Z1 microscope and Axiovision Windows software version 4.4 (Carl Zeiss, Oberkochen, Germany), and mean thickness was calculated.

**Immunohistochemistry (IHC) and In Situ Hybridization (ISH) for CTGF and α-Smooth Muscle Actin**

Total 44 tissue samples from 32 PD patients and 12 pre-dialysis control patients were performed for IHC. Four-μm-thick sections of formalin-fixed, paraffin-embedded tissues were deparaffinated, and rehydrated. The slides were boiled in 0.04 M citrate, 0.12 M, phosphate, pH 5.8, for 10 min at 100 °C. After washing, sections were incubated in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase and incubated in 10% normal goat serum (Dako, Glostrup, Denmark) in PBS to block non-specific binding. Afterwards, the sections were incubated with mouse monoclonal anti-human CTGF antibody (FibroGen, Inc., San Francisco, CA) at 1 μg/ml in PBS for 16 hours at 4°C, followed by reaction with a conjugate of polyclonal goat anti-mouse IgG antibody and horseradish peroxidase-labeled polymer (Histofine Simple Stain, Nichirei, Tokyo, Japan) as a secondary reagent. Enzyme activity was detected using a 3, 3'-diaminobenzidine tetrahydrochloride liquid system (Dako) (30, 32). Immunostaining for α-smooth muscle actin (α-SMA) was performed using mouse monoclonal α-SMA antibody (1A3; Dako) as we described previously (16, 17, 18). Counter staining with hematoxylin was done on the IHC sections of CTGF and α-SMA.

ISH to detect CTGF mRNA was performed on the 10% buffered formalin-fixed paraffin-embedded human peritoneal biopsy tissues using previously described methods (19, 20). Counter staining with hematoxylin was not performed on the ISH sections.
**Enzyme-Linked Immunosorbent Assays (ELISA) for CTGF**

CTGF protein was measured in Na$_2$EDTA–plasma and peritoneal dialysate (PD fluid) samples. Samples were frozen at the time of collection, stored at -80 °C and not subjected to freeze-thaw cycles. CTGF was detected by sandwich ELISAs using monoclonal antibodies against distinct epitopes on the N-terminal and C-terminal halves of human CTGF (FibroGen, Inc.) with similar protocols as we have described previously (33, 34). The same preparation of full length recombinant human CTGF (rhCTGF, FibroGen, Inc.) in appropriate matrices was used for standards in all assays, making the results of the different ELISAs comparable and obviating the need to equalize concentration of the different CTGF forms by molarity. Low, medium and high concentration rhCTGF Quality Control samples, prepared using matrices similar to that of the experimental samples, were included in each assay plate to identify quantitation and detection limits. Acceptable coefficients of variation for the Quality Control replicates were set to be ≤20% for medium and high concentration Quality Control samples and ≤25% for the lowest concentration Quality Control samples. In some of the PD fluid analyses, values were extrapolated from the calibration curve to report “low” values that are above the lower limit of detection (LOD), but below the lower limit of quantitation (LLOQ). The assays used detect N-terminal half fragments of CTGF (N-CTGF, Domains 1 and 2) and/or full length CTGF (W-CTGF, consisting of Domains 1 - 4). We determined sample concentrations of CTGF N-half fragments plus W-CTGF with CTGF ELISAs that use monoclonal antibodies directed against epitopes in Domains 1 and 2. For these assays, the LOD for PD fluid was 1.6 ng/mL and the LLOQ was ~14 ng/mL. For Na$_2$EDTA plasma, the LLOQ = 4.7 ng/mL. We determined sample concentrations of W-CTGF with a CTGF ELISA that uses monoclonal antibodies directed against epitopes in Domains 1 and 3. The LOD for PD fluid was 3.2 ng/mL and the LLOQ was 4.7 ng/mL. For Na$_2$EDTA plasma, the LLOQ = 4.7 ng/mL. The coefficient of variation of intra- and interassay was 6 and 20%, respectively.

