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ORIGINAL ARTICLE

Artificial Kidney / Dialysis

Morphological characteristics in peritoneum in patients with neutral peritoneal dialysis solution

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Abstract Peritoneal dialysis solution (PDS) plays a role in functional and morphological damage to the peritoneum. This study aimed to clarify the effect of neutral PDS in preventing morphological changes by assessing peritoneal damage and comparing morphological alterations between PD patients treated with neutral PDS and acidic PDS. Sixty-one patients participated from seven hospitals. All patients were treated with neutral PDS excluding icodextrin, during their entire PD treatment, and experienced no episode of peritonitis. The thickness of submesothelial compact (SMC) zone and the presence of vasculopathy in the anterior parietal abdominal peritoneum were assessed. The impact of icodextrin, hybrid therapy, and peritoneal rest and lavage in morphological alterations were determined. There was no significant difference in the average SMC thickness between neutral and acidic PDS. The vessel patency in patients using neutral PDS was significantly higher compared to that in acidic PDS at any time during PD. There were no significant suppressive effects from interventions or use of icodextrin with respect to peritoneal morphological injury. A monolayer of mesothelial cell was observed in approximately half the patients, especially in their receiving lavage patients. Neutral PDS, accompanied by other preventive approaches against peritoneal injury, might suppress the development of peritoneal morphological alterations.

Keywords Peritoneal dialysis · Neutral PD solution · Pathological changes · Hybrid therapy · Peritoneal lavage

On behalf of the Peritoneal Biopsy Study Group of the Japanese Society for Peritoneal Dialysis.

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Introduction

Peritoneal dialysis (PD) has been an important renal replacement therapy over past several decades. Conventional PD solution is potentially bioincompatible because of its hyperosmolarity, low pH, and high lactate and glucose concentration [1, 2]. Glucose in conventional PD solution is affected by heat sterilization and converted to the substrate for glucose degradation products (GDPs), such as methylglyoxal, formaldehyde, 3-deoxyglycosone, acetaldehyde, 2-furaldehyde and 5-hydroxymethyl furaldehyde [3, 4]. Furthermore, glucose in PD solution is involved in providing the substrate for nonenzymatic glycation of tissue protein, which is a substance detrimental to peritoneum [5]. Although the acidity of conventional PD solution plays a role of preventing bacteria from growing during storage and glucose degeneration by heat sterilization, acidity causes dysfunction of mesothelial cells and peritoneal fibroblasts, as well as impairment of immune defense in the peritoneal cavity through deteriorating intracellular pH [4, 6]. Additionally, long-term exposure to bioincompatible PD solution results in various functional and morphological alterations in the peritoneum of PD patients.

Morphological characteristics of the peritoneum in PD patient who use bioincompatible PD solution include loss of mesothelial cells, thickening of the peritoneal interstitium, and vasculopathy in small vessels [2, 7]. Ultrafiltration failure associated with increased peritoneal permeability and impairment of immune defense in peritoneal cavity are characteristics of the major functional abnormalities induced by the bioincompatible PD solution [8]. Recently, greater biocompatibility of PD solution has been achieved through a more physiological pH, lower osmolarity, a decreased glucose load, and lower GDP content compared with the conventional acidic PD solutions [9]. A new biocompatible PD solution has been reported to provide some clinical benefits [10]. A higher concentration of cancer antigen (CA)-125 and a lower concentration of hyaluronan in overnight PD effluent have been observed in patients treated with the new biocompatible solution, indicating less mesothelial and interstitial damage [9, 10]. Several new strategies, such as using biocompatible PD solution in combination with peritoneal rest, peritoneal lavage and hemodialysis, have been implemented to prevent and reduce peritoneal injuries. These new strategies are expected to have a positive impact on the clinical and morphological alterations in the PD peritoneum.

The purpose of the present study was to examine the effects of neutral PD solution and several interventions with regard to PD treatment on morphological alterations in PD patients.

Materials and methods

Patients

Peritoneal biopsy specimens were obtained from 61 patients, who were treated with neutral peritoneal dialysate alone during PD therapy. The biopsies were performed during surgery for PD catheter removal prior to transfer to hemodialysis ($n = 60$), and during surgery for non PD-related abdominal disease ($n = 1$). Patients with history of peritonitis during PD therapy were excluded from this study; therefore, any influences by peritonitis on a study patient's peritoneal histology were eliminated in this study. The existence of diabetes was identified from clinical records. Approval for the study was obtained from the local ethics committees, and all patients gave written informed consent.

Peritoneal rest is the drainage of peritoneal effluent every day or every other day in PD patients transferred from PD to hemodialysis and scheduled to undergo PD catheter removal several months after PD cessation. Peritoneal lavage is lavage of the peritoneal cavity with PD solution every day or every other day in PD patients transferred to hemodialysis and scheduled to undergo PD catheter removal several months after PD cessation. The studied PD patients, who were experiencing dialysis that was nearly inadequate or overhydration caused by deterioration of residual renal function, had been undergoing hemodialysis once a week additionally as standard hybrid therapy.

Peritoneal biopsy samples ($n = 80$) were taken from PD patients who were treated with acidic peritoneal dialysate as control group, these were the same specimens used in our previous study [9]. The patients, whose average PD duration was 62.5 ± 43.3 months, had no history of peritonitis during their PD therapy.

Processing of biopsy samples

Forty-eight specimens out of the 61 participated patients were sampled near the site of catheter insertion during the routine surgical procedure of PD catheter removal. In 11 patients who underwent endoscopic surgical procedure and two patients who underwent open abdominal surgery, parietal peritoneal specimens from the anterior abdominal wall were obtained at the opposite site of catheter insertion.

