TGF-β1 Promotes Lymphangiogenesis during Peritoneal Fibrosis

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ABSTRACT
Peritoneal fibrosis (PF) causes ultrafiltration failure (UFF) and is a complicating factor in long-term peritoneal dialysis. Lymphatic reabsorption also may contribute to UFF, but little is known about lymphangiogenesis in patients with UFF and peritonitis. We studied the role of the lymphangiogenesis mediator vascular endothelial growth factor-C (VEGF-C) in human dialysate effluents, peritoneal tissues, and peritoneal mesothelial cells (HPMCs). Dialysate VEGF-C concentration correlated positively with the dialysate-to-plasma ratio of creatinine (D/P Cr) and the dialysate TGF-β1 concentration. Peritoneal tissue from patients with UFF expressed higher levels of VEGF-C, lymphatic endothelial hyaluronan receptor-1 (LYVE-1), and podoplanin mRNA and contained more lymphatic vessels than tissue from patients without UFF. Furthermore, mesothelial cell and macrophage expression of VEGF-C increased in the peritoneal membranes of patients with UFF and peritonitis. In cultured mesothelial cells, TGF-β1 upregulated the expression of VEGF-C mRNA and protein, and this upregulation was suppressed by a TGF-β type I receptor (TGFβR-I) inhibitor. TGF-β1–induced upregulation of VEGF-C mRNA expression in cultured HPMCs correlated with the D/P Cr of the patient from whom the HPMCs were derived (P<0.001). Moreover, treatment with a TGFβR-I inhibitor suppressed the enhanced lymphangiogenesis and VEGF-C expression associated with fibrosis in a rat model of PF. These results suggest that lymphangiogenesis associates with fibrosis through the TGF-β–VEGF-C pathway.


The decrease in ultrafiltration capacity that is associated with the high peritoneal solute transport that is observed after prolonged peritoneal dialysis (PD) treatment is a major reason for its discontinuation.1–4 Several studies have shown that a higher peritoneal solute transport rate is associated with reduced survival of PD patients.1,2,5 The characteristic features of chronic peritoneal damage in PD treatment are associated with submesothelial fibrosis and neangiogenesis.6,7 Analyses of the surface peritoneum showed no significant changes in vessel density with duration of PD.6,8 In addition, the vessel density in patients with ultrafiltration failure (UFF) was significantly higher than the vessel density in normal individuals or non-PD patients, but it was not higher than the vessel density in patients undergoing PD.4 These findings suggest that factors other than increased vascular density may be involved in disease states associated with increased ultrafiltration capacity.
transport of peritoneal membranes. In addition, the relationship between peritoneal fibrosis and UFF remains obscure.

Blood capillaries have a continuous basal lamina with tight interendothelial junctions and are supported by pericytes and smooth muscle cells. In contrast, lymphatic capillaries are thin-walled with a wide lumen and do not contain pericytes or basement membrane. The structures of lymphatic vessels are suitable for the removal of tissue fluid, cells, and macromolecules from the interstitium. If lymphangiogenesis develops in the peritoneal membrane, absorption of the PD fluid could be increased and lead to UFF. An increase in the number of lymphatic vessels has recently been reported in several disease conditions, including tumor metastasis, chronic respiratory inflammatory diseases, wound healing, and renal transplant rejection. We recently reported that lymphangiogenesis had developed in tubulointerstitial fibrosis of human renal biopsy specimens, and we also reported the mechanisms of lymphangiogenesis in rat unilateral ureteral obstruction models.

The lymphatic absorption rate, which is measured by the rate at which intraperitoneally administered radioactive serum albumin or macromolecule dextran 70 disappears, is significantly higher in patients with UFF, and lymphatic reabsorption is considered to be one of the causes of UFF. However, the results from these clinical approaches have been controversial. In addition, little is known about the pathology and the process of lymphangiogenesis in patients with UFF and peritonitis.

In this study, we investigated lymphangiogenesis and the expression of vascular endothelial growth factor-C (VEGF-C), which is a potentially important mediator of lymphangiogenesis, in human peritoneal tissues, PD effluent, and peritoneal mesothelial cells. We also explored VEGF-C induction by TGF-β1 in the human mesothelial cell line (Met-5A) and cultured human peritoneal mesothelial cells (HPMCs) from the spent PD effluent of patients with varying rates of peritoneal transport. Finally, we explored the relationship between peritoneal fibrosis and lymphangiogenesis in rats that were administered chlorhexidine gluconate (CG) into the abdominal cavity, which provides a model of chemically induced peritoneal inflammation/fibrosis. This work is the first report to show that lymphangiogenesis is linked to the peritoneal fibrosis that is often associated with a high peritoneal transport rate.

**Figure 1.** VEGF-C concentration in human PD effluent correlates with TGF-β1 concentration in PD effluent and the peritoneal transport rate (D/P Cr). (A) Positive correlation between the VEGF-C concentration in the PD effluent of 4-hour dwelled samples and the D/P Cr. (B) Positive correlation between VEGF-C and TGF-β1 concentrations in the PD effluent of 4-hour dwelled samples. (C) Positive correlation between the VEGF-C concentration in overnight dwelled PD effluent samples and D/P Cr.