**Calculations for Local Peritoneal Production of CTGF**

Local peritoneal production of CTGF was defined as the difference between the measured and expected dialysate concentration calculated from the peritoneal transport line of each patient using the methods by Zweers, MM et al (59, 60). The peritoneal transport line was computed for each patient based on the least squares regression analysis of the D/S ratio of β2-microglobulin [molecular weight (MW) 11,800 Da], albumin (MW 69,000), IgG (MW 150,000) and α2-macroglobulin (MW 820,000) and their molecular weights when plotted on a double logarithmic scale. The expected amount of CTGF protein (MW 22,000 as described below) transported from the circulation to the peritoneal cavity was estimated using the peritoneal transport line of each patient. The slope of this line represents the size selectivity of the peritoneal membrane. “Local peritoneal CTGF production index” of each individual is calculated from the difference between measured D/P CTGF and expected D/P CTGF values transported from the circulation to the peritoneal cavity.
Cell Culture Study
A human mesothelial cell line (Met-5A), which was derived after transfection with pRSV-T plasmid from pleural fluid of non-cancerous patients, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained according to ATCC guidelines. Briefly, Met-5A cells were grown in Medium 199 containing Earle's BSS, L-glutamine and sodium bicarbonate (Sigma, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Sigma), 20 mM HEPES (Dojindo, Kumamoto, Japan), 3.3 nM epidermal growth factor (EGF; R&D System, Minneapolis, MN), 400 nM hydrocortisone (Sigma), and 870 nM zinc-free insulin (Sigma) in humidified air with 5% CO₂ at 37°C. Human peritoneal mesothelial cells (HPMC) from spent peritoneal dialysis effluent of glucose based pH neutral peritoneal dialysis solution were obtained by centrifugation of dialysis fluid taken randomly from the clinically stable patients with a variety of peritoneal permeability undergoing nocturnal exchanges using modified methods described previously (5, 6, 9, 26, 50, 56). HPMC were cultured using two different conditions.

1) Cellular components were isolated using low speed (200 g) centrifugation, washed with RPMI 1640 (Sigma) and then cultured in RPMI 1640 containing L-glutamine (Sigma) supplemented with 15% FBS (Sigma), insulin/transferrin/selenium A (Invitrogen, Tokyo, Japan), 10⁻⁵ M 2-mercaptoethanol (Wako, Osaka, Japan), 3.3 nM EGF (R&D) and 400 μg/l Hydrocortisone (Sigma) in humidified air with 5% CO₂ at 37°C. Non-adherent material was removed the next day with two brief washes with RPMI 1640 and the adherent population was incubated in fresh culture medium. The cells reached confluence in 7-10 days, and were split two to three times and cultured. Under sub-confluent conditions, HPMC and Met-5A were washed twice with PBS, and culture medium was replaced with serum-free medium for 24 hours in order to render the cells quiescent. Subsequently, cultures were incubated with 5 ng/mL recombinant human TGF-β1 (R&D), which was diluted in serum-free medium. Cells were harvested at 0 (basal condition), 3, 6, 12 and 24 hours (n=4 dishes at each time point of each patient). All experiments were performed during 3rd to 4th passage. To explore correlation between amplification of CTGF expression by TGF-β and D/P Cr, we assessed the increase of CTGF mRNA after 12 and 24hrs incubation with TGF-β1.

2) In order to evaluate CTGF mRNA expression in cell culture conditions without EGF, the harvested HPMC were cultured on dishes pre-coated with type I collagen (Iwaki, Tokyo, Japan, Cat. #4010-010) (n=4 dishes of each patient). Characterization of mesothelial cells was based on both morphology and positive immunofluorescence staining with mouse anti-human Cytokeratin 18 (Dako) and rabbit anti-ZO-1 (Zymed Laboratories, South San Francisco, CA) and absence of staining for CD68 (PG-M1; Dako), CD31 (JC/70A; Dako) and α-SMA (Dako).

RNA Preparation from Peritoneal Tissues and Cultured Mesothelial Cells and Quantitative Polymerase Chain Reaction (PCR)
RNA preparation from human peritoneal tissues and cultured mesothelial cells were done using the RNeasy Fibrous Tissue Mini Kit or RNeasy Mini Kit (Qiagen) as described previously.
First-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. A total of 1 μg of RNA was reverse transcribed. To validate gene expression changes, quantitative PCR analysis was performed with an Applied Biosystems Prism 7500HT Sequence Detection System using TaqMan Gene Expression Assays for CTGF (assay identification number Hs00170014_m1), bone morphogenic protein (BMP)-4 (Hs00370078_m1) and 18S ribosomal RNA (4319413E) according to the manufacturer’s specifications (Applied Biosystems Inc., Foster City, CA). The thermal cycler conditions were as follows: hold for 10 min at 95°C, followed by two-step PCR for 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate. Amplification data were analyzed with Applied Biosystems Sequence Detection Software version 1.3.1 (Applied Biosystems). To normalize the relative expression of the CTGF mRNA against the 18S ribosomal RNA control, standard curves were prepared in each experiment.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting Analyses

**CTGF.** CTGFs present in PD fluid samples from 2 different patients were isolated by immunoprecipitation (IP), resolved by SDS-PAGE and detected by Western blotting to determine molecular weights. A CTGF monoclonal antibody against Domain 1 (2 μg/mL in 1% BSA, 0.05% Tween; FibroGen, Inc.) was added to 0.5-mL samples of PD fluid and immune complexes were allowed to form by rotating the samples at 4°C for 30 minutes. Immune complexes were collected on Protein A sepharose (Sigma, St. Louis, Mo; Cat. #P-9424) after 6 hours at 4°C with rotation, washed twice with D-PBS (Ca²⁺-, Mg²⁺-free, Mediatech, Herndon, VA; Cat. #21-031-CV), and eluted using Laemmli sample buffer without DTT or β-mercaptoethanol. Unreduced samples were heated at 70°C for 10 minutes, resolved by SDS-PAGE (4-12% non-reducing gradient gel, Invitrogen Corp., Carlsbad, CA; Cat. #NP0335BOX) and proteins were transferred to nitrocellulose (8 minute transfer; Invitrogen iBlot). Blots were blocked with 1% BSA-0.05% Tween 20 at 4 °C overnight and total CTGF species (W-CTGF and N-CTGF) were detected by exposing the blot to a CTGF monoclonal antibody against Domain 2 (FibroGen, Inc.) at 2 μg/mL in 1% BSA, 0.05% Tween 20 in D-PBS for 4 hours at room temperature with agitation. CTGF bands were visualized by treating blots with goat anti-Mouse IgG-HRP (1:5,000) and Pierce Luminescence reagents (Pierce, Lockford, IL) at room temperature followed by exposing blot to X-ray film. For comparison, Chinese Hamster ovary (CHO) cell-derived W- and N- rhCTGFs were included on the gel and molecular weights were determined by comparing mobilities of CTGF bands and molecular weight standards (Bio-Rad Lab, Hercules, CA, Cat. #161-0374).

**TGF-β type II receptor.** In order to detect TGF-β type II receptor on HPMC from 6 patients in the highest and lowest PET category each, we modified our previously reported technique (21). Briefly, protein amount of lysates was measured by BCA Protein Assay Kit (Pierce, Lockford, IL, USA). Lysates, in which protein amounts were adjusted, were mixed with sample buffer for SDS-PAGE and separated under nonreducing condition.
on 20% gels. Separated proteins were transferred to nitrocellulose membrane (Bio-Rad Lab., Hercules, CA), and membranes were blocked with 5% (wt/vol) nonfat milk in PBS (PBS-M). Membranes were then probed with a polyclonal goat anti-TGF-β type II receptor polyclonal antibody (R&D Systems, Minneapolis, MN, USA) diluted in PBS-M, washed in PBS containing 0.1% Tween 20, and then probed with horseradish peroxidase-conjugated rabbit anti-goat IgG (Cappel, Durham, NC, USA) absorbed with normal human serum (1:1/v:v). After they were washed again in PBS containing 0.1% Tween 20, bands were developed using enhanced chemiluminescence (GE Healthcare Bio-Sciences KK, Tokyo, Japan) and captured on LAS-300 image analysis system (FujiFilm Corp., Tokyo, Japan).

**Statistical Analysis**

Values are expressed as means ± SD. Differences between groups were analyzed by Student’s *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests. Correlations were assessed by the linear regression. Differences were considered to be statistically significant if *P*<0.05. All analyses were performed using SPSS (Chicago, IL, USA). Polynomial regression analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA, USA).

**RESULTS**

**CTGF concentration in the plasma and peritoneal effluent and local peritoneal CTGF production in the Peritoneal Equilibration Tests (PET)**

To address the possibility that CTGF content in the PD effluent could be a biomarker of peritoneal dialysis, we measured both plasma and dialysate CTGF (N-CTGF and/or W-CTGF) by sandwich ELISA. Plasma CTGF levels were significantly increased in PD patients when compared to healthy controls (121.97±47.36 vs. 5.76±1.50 ng/ml, *p*<0.0001, Figure 1A). There was no correlation between plasma CTGF levels and the peritoneal transport rate D/P Cr in the PD patients (Figure 1B). We found a positive correlation between CTGF concentration in PD effluent from 4 hr dwelling times and the ratio of creatinine concentrations in dialysate and plasma (D/P Cr), an index of peritoneal transport (R=0.653, *p*<0.001, Figure 1C). For calculation of peritoneal transport of CTGF, we performed immunoprecipitation and Western Blot analysis to determine molecular weights and species of CTGF in peritoneal effluent. The intact form of CTGF (36-38 kD) was not identified, whereas CTGF reactive fragments of 22kD and diffuse 25-28kD were demonstrated (Figure 1D). Most of the measured CTGF effluent dialysate concentrations (point “a” of Figure 1E) were significantly greater than CTGF levels that could be expected due to simple diffusion from circulation to the dialysate using the peritoneal transport line (point “b”). The difference defines the potential extent of local peritoneal CTGF production, designated as “local CTGF production index” (Figure 1E).
CTGF is associated with high peritoneal transport