**Table 1.** Peritoneal biopsy cases evaluated for VEGF-C, LYVE-1, and podoplanin mRNA expression

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<td>Average thickness of peritoneum, μm</td>
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<td>155.7 ± 93.1</td>
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Values are means ± SD. Normal renal function indicates living kidney donors with normal renal function. Predialysis uremia indicates peritoneal tissues that were taken at the time when a PD catheter was inserted because of advanced renal failure. Incident indicates that peritoneal tissues were taken when the catheter was removed because of reasons other than UFF.
RESULTS

VEGF-C Concentration in the Peritoneal Effluent Correlated with the Peritoneal Transport Rate

We found a positive correlation between VEGF-C concentration in the PD effluent of 4-hour dwelled samples and dialysate-to-plasma ratio of creatinine (D/P Cr; \( R=0.663, P<0.001 \)) (Figure 1A). We also measured dialysate TGF-β1 levels. There was a positive correlation between VEGF-C and TGF-β1 concentration in the PD effluent of 4-hour dwelled samples (\( R=0.772, P<0.001 \)) (Figure 1B). We also assessed VEGF-C concentration in the overnight dwelled PD effluent.
of 83 patients; there was a positive correlation between the dialysate VEGF-C concentration and the D/P Cr ratio ($R=0.417$, $P<0.001$) (Figure 1C).

**VEGF-C, Lymphatic Endothelial Hyaluronan Receptor-1, and Podoplanin mRNA Expression Were Correlated with Peritoneal Thickness in Human Peritoneal Biopsy Samples**

We next investigated the mRNA expression of lymphatic markers (lymphatic endothelial hyaluronan receptor-1 [LYVE-1] and podoplanin) and VEGF-C in the peritoneal membrane of human biopsy samples (Table 1). The peritoneal membrane in the predialysis uremia group (160.0±51.8 μm) was thicker than the peritoneal membrane in living kidney donors with normal renal function (82.0±22.9 μm). The peritoneum from patients with UFF and peritonitis conditions (referred to hereafter as UFF peritoneum and peritonitis peritoneum, respectively) was extremely thick (295.2±125.5 and 311.2±169.6 μm, respectively). The mRNA expression of VEGF-C, LYVE-1, and podoplanin was significantly higher in the UFF peritoneum than the membranes of peritoneum from patients with normal renal function, patients with predialysis uremia, or patients undergoing PD without UFF (Figure 2, A–C). There were correlations between VEGF-C, LYVE-1, and podoplanin mRNA expression and the thickness of the submesothelial compact zone of peritoneal membranes in all patients other than peritonitis patients (Figure 2, D–F). Cases with peritonitis were excluded from this analysis, because as described previously, peritoneum can be thickened by acute inflammatory changes with edema.33 Moreover, there were positive correlations between VEGF-C, LYVE-1, and podoplanin mRNA expression in peritoneal membranes (Figure 2, G–I).

**Lymphatic Vessels and VEGF-C Expression Increased in UFF Human Peritoneum Analyzed by Immunohistochemistry**

We evaluated lymphatic vessels, blood vessels, and expression of VEGF-C in both UFF and predialysis peritoneum by immunohistochemistry (IHC). LYVE-1–positive lymphatic vessels, pathologische anatomie leiden endothelium–positive blood vessels, and VEGF-C expression were widely observed in UFF peritoneum, but in contrast, they were barely detected in predialysis peritoneum (Supplemental Figure 1). VEGF-C was expressed in cytokeratin-positive mesothelial cells and CD68-positive macrophages, and its expression was enhanced in UFF and peritonitis peritoneal membranes (Figure 3). These findings are consistent with the quantitative PCR data (Figure 2).

**TGF-β1–Induced Upregulation of VEGF-C in Cultured Met-5A Mesothelial Cell Line and HPMCs**

The time course of VEGF-C expression in response to TGF-β1 treatment was studied in both HPMCs and Met-5A cells. Samples were taken after 3, 6, 12, and 24 hours of exposure to TGF-β1 (5 ng/ml). In the Met-5A cells, VEGF-C protein was secreted into the culture supernatants under serum-free conditions without TGF-β1 incubation. The amount of VEGF-C protein secreted was significantly increased by TGF-β1 incubation for 6 ($P<0.05$), 12, and 24 hours ($P<0.001$) (Figure 4A). VEGF-C mRNA
induction by TGF-β1 also increased over time and peaked at 12 hours (Figure 4B). Both VEGF-C protein and mRNA induction by TGF-β1 in Met-5A cells were suppressed by a TGF-β type I receptor inhibitor (TGFβRI inhibitor; LY364947) in a dose-dependent manner (Figure 4, C and D). Incubation of HPMCs derived from 29 patients with variable peritoneal membrane transport (Table 2) with TGF-β1 increased VEGF-C protein and mRNA expression (Figure 5, A and B).
VEGF-C mRNA expression peaked at 12 hours in 8 of the HPMCs and 24 hours in 21 of the HPMCs. Peak values for VEGF-C mRNA at 12 or 24 hours in all 29 HPMCs showed a correlation with the D/P Cr values of the patients from whom they were derived \((R = 0.610, P < 0.001)\) (Figure 5C). No significant correlation was found between the extent of the TGF-β1–induced increase in VEGF-C mRNA in the HPMCs and the duration of PD treatment of the patients from whom the HPMCs were derived (Figure 5D).

### Lymphangiogenesis Developed in a Rat CG Model of Peritoneal Fibrosis

Immunohistochemical analysis indicated that the expression of LYVE-1–positive lymphatic vessels, with a pattern of expression that is similar to the pattern of podoplanin-positive vessels, was increased in both the parietal peritoneum and the diaphragm of CG models compared with controls (Figures 6 and 7A). Furthermore, lymphangiogenesis was more pronounced in the diaphragm than in the parietal peritoneal wall (Figure 6). TGF-β1, VEGF-C, LYVE-1, and VEGF receptor-3 (VEGFR-3) mRNA expression were also increased in both the parietal peritoneum and diaphragm in the CG model compared with controls (Figure 6). VEGFR-3 expression, which was detected in the lymphatics, was also high in the CG model, especially in the diaphragm (Figure 6 and Supplemental Figure 2). Double staining indicated that VEGF-C was expressed by cytokeratin-positive mesothelial cells and ED-1–positive macrophages (Figure 7).

### TGFβ-R-I Treatment Suppressed Lymphangiogenesis in the CG Model

We investigated the effects of a TGFβ-R-I inhibitor in the rat CG model (Figures 8–10). Analysis of the parietal peritoneum indicated that the thickness of the peritoneum was significantly reduced by TGFβ-R-I inhibitor treatment. Quantitative immunohistochemical assessment showed that α-smooth muscle actin (α-SMA) expression, type III collagen deposition, and the number of ED-1–positive macrophages were suppressed by TGFβ-R-I inhibitor treatment (Figures 8 and 9). In addition, VEGF-C expression and LYVE-1–positive areas were significantly suppressed by TGFβ-R-I inhibitor treatment (Figures 8 and 9). Uregulation of type III collagen, VEGF-C, LYVE-1, and podoplanin mRNA was significantly inhibited by TGFβ-R-I inhibitor \((P < 0.05)\) (Figure 10). Moreover, diaphragm thickness \((P < 0.01)\) as well as the number of LYVE-1–positive areas \((P < 0.05)\) and VEGF-C \((P < 0.001)\) and LYVE-1 \((P < 0.05)\) mRNA expression in the diaphragm were all significantly suppressed by TGFβ-R-I inhibitor treatment (Supplemental Figure 3).

### Function of Lymphatic Vessels

We analyzed lymphatic vessel function using the modified method of lymphangiography as described previously.34 We detected passage of FITC dextran (molecular weight=2,000,000), which can only be absorbed through the lymphatic vessels in the diaphragm, by immunofluorescence microscopy (Figure 11A), and we detected positive levels of FITC in the serum, which were especially high in the rats without treatment in the CG model (Figure 11D). The presence of FITC levels in the blood and analysis of microscopic findings including serial sections (Supplemental Figures 4D and 5) indicated that FITC dextran was absorbed by the lymphatic vessels and drained into the venous circulation by the lymphatic and thoracic ducts. Passage through the parietal lymphatic vessels was not prominent in the parietal peritoneum in the CG rats (Supplemental Figure 6). Inhibition of lymphangiogenesis by celecoxib35,36 reduced the serum FITC dextran levels, suggesting the reduction of the absorption volume.

### DISCUSSION

Ultrafiltration dysfunction often results from a combination of increased vascular surface area and decreased osmotic conductance.37,38 Dysfunction of the water channel aquaporine-1 (so-called ultrasmall pore39,40) is an important cause of UFF; in that this water channel alters osmotic conductance. Hypo-permeable peritoneum with a loss of peritoneal surface area is a rare cause of UFF. Lymphatic absorption was reported to be important in patients on short-term PD with UFF.38 However, little is known about the pathology, process, and mechanisms of lymphangiogenesis in patients with UFF and peritonitis.

In the present study, we first found that the VEGF-C content in the PD effluent of 4-hour dwelled samples and overnight dwelled samples correlated with the peritoneal membrane transport rate. Our data are consistent with the recent report by Yang et al.,41 which showed that VEGF-C could possibly be a biomarker for UFF; however, unlike our study, they did not investigate lymphangiogenesis. Our studies of human peritoneal membranes showed that VEGF-C, LYVE-1, and podoplanin mRNA expression were higher in UFF peritoneum than control membranes and that the expression level of VEGF-C and the number of lymphatic vessels correlated with one another. These findings suggest that lymphangiogenesis develops in patients with a high peritoneal transport rate. Markers of lymphatics as well as VEGF-C mRNA levels tended to be increased in the state of peritonitis, which is an important factor for the induction of peritoneal damage and fibrosis (Figure 2). The extent of lymphangiogenesis may be related to the duration of infection and inflammation, which may also be the case in acute and chronic tubulointerstitial nephritis.22 Thus, we
reported that lymphangiogenesis developed in chronic but not acute tubulointerstitial nephritis, which indicates that the duration of inflammation together with fibrotic process may be important for lymphangiogenesis.\textsuperscript{22} We propose that lymphangiogenesis is linked with the fibrotic process in the peritoneal membrane, because it is in other disorders, including renal fibrosis\textsuperscript{22,23,42} and lung fibrosis.\textsuperscript{18} Our experiments clearly showed that TGF-\textbeta1 functions as an inducer of lymphatic growth in peritoneum as well as an inducer of inflammatory cytokines.\textsuperscript{16,17,43–46} Lymphangiogenic induction by TGF-\textbeta1 may be one of the key mechanisms of the UFF that is associated with peritoneal fibrosis.