**Figure 1.** Concentration of CTGF in the plasma and dialysate levels in the Peritoneal Equilibration Tests (PET).

- **A)** Plasma CTGF levels in healthy control individuals (n=12) and in PD patients (n=23). ### P < 0.0001.
- **B)** There is no relationship between plasma CTGF levels and the ratio of creatinine concentrations in dialysate and plasma at 4 hours (D/P Cr) in PD patients.
- **C)** There is a positive correlation between CTGF concentration in the PD effluent of 4 hr dwelling and peritoneal transport rate (D/P Cr).
- **D)** Immunoprecipitation and Western Blot analysis reveals CTGF reactive fragments of 22kD and diffuse 25-28kD in the spent peritoneal dialysate.
- **E)** Representative case of regression line based on the power relationship between the D/P ratio of β2-microglobulin, albumin, IgG and α2-macroglobulin (●) and their molecular weights. The measured dialysate-to-plasma ratio of CTGF (D/P CTGF; ○, A) is given at the molecular weight of CTGF in the dialysate (22,000). Local peritoneal CTGF production index is calculated from the difference between measured D/P CTGF (a) from the regression line of each patient and expected D/P CTGF values (b) transported from the circulation to the peritoneal cavity. MW, molecular weight.
- **F)** Local peritoneal CTGF production index calculated from the regression line from the each patient is significantly correlated with D/P Cr.
CTGF is associated with high peritoneal transport

Local CTGF production indices correlated well with D/P Cr (R=0.723, p<0.0001, Figure 1F). W-CTGF in the spent PD effluent was not detected by ELISA, which is consistent with immunoprecipitation and Western Blot results.

**Concentration of CTGF in the over night human PD effluent**

We further assessed CTGF concentration in over night dwelled peritoneal dialysis effluent of 155 patients, and evaluated relationships with peritoneal transport rate and duration of treatment. There was a positive correlation between dialysate CTGF concentration and the ratio of creatinine concentrations in dialysate and plasma (D/P Cr) (Figure 2A, R= 0.603), and a correlation with duration of PD treatment (Figure 2B, R=0.264).

![Figure 2](image)

**Figure 2.** Concentration of CTGF in the overnight human PD effluent. There was a positive correlation between dialysate CTGF concentration and dialysate to plasma creatinine ratio (D/P Cr) (A), and a correlation with duration of PD treatment (B).

(A) \(Y = 7.377 - 0.011X + 40.84X^2 + 88.72X^3, n = 144, R = 0.6031, R^2 = 0.3637, 95\%\) confidence interval in gray. (B) \(Y = 14.06 + 1.347X, n=155, R= 0.264, R^2= 6.99\times10^{-2}, p<0.001.\)

**CTGF mRNA expression and correlation with peritoneal thickness and the number of blood vessels in human peritoneal biopsy samples**

We next investigated CTGF expression in the peritoneal membrane before and after treatment with PD. Peritoneal membrane in the group of pre-PD renal failure (157.9 ± 17.9 μm) was thicker compared with the normal peritoneum reported by Williams (50 μm) and Honda (62.4 ± 52.0 μm) (17, 49). In UF and peritonitis conditions, the peritoneum was remarkably thickened (308.6 ± 48.8 and 432.1 ± 144.4 μm respectively). CTGF mRNA expression assessed by real-time PCR was 11.4-fold higher in peritoneal
Figure 3. CTGF mRNA expression detected by real-time PCR in human peritoneal biopsy samples and correlation with peritoneal thickness and the number of blood vessels. (A) CTGF mRNA expression in peritoneal biopsy samples (n=61) was assessed by real-time PCR. Control; Peritoneal tissues were taken at time when PD catheter was inserted because of renal failure. UFF; Cases of Ultra-Filtration Failure, Incidental; Peritoneal tissues were taken when the catheter was removed because of reasons other than UFF, ## $P < 0.001$ compared against controls. (B) Correlation between CTGF mRNA expression and thickness of submesothelial compact zone in the groups of control and incidental patients. (C) Correlation between CTGF mRNA expression and thickness of submesothelial compact zone in all groups other than peritonitis group. (D) There is no correlation between CTGF mRNA expression and density of blood vessels of the peritoneum in the groups of control and incidental patients. (E) There is no correlation between CTGF mRNA expression and density of blood vessels of the peritoneum in all groups other than peritonitis group.
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**Figure 4.** CTGF mRNA and protein expression in the peritoneal tissues by in situ hybridization (ISH) and immuno-histochemistry (IHC).

CTGF mRNA (A) and protein (B) were weakly detected in the mesothelial cells (arrow) and vascular wall of peritoneal tissues (open arrow) from the patients with pre-dialysis chronic renal failure. In the patients whose catheters removed incidentally (C, D), CTGF was demonstrated in the mesothelial cells (arrow) and some fibroblast like cells (arrowhead). Open arrow heads indicate the positive expression in the vessels. In advanced fibrotic peritoneum (E, F), mesothelial cells were partly lost on the surface of the peritoneal membrane. CTGF mRNA (E) and protein (F) were strongly detected in the mesothelial cells (arrow) and fibroblast like spindle shaped cells (arrowhead). Counter staining was not performed on the ISH sections. Most of these spindle shaped cells were α-SMA positive cells (inset, α-SMA IHC counter stained with hematoxylin). Scale bars: 100 μm.
membranes with UFF vs. biopsy samples at insertion of PD catheter (pre-PD renal failure peritoneum) (Figure 3A). There was a correlation between CTGF mRNA expression and thickness of submesothelial compact zone of the peritoneum in the groups of control and incidental patients (R=0.61, p<0.0001) and in the all patients other than peritonitis (R=0.57, p<0.0001) (Figure 3B, 3C). However, we could not find a relationship between CTGF mRNA expression and blood vessel density of the peritoneum in either group (Figure 3D, 3E).

Localization of CTGF mRNA and protein expression in the peritoneal tissues
ISH and IHC weakly detected CTGF mRNA and protein in the mesothelial cells and vascular wall of peritoneal tissues from patients with chronic renal failure before initiation of dialysis (Figure 4A, B). In the patients whose catheters were removed incidentally, CTGF was identified in the mesothelial cells and vessel walls (Figure 4C, D). The extent of CTGF expression was similar or slightly increased when compared with pre-PD renal failure group. In the advanced fibrotic peritoneum, mesothelial cells were partially detached from the surface of the peritoneum. However, CTGF mRNA and protein were strongly detected in the mesothelial cells and in fibroblast-like spindle shaped cells (Figure 4E, F). Immunostaining for α-SMA suggested these CTGF-positive spindle shaped cells were fibroblasts (Figure 4E inset).

Morphological features and CTGF mRNA expression in the cultured human peritoneal mesothelial cells (HPMC) under basal condition
Human mesothelial cells (HPMC) were isolated from the spent peritoneal dialysis effluent of 32 PD patients and cultured on the collagen-I coated dish. We could not find obvious morphological differences in cells from patients of variable peritoneal transport rates (Figure 5A; PET low, 5B; PET high). All cells appeared cobblestone-shaped and were positive for ZO-1 and cytokeratin-18, but negative for α-SMA, CD68, CD31 and Factor VIII by IHC analysis. In contrast, HPMC grew rapidly in culture on the non-coated dishes in the presence of EGF, adopting a spindle fibroblast-like shape as previously reported (26). We studied the CTGF mRNA content of HPMC in both culture conditions. There was no significant correlation between the rate of peritoneal membrane transport (D/P Cr) in the PD patient of origin and the basal CTGF mRNA expression under EGF-positive (p=0.601) or negative (p=0.452) culture conditions with or without type I collagen. Also no correlation between basal CTGF mRNA expression and duration of PD treatment (EGF positive condition; p = 0.748, EGF negative condition; p=0.822) was found. We confirmed that all of the spindle-shaped HPMC cultured in EGF-containing medium reversibly modified into the polygonal, cobblestone-shaped, epitheloid morphology after seeding and culture on collagen dishes.
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Figure 5. Morphological differences in the cultured human peritoneal mesothelial cells (HPMC) under basal conditions. Morphological differences of mesothelial cells were not demonstrated between mesothelial cells from dialysate effluent of high permeable peritoneal function (PET high; B) and low permeable peritoneal function (PET low; A).

Figure 6. CTGF mRNA expression after incubation with TGF-β1 in Met-5A (mesothelial cell line) and HPMC. CTGF mRNA expression was studied 3, 6, 12, and 24 hours post TGF-β1 stimulation (5ng/ml). (A) TGF-β1 (5ng/ml) stimulation on Met-5A (n = 4 dishes of each time point). (B) CTGF mRNA expression after TGF-β1 stimulation on the HPMC from the spent PD fluid of the patient with high permeability (PET high) and with low permeability (PET low) (n = 4 dishes of each time point). Figures indicate one of the representative cases from each category.

*P < 0.05, **P < 0.005, ## P < 0.001, ### P < 0.0001 vs 0 hr.

CTGF mRNA expression after incubation with TGF-β1 in cultured human peritoneal mesothelial cells (HPMC) and Met-5A mesothelial cell-line

The time course of CTGF mRNA expression in response to TGF-β1-treatment was studied in both HPMC and Met-5A cells. Samples were taken after 3, 6, 12 and 24 hours of exposure to TGF-β1 (5 ng/ml). In 24 HPMC derived from 32 patients and Met-5A cells,
CTGF mRNA expression was increased and peaked at 12 hours. In another 8 strains of HPMC CTGF mRNA expression peaked at 24 hours after incubation with TGF-β1 (Figure 6A, B). Therefore, we evaluated the increment of CTGF induction by TGF-β1 at both 12 hr and 24 hr post TGF-β1 exposure. Increase of CTGF mRNA at 12 hours (fold) showed a good correlation with D/P Cr in both the PD patients group treated less than 2 yrs with PD (r=0.802, p<0.0001) and all patients group (R=0.668, p<0.0001, Figure 7A, B). This suggests that mesothelial cells from high peritoneal solute transport groups induce higher levels of CTGF by TGF-β1. Correlation was higher at 12 hours than at 24 hours (Figure 7).

No significant correlation between the extent of TGF-β1-induced increase of CTGF mRNA expression in HPMC and the duration of PD treatment of the patients from which the HPMC were derived was found (Figure 8). Western blotting did not reveal differences in the levels of TGF-β type II receptor expression on the HPMC of patients with high peritoneal transport compared to those with low peritoneal transport, therefore excluding this as a possible factor in differential induction of CTGF expression by TGF-β in these groups of HPMC (Figure 9).