**Figure 5.** The increase in VEGF-C expression by TGF-\textbeta1 in HPMCs correlates with D/P Cr. HPMCs from the spent PD effluent of patients with high peritoneal permeability (PET high category) and low permeability (PET low average category) were stimulated with TGF-\textbeta1. (A) VEGF-C protein levels in the supernatant were determined by ELISA, and (B) VEGF-C mRNA levels were determined by real-time PCR. The figures show representative cases from each category. VEGF-C mRNA was increased by TGF-\textbeta1, and it peaked at 12 hours in 8 of the HPMCs and 24 hours in 21 of the HPMCs derived from 29 PD patients. (C) Positive correlation between peritoneal permeability (D/P Cr) and the peak values of enhanced VEGF-C mRNA expression at 12 or 24 hours after stimulation with TGF-\textbeta1 (5 ng/ml). (D) No significant correlation was observed between the duration of PD treatment and the peak values of increased VEGF-C mRNA expression at 12 or 24 hours after stimulation with TGF-\textbeta1 (5 ng/ml).
Figure 6. Expression of LYVE-1 increases in the parietal peritoneum and diaphragm of CG model rats. Staining of (A and B) the parietal peritoneum and (C and D) the diaphragm of (A and C) control and (B and D) CG model rats showed an increased expression of LYVE-1–positive lymphatic vessels (arrows) in the CG model. Strong expression of lymphatic vessels was seen in the diaphragm of the CG rats. Scale bars, 200 μm. (E) TGF-β1, VEGF-C, LYVE-1, and VEGFR-3 mRNA expressions in both the parietal peritoneum and the diaphragm were increased in the CG model compared with the control. Control, n=5; CG models, n=7 for each group. P, peritoneal side; PL, pleural side.
growth factor and bone morphogenic protein-4 expression. Similar to the previous study, VEGF-C induction was also enhanced in HPMCs of patients with a higher peritoneal transport rate, which suggests that the function of HPMCs from patients with higher peritoneal transport rates had changed. In contrast, VEGF-C is not upregulated by TGF-β1 in fibroblasts, despite the presence of basal VEGF-C expression. TGF-β1 in the peritoneal effluent was reported to be elevated in the PD effluent and correlate with D/P Cr, which we confirmed in our cohort (R=0.487, P<0.01) (Supplemental Figure 7). Furthermore, we found a significant correlation between the content of TGF-β1 and VEGF-C in the PD effluent (Figure 1B), which was consistent with the observed relationship between peritoneal thickness and the expression of VEGF-C and lymphatic vessels (Figure 2). There are several mechanisms by which TGF-β levels can be increased in dialysate. In this regard, prevention of TGF-β induction may reduce fibrosis and lymphangiogenesis, resulting in the prevention of peritoneal membrane failure.

In the normal abdominal cavity, lymphatics in the diaphragm form a specialized system that drains fluid from the peritoneal cavity and returns it to the vascular system. The net ultrafiltration volume at the end of a PD exchange equals the cumulative net transcapillary water transport minus lymphatic absorption during the exchange. We showed that lymphatic vessels are present in both the peritoneal and pleural sides of the normal rat diaphragm but are rarely detectable in the normal rat peritoneal wall. In the CG model, lymphangiogenesis developed in association with the upregulation of TGF-β1 and VEGFR-3. VEGFR-3 was mainly expressed by lymphatic vessels, and its expression was also increased after intra-peritoneal administration of CG (Figure 6 and Supplemental Figure 2). Consistent with the human studies, VEGF-C was mainly expressed by mesothelial cells and macrophages (Figure 7). Indeed, fibrotic processes, reflected by peritoneal thickness and α-SMA and type III collagen expression, were suppressed by the TGFBR-1 inhibitor. In addition, VEGF-C expression and lymphangiogenesis in both the parietal peritoneum and the diaphragm were also suppressed by the TGFBR-1 inhibitor (Figure 8 and Supplemental Figure 3). These findings indicate that peritoneal lymphangiogenesis is linked with TGF-β1 and VEGF-C expression. The inhibition of lymphangiogenesis by the TGFBR-1 inhibitor suggests that TGF-β1 is a key mediator of lymphangiogenesis in PD.

Using immunohistochemical and cell culture studies, we clearly showed that mesothelial cells and macrophages expressed VEGF-C, which indicates that these cells are at least the sources of growth factors for lymphangiogenesis in the peritoneum (Figures 3, 4, and 7). VEGF-C was induced by TGF-β1 in HPMCs and a cultured mesothelial cell line; the latter induction was suppressed by the TGFBR-1 inhibitor in a dose-dependent manner. In addition, we recently showed that TGF-β induces VEGF-C production by macrophages. These findings suggest that TGF-β1 is an important inducer of VEGF-C, leading to the lymphangiogenesis that is associated with peritoneal fibrosis in PD patients. Interestingly, TGF-β1-induced VEGF-C expression in mesothelial cells was enhanced in the mesothelial cells obtained from patients with a high peritoneal transport rate. We recently reported that connective tissue growth factor induction by TGF-β was higher in HPMCs derived from patients with high peritoneal transport. In these previous experiments, we confirmed that there was no difference in the level of TGF-β type II receptor expression on HPMCs of patients with high peritoneal transport compared with patients with low peritoneal transport and that an imbalance of TGF-β signaling altered connective tissue

Figure 7. Double immunofluorescent staining of frozen sections of a CG rat diaphragm shows that VEGF-C is expressed by cytokeratin-positive mesothelial cells and ED-1-positive macrophages. (A) The expression pattern of LYVE-1 (green) was similar to the expression pattern of podoplanin (red). (B) VEGF-C (red) was expressed in cytokeratin-positive mesothelial cells (green). (C) VEGF-C (red) was expressed in ED-1-positive macrophages (green). Arrows and arrowheads of the same color indicate the same cells. Nuclei were counterstained with 4',6-diamino-2-phenylindole (blue). (Insets) Magnification of the white boxed area. Scale bars, 50 μm.
in the development of lymphangiogenesis in the CG rat model. The limitation in the CG model is the difficulty in assessing peritoneal permeability because of severe inflammation, such as in bacterial peritonitis in humans. Therefore, we performed FITC dextran lymphangiography to evaluate the function of the lymphatic vessels and found that these lymphatics were functional (Figure 11). Reduc- tion of FITC dextran levels in the serum by inhibition of lymphangiogenesis indicates that the lymphatic vessels might be involved in the control of effluent volume in PD.

In clinical settings, TGF-β1, with levels in dialysate that are known to be increased by a glucose-based dialysis solution and episodes of peritonitis, may induce VEGF-C production by mesothelial cells and macrophages, thereby leading to lymphangiogenesis in the peritoneal membrane in PD patients, especially after long-term PD treatment (Figure 12). Future studies are required to determine whether specific inhibition of lymphatic vessels can ameliorate ultrafiltration failure in patients undergoing PD.

**CONCISE METHODS**

**Patient Profiles**

All of the studies were approved by the Ethics Committee for Human Research of the Faculty of Medicine, Nagoya University (approval #298: peritoneal fluid experiment; approval #299: peritoneal tissue experiments), and all patients provided informed consent before participation in the study.

**Peritoneal Transport of VEGF-C in PD Patients**

The VEGF-C concentration in peritoneal effluent was measured in overnight dwelled (8.95 ± 1.63 h) samples collected from 83 PD patients (27 women and 56 men) who were treated between July of 2005 and April of 2008 at the Department of Nephrology and Renal Replacement Therapy of Nagoya University Hospital (Nagoya, Japan) and affiliated hospitals including Handa Municipal Hospital (Handa, Japan), Chubu Rosai Hospital (Nagoya, Japan), Yokkaichi Municipal Hospital (Yokkaichi, Japan), Kounan-Kousei Hospital (Kounan, Japan), Daiyukai-Daiichi Hospital (Ichinomiya, Japan), and Nagoya Kyoritsu Hospital (Nagoya, Japan). The mean age of all patients was 55.9 ± 13.5 (range=28–89) years, and the mean duration of PD treatment was 31.9 ± 32.0 (range=1–132) months. Diabetic nephropathy was the cause of ESRD in 27 PD patients (32.5%). All patients were free from peritonitis for at least 1 month before the study, and patients with other diseases, such as liver or lung diseases and malignancy, were excluded. Patients undergoing combination therapy (hemodialysis + PD) were not included in this study. Peritoneal transport was assessed based on D/P Cr, and the average value was 0.67 ± 0.14 (range=0.28–0.96). The correlation between VEGF-C concentration in the PD effluent and D/P Cr was analyzed. In addition, we measured VEGF-C and TGF-β1 concentration in dialysate samples at 4 hours of peritoneal equilibration tests (PETs) collected from 47 PD patients (16 women and 31 men) treated between November of 2008 and June of 2009 at the Nagoya University Hospital and Handa Municipal Hospital. Fast PET was performed using 2.27% glucose-based dialysis solutions (Dianeal-N PD-4; Baxter) as described in the work by Twardowski. The mean age of all patients was 52.3 ± 10.9
impaired UFF, which was defined by the use of more than four hypertonic bags (2.27% glucose and 3.86% glucose or icodextrin) per 24 hours to maintain fluid balance. Six patients were peritonitis-positive; 23 patients (incident) had their catheters removed because of transplantation, mental disorders, severe exit site infection, or difficulty in carrying out the bag exchanges (Table 1). The correlations between VEGF-C, LYVE-1, and podoplanin mRNA expression and peritoneal membrane thickness were evaluated.

**VEGF-C Production in Human Mesothelial Cells**

HPMCs were isolated from spent PD effluent taken from 29 clinically stable patients (Table 2) and cultured by use of a modified method as described previously. Basal and TGF-β1–induced VEGF-C mRNA expressions were studied.

**Processing of Biopsy Samples and Morphologic Analysis**

Samples of parietal peritoneum were biopsied in the standard manner and processed as reported previously. The tissue samples were fixed with 10% buffered formalin overnight, routinely processed for light microscopy, and embedded in paraffin; 4-μm-thick sections were cut and stained with hematoxylin and eosin and Masson’s trichrome. Before analysis of peritoneal thickness, each specimen was assessed for size and the site and direction of the peritoneum. The adequacy of the samples was then judged as described by Honda et al., in which less than 50% of the samples were considered to be appropriate. It was possible to measure the thickness of 45 of 75 samples in our study. To assess the extent of peritoneal thickening, the submesothelial compact zone, which was the zone of peritoneal fibrosis, was defined as the zone between the basal border of the surface of the mesothelial cells and the upper border of the peritoneal adipose tissues. We measured peritoneal thickness at five random points using a Zeiss Z1 microscope and AxioVision Windows software version 4.4 (Carl Zeiss, Oberkochen, Germany), and mean thickness was calculated.