**BMP-4 mRNA expression before and after incubation with TGF-β1 in cultured human peritoneal mesothelial cells (HPMC)**

To characterize the BMP-4 expression after exposure to TGF-β1, we examined the time course of BMP-4 mRNA in HPMC. TGF-β1 induced a transient decrease of BMP-4 expression in HPMC of patients with low/low average peritoneal transport. In contrast, BMP-4 mRNA expression continued to be downregulated at 12 to 24 hrs after incubation with TGF-β1 in HPMC from the high peritoneal transport patients (Figure 10A). We found a good inverse correlation between D/P Cr and BMP-4 mRNA levels 12 hrs after exposure to TGF-β1 in both the PD patients groups treated less than 2 yrs (R= - 0.678, p<0.001) and all patients group (R= − 0.500, p<0.01), which was reciprocal to the CTGF response on TGF-β1 exposure (Figure 10B, C). No correlation was observed between D/P Cr and basal BMP-4 mRNA expression both with and without presence of EGF (p=0.474).

**DISCUSSION**

Although there is increasing evidence that peritoneal membrane failure is associated with fibrosis and neoangiogenesis, the precise mechanisms of interactions between peritoneal fibrosis and ultra-filtration failure have not been defined. Peritoneal dialysis treatment itself had a strong impact on the progression of peritoneal fibrosis/sclerosis (17), and the sub-mesothelial compact zone thickness increased significantly with duration of PD therapy and was remarkable in the state of UFF (49). In our analysis, CTGF mRNA was significantly increased in the UFF group and was correlated with thickness of the peritoneum in all groups other than peritonitis. We excluded the cases
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with peritonitis in this analysis, because peritoneum can be thickened by the acute inflammatory changes with strong cell infiltration, exudation of fibrin and edema (35, 38). By immunohistochemistry and in-situ hybridization, CTGF protein and mRNA were detected in the mesothelial cells and fibroblasts in the thickened peritoneal membrane associated with high peritoneal transport. These findings indicate that CTGF is likely to be involved in peritoneal fibrosis and UFF.

Higher levels of plasma CTGF in PD patients may be affected by several factors, such as 1) accumulation by reduced renal clearance; 2) production by proliferative renal cells, preretinal tissues, blood vessel endothelial cells and peritoneal tissues (16, 33, 34, 36); 3) absorption of PD fluid, which contains CTGF, through lymphatic vessels (29, 42). In this respect, local peritoneal CTGF production may contribute to the high levels of plasma CTGF. CTGF in PD effluent might be derived from the circulation and/or be locally produced in the peritoneum, especially by mesothelial cells and fibroblasts which

Figure 7. The relationship between “CTGF amplification ratio” and peritoneal permeability.
Relationship between peritoneal permeability (D/P Cr) and increment of CTGF mRNA expression after stimulation with TGF-β1 in cultured mesothelial cells from the spent PD effluent.
(A) 12 h after stimulation with TGF-β1 (5 ng/ml), patients treated for less than 2 years
(B) 12 h after stimulation with TGF-β1 (5 ng/ml), all patients
(C) 24 h after stimulation with TGF-β1 (5 ng/ml), patients treated for less than 2 years
(D) 24 h after stimulation with TGF-β1 (5 ng/ml), all patients
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is consistent with the ISH and IHC results. In contrast to the rapid removal of the small molecular weight solutes such as urea and creatinine into the PD fluid, the extent of low molecular weight protein transfer from circulation to PD fluid is dependent on several factors including dwelling time and molecular weight (23). Diffusion of full-length and fragmentary CTGF from circulation to PD fluid can be expected to increase linearly with time. Based on these concepts, local peritoneal production of CTGF can be calculated by the difference between the measured and expected dialysate concentration using the peritoneal transport line determined for each patient. IP/Western blotting showed that CTGF proteins in the peritoneal effluent were 22 kDa and 25-28 kDa peptides, a proteolytically processed form of CTGF. Smaller fragments of CTGF can more easily diffuse from the circulation to the peritoneal fluid. Therefore, local production of CTGF using individual peritoneal transport lines was calculated at the MW 22 kDa to estimate the minimum local production rate. In addition, charge may affect the D/P

Figure 8. The relationship between “CTGF amplification ratio” and duration of PD treatment. Relationship between duration of treatment with PD and increment of CTGF mRNA expression in cultured mesothelial cells from the spent PD effluent.
(A) 12 h after stimulation with TGF-β1 (5 ng/ml), patients treated for less than 2 years
(B) 12 h after stimulation with TGF-β1 (5 ng/ml), all patients
(C) 24 h after stimulation with TGF-β1 (5 ng/ml), patients treated for less than 2 years
(D) 24 h after stimulation with TGF-β1 (5 ng/ml), all patients
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ratio. β2-Microglobulin, albumin and α2-macroglobulin are negatively charged, and their isoelectric points are 6.5, 6.2 and 6.4, respectively. In contrast, CTGF is positively charged with an isoelectric point of 8.0 (http://www.ensembl.org/index.html) and therefore is prone to adhere to the peritoneal membrane, which is negatively charged by glycosaminoglycans and proteoglycans (14, 55). CTGF is generally considered to be a “sticky” protein and the C-terminal half of CTGF is known to bind heparin sulfate proteoglycans (15). A recent report has further shown that the N-terminal half of CTGF can bind aggrecan (2). In this respect, the actual local CTGF production may be even higher than the calculated local production of CTGF. Another complication to consider is that after initiation of PD, the amount of glycosaminoglycans and proteoglycans may change (55). Presently it is not possible to correct for these factors in the formula of calculation. Nevertheless, our calculations indicate that local peritoneal CTGF production correlates well with peritoneal transport D/P Cr (R=0.723, p<0.0001), suggesting that peritoneal membranes in patients with higher peritoneal transport rates are characterized by production of larger amounts of CTGF. This is consistent with the positive correlation between D/P Cr and TGF-β induced CTGF production we observed in cultured mesothelial cells from spent effluents, and strong expression of CTGF demonstrated by IHC and ISH in the UFF group. Therefore, because abnormally elevated CTGF production correlates with peritoneal fibrosis, use of CTGF as a biomarker by measuring CTGF content in PD effluent could provide important information about peritoneal transport abnormalities and activity of the peritoneal fibrotic process. TGF-β was also reported to be elevated in the PD effluent and was correlated with peritoneal transport D/P (24). There are many mechanisms including exposure to glucose, AGE, glucose degradation products, IL-1 and Angiotensin II, to up-regulate TGF-β in the mesothelial cells of the PD patients (3, 27, 47). Thus, high levels of both TGF-β and CTGF in PD effluent of high transport patients might strongly enhance profibrotic activity in the peritoneal cavity.

Mesothelial cells are the main components of peritoneum and play an important role in peritoneal homeostasis including antigen presentation, clearance of fibrin, synthesis of

Figure 9. Western blotting of TGF-β type II receptor on the cultured human peritoneal mesothelial cells (HPMC) Lanes 1 to 4 are from different patients with the high category and lanes 5 to 8 were from the low category of the PET. There are no differences between two groups. Representative cases are shown.
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**Figure 10.** BMP-4 mRNA expression after incubation with TGF-β1 in the cultured HPMC.

**A.** BMP-4 mRNA expression is downregulated from 3 hrs to 24 hrs after incubation with TGF-β1 in the PET high category patient. In contrast, BMP-4 is decreased transiently and recovered at 12 and 24 h in the PET low category patient. Representative cases are shown. *P < 0.05, **P < 0.005, ###P < 0.0001 vs 0 h.

**B.** Relationship between peritoneal permeability (D/P Cr) and increment of BMP-4 mRNA expression in cultured mesothelial cells from the spent PD effluent. 12 h after stimulation with TGF-β1 (5 ng/ml), patients treated for less than 2 years.