**Cell Culture Study**

A human mesothelial cell line (Met-5A) was purchased from the American Type Culture Collection (Manassas, VA) and maintained as reported previously. HPMCs from spent PD effluent were obtained by centrifugation of dialysis fluid taken randomly from clinically stable patients who had a variety of peritoneal permeabilities and were undergoing nocturnal exchanges using modified methods as
described previously. Cellular components were isolated using low-speed (200×g) centrifugation, washed with RPMI 1640 (Sigma, Tokyo, Japan), and then cultured in RPMI 1640 containing l-glutamine (Sigma) supplemented with 15% FBS (Sigma), insulin/transferrin/ selenium A (Invitrogen, Tokyo, Japan), 10 μM 2-mercaptoethanol (Wako, Osaka, Japan), 3.3 nM EGF (R&D Systems, Minneapolis, MN), and 400 μg/L hydrocortisone (Sigma) in humidified air with 5% CO_2 at 37°C. Nonadherent 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 they were then split two to three times and cultured. Subconfluent HPMCs and Met-5A were washed two times with PBS, and the culture medium was replaced with serum-free medium for 24 hours.

Subsequently, the 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=3 dishes of cells from each patient at each time point). All experiments were performed during the second to seventh passage. To explore the correlation between the enhancement of VEGF-C expression by TGF-β and D/P Cr, we assessed the increase in VEGF-C mRNA after 12- or 24-hour incubation with TGF-β1 as described previously. TGF-β1 inhibition studies were performed by incubating Met-5A cells with 5 ng/ml TGF-β1 combined with a selective inhibitor of TGFβR-1 (Calbiochem, La Jolla, CA) for 12 hours.

Animal Model

All animal studies were carried out in accordance with the Animal Experimentation Guidelines of Nagoya University Graduate School of Medicine (Nagoya, Japan). Eight-week-old male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) that initially weighed 240–260 g were used throughout the study. The animals were maintained under conventional laboratory conditions and given free access to food and water. Rats were given an intraperitoneal injection of 3 ml/200 g body wt of 0.04% CG (Wako, Japan) and 10% ethanol (Wako) dissolved in saline every other day. These rats were randomly assigned to a treatment or saline group. The rats in the treatment group (n=7) were given a daily intraperitoneal injection of 3 μg/g body wt TGFβR-I inhibitor dissolved in saline and DMSO (140 mg/ml; Wako). On the day of CG injection, the rats were injected with the TGFβR-I inhibitor 2 hours before injection of CG. The rats in the saline group (n=7) were given a daily intraperitoneal injection of saline and DMSO in a similar manner. Five control rats were injected daily with the same dosage of saline without CG or TGFβR-I inhibitor. All rats were euthanized on day 16. All injections and euthanizations were performed under anesthesia with diethyl ether (Wako). Parietal peritoneal and diaphragmatic samples were procured, and the harvested samples were used for analysis of peritoneal thickness, immunohistochemical analysis of VEGF-C, VEGFR-3, LYVE-1, podoplanin, ED-1, type III collagen, α-SMA, and cytokeratin, and analysis of the mRNA expression of VEGF-C, VEGFR-3, LYVE-1, podoplanin, and type III collagen.

Analysis of Lymphatic Vessel Function

We analyzed lymphatic vessel function using the modified method of lymphangiography as described previously. We intraperitoneally administered a total of 5 ml FITC-labeled dextran 2,000 (10 mg/ml concentration; molecular weight=2,000,000; Sigma). Twenty minutes later, blood was drawn, and the animals were euthanized. Serum samples were immediately separated from the blood, and absorbance was measured at 493 nm by spectrophotometry; blood from nonadministered rats was used as a normal control. The required time for all procedures by measurement with the spectrophotometer was fixed to 3 hours. The diaphragm and parietal peritoneum were harvested and snap frozen in OCT compound (Sakura Fine Technical, Tokyo, Japan). FITC dextran was mainly taken up by the lymphatic vessels in the diaphragm and passed to the central collectors of lymphatics. The detection of the FITC signal in the blood taken from the rats...
Figure 11. Drainage of FITC dextran administered into the abdominal cavity is suppressed by inhibition of lymphangiogenesis with cyclooxygenase-2 inhibitor. (A, upper panels) Immunohistochemical staining of LYVE-1 in the diaphragm of control rats, celecoxib-treated (daily oral administration of 50 mg/kg body wt) CG rats, and untreated CG rats. (A, lower panels) Rats were given intraperitoneal injections of 50 mg FITC dextran (molecular weight=2,000,000). Twenty minutes later, blood, peritoneal, and diaphragmatic samples were obtained. Immunohistochemical findings in the diaphragm of control, untreated, and celecoxib-treated rats were recorded. Scale bars, 100 μm. Arrow indicates the accumulation of FITC dextran in the central collector of lymphatic vessel. Quantification of positive area determined by MetaMorph 6.3 image software (Universal Imaging, West Chester, PA) in the diaphragm for (B) LYVE-1 and (C) FITC dextran showed that celecoxib treatment significantly reduced both positive areas compared with the untreated CG rats. (D) To assess the amount of FITC dextran in blood drawn at euthanization, absorbance of serum samples was measured at 493 nm by spectrophotometry. For preparation of the standard curve, FITC dextran was reconstituted and diluted with normal rat serum. Concentrations of FITC dextran in the serum were significantly decreased in celecoxib-treated CG rats compared with untreated CG rats. Real-time PCR analysis of (E and H) LYVE-1, (F and I) podoplanin, and (G and J) VEGF-C mRNA in (E–G) the rat diaphragm and (H–J) the parietal peritoneum indicated that increased expression of LYVE-1, podoplanin, and VEGF-C mRNA in CG rats was significantly suppressed by celecoxib treatment. Control, n=5; CG models with no treat, n=6; CG models treated with celecoxib, n=6.

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administered intraperitoneally with FITC dextran of molecular weight of 2,000,000 indicates that the FITC dextran was absorbed through the lymphatic vessels and drained into the blood circulation through the thoracic duct.\(^{59}\)

**Histology and IHC**

Routine histologic and IHC analyses of human and rat tissues were performed and analyzed as described previously.\(^{22,23,33,58,60}\) VEGF-C expression was analyzed and semiquantitatively classified as follows: 0, no staining; 1, mild staining; 2, moderate staining; 3, pronounced staining. The antibodies used are listed in Supplemental Table 1.

**ELISA**

VEGF-C and total (both active and latent forms) TGF-\(\beta\)1 protein levels in peritoneal dialysate (PD fluid) samples and cell culture supernatant were measured using the Human VEGF-C (IBL, Takasaki, Japan) and the TGF-\(\beta\)1 (R&D) ELISA Kit, respectively, according to the manufacturers’ instructions. Samples were frozen at the time of collection and stored at \(-80^\circ\text{C}\). Samples were not subjected to freeze–thaw cycles.

**RNA Preparation from Peritoneal Tissues and Cultured Mesothelial Cells and PCR Analysis**

Human and rat peritoneal tissues were immersed in RNA later (Ambion, Austin, TX) for 1 day or more. RNA preparation and the synthesis of first-strand cDNA were performed as described previously.\(^{22,23,33,58,60}\) Total RNA (1 \(\mu\)g) was then reverse transcribed. Real-time PCR analysis was performed with an Applied Biosystems Prism 7500HT sequence detection system using TaqMan gene expression assays as described previously.\(^{28}\) The TaqMan Gene Expression Assays (Applied Biosystems Inc.) used are described in Supplemental Table 2; 18S ribosomal RNA was used as an endogenous control.\(^{23,33,58,60}\)

**Statistical Analyses**

Values are expressed as means \(\pm\) SDs. Differences between two groups were analyzed by \(t\) test or Mann–Whitney test. Comparisons among groups were performed by one-way ANOVA followed by Dunnett or Kruskal–Wallis multiple comparison test. Pearson correlation coefficient was used to analyze the correlations. Differences were considered to be statistically significant if \(P<0.05\). All analyses were performed using SPSS software (SPSS, Chicago, IL).

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

None.

**REFERENCES**


29. Flesner M: Effective lymphatic absorption rate is not a useful or accurate term to use in the physiology of peritoneal dialysis. Perit Dial Int 24: 313–316, 2004


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Supplementary Figures and Tables

Transforming growth factor-β1 promotes lymphangiogenesis during peritoneal fibrosis

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Yoshihisa Matsukawa², Tomohiro Mizuno³, Yukihiro Noda³,
Hayato Nishimura⁴, Ryosuke Nishio⁵, Shoichi Maruyama¹,
Enyu Imai¹, Seiichi Matsuo¹ and Yoshifumi Takei⁶

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³ Division of Clinical Sciences and Neuropharmacology,
Meijyo University Graduate School of Medicine, Nagoya, Japan
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⁵ Department of Emergency Medicine, Kyoto University Hospital, Kyoto, Japan
⁶ Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan
Supplementary Figure 1. The number of lymphatic vessels and VEGF-C expression were increased in UFF-peritoneum compared to pre-dialysis uremia peritoneum.

Peritoneum was analyzed by immunohistochemistry. Lymphatic vessels were stained with an anti-LYVE-1 antibody. Blood vessels were stained with an anti-Pathologische Anatomie Leiden-Endothelium (PAL-E) antibody. Some mononuclear cells and non-vascular structures were revealed in immunostaining of LYVE-1 (D, G-J). These cells may be derived from other cells, such as macrophages, which may potentially transform to the lymphatic vessels (Ref. 1-3).

A and B: PAL-E, C and D: LYVE-1, E and F: VEGF-C, G-J: double staining of LYVE-1 (red) and PAL-E (blue). (A, C and E) and (B, D and F) were serial sections of the same patient. Scale bars, 200 μm.

References
Pre-dialysis uremia

A

PAL-E

C

LYVE-1

E

VEGF-C

G

PAL-E / LYVE-1

I

Supplementary Figure 1
Supplementary Figure 2.

Analysis of VEGF receptor-3 (VEGFR-3) expression in the diaphragm of control and CG model rats and in human peritoneal mesothelial cells and normal human lymphatic microvascular endothelial cells.

(A-E) Immunofluorescent staining of VEGFR-3 (green) in the diaphragms of representative control (A and C) and CG model (B and D) rats. Nuclei were counterstained with DAPI (blue). The level of VEGFR-3 expression, which was detected in the lymphatics, was increased in the CG model.

E: Double staining of VEGFR-3 and podoplanin in the diaphragm of a CG rat. Scale bars, 200 μm.