**C.** Relationship between peritoneal permeability (D/P Cr) and increment of BMP-4 mRNA expression in cultured mesothelial cells from the spent PD effluent. 12 hours after stimulation with TGF-β1 (5 ng/ml), all patients.
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cytokines, growth factors, and matrix proteins (56). There is increasing data about the role of mesothelial cells in determining the functional alteration of peritoneum during PD. Human peritoneal mesothelial cells isolated from spent dialysate of PD patients cannot be termed “normal”. Nevertheless, studies using PD-derived HPMC may provide essential data of the physiologic status of renal failure and the patient’s own peritoneal status during PD (56). Cells obtained from spent dialysis effluent from PD patients were reported to be enlarged, multivacuolated and to have reduced microvilli density and dysfunctional mitochondria in longterm PD therapy (8, 51, 56). Investigations into the role of mesothelial cells in the structural and functional alterations of the peritoneum during PD have shown that mesothelial cells lose epithelial phenotype and acquire myo-fibroblast phenotype by an epithelial-to-mesenchymal transition (EMT) (3, 50). The prevalence of nonepitheloid cells by culture on the type I collagen plates in the absence of EGF conditions was reported to be related to the duration of CAPD (50). During the first 2 PD years, EMT of mesothelial cells is a frequent morphological change in the peritoneal membrane (50). In addition, the prevalence of nonepitheloid cells were detected in the high transport status (13). However, in our experiments all patients’ mesothelial cells from the spent dialysis fluid cultured on the type I collagen dishes showed a cobblestone-like appearance with positive staining for cytokeratin and ZO-1, which suggests that mesothelial cells in our experimental setup display a reduced fibrogenic phenotype compared to those in previous reports (50). These differences may be related to the usage of neutral pH dialysate in our patient groups. In contrast, mesothelial cells grew rapidly in culture when provided with serum, EGF, and hydrocortisone, adopting a fibroblast shape and forming parallel, multilayered arrays as reported previously (9). EGF was reported to transform cultured HPMC to fibroblast phenotype (26). When cells were split and subsequently cultured on a collagen matrix in absence of EGF, we established that these cells reverse their phenotype into polygonal, cobble-stone epitheloid morphology with positive cytokeratin and ZO-1 staining. These findings suggest that EMT of mesothelial cells is reversible and may be followed by mesenchymal-to-epithelial transition under proper conditions. Therefore, we explored basal CTGF expression in both mesothelial cells cultured on the type I collagen dishes without EGF conditions and cultured on the non-coated dishes with EGF positive conditions. In both conditions CTGF basal expression before TGF-β1 stimulation was not different for mesothelial cells from patients with either high or low peritoneal solute transport. However, CTGF induction by TGF-β is quite different for these two groups. CTGF mRNA is shown to be up-regulated by TGF-β in the cultured mesothelial cells from spent PD effluent in our experiments as previously reported for rat (44) and human mesothelial cells derived from omentum (57). These findings are consistent with the notion that TGF-β is an important inducer of CTGF expression in mesothelial cells. An increased susceptibility to TGF-β exposure, leading to increased production of CTGF in mesothelial cells, may be related to the extent of fibrosis of the peritoneal membrane and corresponds with the observed phenotypic changes in patients with high peritoneal solute transport. Identification of the factors which regulate enhanced
responsiveness to TGF-β may help to identify new strategies to prevent peritoneal fibrosis. First, we examined the expression of TGF-β type II receptor in the HPMC and found no differences between high and low peritoneal transport categories. To further characterize the difference we evaluated BMP-4 expression in HPMC. We demonstrated reciprocal expression patterns of BMP-4 and CTGF after exposure to TGF-β1. CTGF was previously shown to bind directly to TGF-β and BMP-4 through its CR domain (i.e., domain 2) and regulate opposite effects. Recently BMP-4 was reported to inhibit TGF-β2-induced expression of extracellular matrix protein and to have antagonistic effects on aldosterone signaling in the mesangial cells (1, 31, 32, 37, 58). Imbalance of TGF-β signaling leading to altered CTGF and BMP-4 expression in the mesothelial cells may play an important role in the regulation of fibrogenic and anti-fibrogenic activity in the different PET category. Future studies are necessary to investigate whether overexpression of BMP-4 can compensate the dysregulation of TGF-β and CTGF signaling pathway in vitro and in vivo, which may lead to new strategies to control peritoneal transport. In addition, regulation with other anti-fibrogenic factors including BMP-7 and hepatocyte growth factor will have to be established in the future (46, 54).

Peritoneal neoangiogenesis is a major factor in development of peritoneal membrane failure, and local production of VEGF during PD has been proposed to play a central role in increased solute transport and ultra-filtration failure. VEGF has been shown to stimulate CTGF mRNA and protein production in bovine retinal endothelial cells and pericytes (43), and under certain circumstances, also CTGF can exert angiogenic effects, as was demonstrated by the induction of neovascularization in rat corneal micropocket implants (4) and in chicken chorio-allantoic membranes (41). In contrast, Inoki and colleagues reported that CTGF inhibited VEGF-induced angiogenesis (1, 18). In our studies, we did not observe any relation between CTGF expression and the number of vessels in the peritoneal membrane. This suggests that CTGF expression level may not be a major determinant of angiogenesis in the peritoneal membrane.

In summary, we report that high peritoneal transport state correlates with increased peritoneal CTGF expression and higher CTGF expression in response to TGF-β in mesothelial cells. CTGF content in spent dialysate might be a biomarker for development of peritoneal fibrosis in PD patients. Functional alteration of mesothelial cells, as exemplified by the altered balance of CTGF and BMP-4 expression induced by TGF-β, may be involved in progression of peritoneal fibrosis in the high transport state. Phenotypic control of the mesothelial cells could be a potential therapeutic target of peritoneal fibrosis and membrane failure.
ACKNOWLEDGEMENTS
The technical assistance of Mr. Norihiko Suzuki, Ms. Keiko Higashide, Ms. Naoko Asano and Ms. Yuriko Sawa (Department of Nephrology, Nagoya University, Nagoya, Japan) is gratefully acknowledged. We thank Dongxia Li, Dan Yi, Thomas Crowley and Jay Leone (FibroGen, San Francisco, CA) for the technical assistance on the ELISA and Western blotting analysis.

GRANTS
This work was supported by in part by a grant-in-aid for Scientific Research from the Ministry Education, Science, and Culture, Japan (# 20590972), the 2007 research grant from the Aichi Kidney Foundation and Baxter Japan PD Grant 2009.

DISCLOSURES
N. Oliver is an employee of FibroGen Inc. (San Francisco, CA), supplier of anti-CTGF antibodies; R. Goldschmeding has received research support grants, and is currently also an employee of FibroGen Inc.

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