F: RT-PCR analysis of the mRNA expression of VEGFR-3, in human peritoneal mesothelial cells (HPMC) from patients with high and low peritoneal transport, and in normal human lymphatic microvascular endothelial cells (HMVEC-LLy). GAPDH was used as a loading control. VEGFR-3 mRNA was only detected in HMVEC-LLy cells.

Methods
HMVEC-LLy cells were purchased from Lonza Bioscience (Basel, Switzerland). The reverse transcription polymerase chain reaction (RT-PCR) was performed using the Hot-StarTaq PCR kit (Qiagen), as described previously (1, 2). PCR cycling conditions were as follows: initial denaturation (15 min at 94 °C) followed by 35 cycles of denaturation (1 min at 94 °C), annealing (45 s at 62 °C), and elongation (1 min at 72 °C). After the last cycle, a final extension (7 min at 72 °C) was added and thereafter the samples were kept at 4 °C. PCR products were electrophoresed on 2% agarose gels in Tris acetate EDTA buffer, followed by staining with ethidium bromide.

The sequences of the primers used were;

Human VEGFR-3 (Ref. 3)
forward: 5’-CCCACGCAGACATCAAGACG-3’
reverse: 5’-TGCAGAACTCCACGATCACC-3’ (380bp)

Human GAPDH (Ref. 1)
forward: 5’-ATCATCCCTGCCTCTACTGG-3’
reverse: 5’-CCCTCCGACGCCTGCTTCAC-3’ (188bp)

References
Supplementary Figure 2

1. HPMC (PET low-average category)
2. HPMC (PET High category)
3. HMVEC-LLy

HPMC: human peritoneal mesothelial cells
HMVEC-LLy: normal human lymphatic microvasculocellular endothelial cells
Supplementary Figure 3.
Immunohistochemical analyses, and real-time PCR analyses of selected mRNAs, in the diaphragm of TGFβR-I inhibitor-treated and untreated (saline) rats. Lymphangiogenesis was suppressed by the TGFβR-I inhibitor.

**A**: LYVE-1 staining of inhibitor-treated, untreated and control rats. Scale bars, 200 μm.

**B**: The thickness of the diaphragm was significantly reduced by TGFβR-I inhibitor treatment of CG model rats.

**C**: The size of the LYVE-1-positive area, as analyzed by MetaMorph, was significantly reduced by treatment with the TGFβR-I inhibitor.

**D and E**: The increased expression of VEGF-C and LYVE-1 mRNA in the CG model rats was suppressed by TGFβR-I inhibitor treatment.
Supplementary Figure 4.

The CG model in mice is similar to that in rats. Serial section analysis showed that the lymphatic vessels were connected to the central collector of the lymphatic vessels. Lymphangiogenesis developed in both the parietal peritoneum and diaphragm, which was similar to the rat CG model (A–C). Lymphatic vessels grown in the diaphragm were connected to the central collector of lymphatic vessels (D). Blue arrows in (D-4) of serial sections indicate the possible route of the passage for lymphatic fluid. (A, B) mouse diaphragm; (C) mouse parietal peritoneum.

Supplementary Figure 4
Supplementary Figure 4 D

Enlargement of the rectangular frame of Fig. 4D-4.
Arrow: central collector of lymphatic vessel
Arrowhead: artery

Scale bar, 200 μm.
Supplementary Figure 5.

Newly synthesized lymphatic vessels with entrance were revealed in the diaphragm.
On analysis of the serial sections, newly synthesized lymphatic vessels with entrance (red arrows) were revealed in the fibrotic diaphragm. Black arrows indicate the edges or surroundings of the entrance of the lymphatic vessels.
* (in 4-6) and ▲(in 13-16) indicate the connections of newly synthesized lymphatic vessel system.
Supplementary Figure 5

Scale bar, 200 μm.
Supplementary Figure 6.
There was barely detectable FITC-dextran in the parietal peritoneum, except on the surface of the peritoneum, as seen by immunofluorescence microscopy after administration of 50 mg FITC-dextran intraperitoneally.
Left panel: control.
Middle panel: CG model with no treatment
Right panel: CG model treated with COX-2 inhibitor, Celecoxib
Scale bar, 100 μm.

Supplementary Figure 6
Supplementary Figure 7.

The concentration of TGF-\(\beta1\) protein in human PD effluent was correlated with D/P Cr. There was a positive correlation between TGF-\(\beta1\) concentration in the PD effluent of 4-h-dwelled samples and D/P Cr.
**Supplementary Table 1.**

List of the antibodies used

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<td>rabbit anti-human LYVE-1 antibody</td>
<td>Acris Antibodies GmbH, Hiddenhausen, Germany</td>
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<td>rabbit anti-mouse LYVE-1 antibody</td>
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<td>rabbit anti-VEGF-C antibody</td>
<td>Zymed Laboratories, South San Francisco, CA</td>
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<tr>
<td>mouse anti-cytokeratin antibody</td>
<td>Dako, Glostrup, Denmark</td>
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<td>mouse anti-rat podoplanin antibody</td>
<td>Relia Tech GmbH, Braunschweig, Germany</td>
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<td>goat anti-VEGFR3 (Flt-4) antibody</td>
<td>R&amp;D System, Minneapolis, MN</td>
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<td>mouse anti-rat monocyte/macrophage antibody (ED1)</td>
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<td>DAPI (diamidino-2-phenylindole)</td>
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Supplementary Table 2.
Primers used for real-time PCR (TaqMan Gene Expression Assays)

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