Samples of the parietal peritoneum were biopsied in the usual manner. Briefly, the peritoneal tissue was cut by scalpel measuring approximately 1 cm in size and up to 5 mm in depth. Each tissue sample was placed on a small board or filter paper with the mesothelial surface uppermost, been extended to the same size as in situ, and fixed with 20 % buffered formalin. After overnight fixation at

room temperature, the samples were routinely processed for light microscopy and embedded in paraffin. The 4-μm sections were cut routinely and stained with hematoxylin and eosin and Masson trichrome.

Sample analysis methods

The samples were assessed by microscopy using a standardized method described next. Two experienced examiners, one pathologist (K. H.) and one nephrologist (C. H.), who were unaware of patients' clinical backgrounds, evaluated the samples independently.

Adequacy of specimen for histologic evaluation

The adequacy of each specimen for histologic evaluation of peritoneal thickness and vasculopathy was determined independently. For measurement of peritoneal thickness and vasculopathy, each specimen was assessed in terms of size, site, and direction of the specimen, and classified as an "adequate" or "inadequate specimen". An adequate specimen had a sampling size that was large enough and contained several layers of peritoneum (mesothelial, submesothelial, and adipose tissue layers). The direction of embedding was almost vertical so that the thickness of the submesothelial layer could be measured properly. For the evaluation of vasculopathy, an adequate specimen must contain a post-capillary venule (PCV) at the size of 25–50 μm in external diameter. The number of adequate and inadequate specimens for the evaluation of peritoneal thickness was 30 and 31, respectively, and for the evaluation of vasculopathy was 39 and 22, respectively. In patients using acidic PD solution, 40 samples from the 80 PD patients were adequate for the evaluation of peritoneal thickness and 76 samples from the 80 PD patients were adequate for the evaluation of vasculopathy.

Evaluation of peritoneal fibrosis

The extent of peritoneal fibrosis was determined by the thickness of submesothelial interstitial layer (submesothelial compact zone: SMC) between the basal border of the surface mesothelial cells and the upper border of the peritoneal adipose tissue. The thickness of the mesothelial cell layer was excluded from measurement when it was present on the peritoneal surface. When the submesothelial interstitium was continuous to underlying dense connective tissue (abdominal fascia) without peritoneal adipose tissue, the peritoneal thickness could not be measured. Five portions were randomly selected for the measurement of submesothelial (SMC) thickness (Fig. 1a). The thickness was measured by a micrometer on a microscopic lens or by an image analyzer, and then the average SMC thickness was calculated. The portion where the peritoneum appeared severely fibrotic as a result of tangential embedding or miscellaneous inflammatory reactions was excluded from measurement.

Evaluation of vasculopathy

The extent of vasculopathy was determined by the severity of luminal narrowing at the level of the PCVs. For evaluation of the severity of luminal narrowing, the ratio of luminal diameter to vessel external diameter (*L/V*) was determined (Fig. 1b), which represents the extent of patency of a blood vessel, according to our previous report [7]. In general, hyalinizing vasculopathy in PD patients is usually observed at the PCV or capillary level. For morphologic measurement, we selected a PCV whose diameter ranged from 25 to 50 μm, since the *L/V* was influenced by the level of blood vessel examined [11]. The measurement was obtained on the short axis to avoid the artificial effect of elongated distance as a result of tangential cutting of the

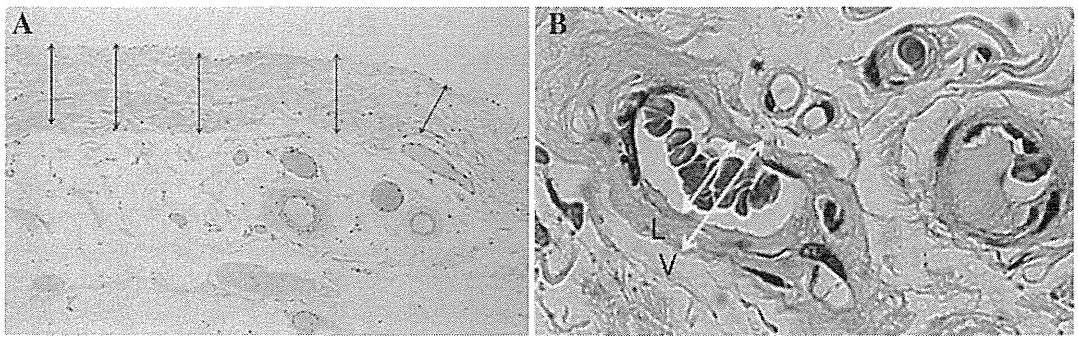

Fig. 1 **a** For the evaluation of peritoneal fibrosis, the peritoneal thicknesses at randomly selected five points were measured, and then the average peritoneal thickness (APT) was calculated. The average of two APT values determined by two examiners was taken as a representative APT of that case. **b** For the evaluation of vascular patency, the average ratio of lumen-to-vessel (*L/V*) diameter was calculated at five randomly selected post-capillary venules (PCVs) with external diameters of 25–50 μm

vessel during histologic preparation. When different vessels showed different severities of vasculopathy, five vessels of each specimen were chosen for measurement. The average of two thicknesses of vascular wall and *L/V* measured by two examiners was taken as the representative value of that case.

Evaluation of surface coverage with mesothelial cells

Forty-three out of 61 specimens were assessable for surface coverage. For semi-quantitative assessment, we classified four grades according to the coverage with mesothelial cells: grade 0, none: 1, <25 %; 2, 25–50 %; 3, >50 %.

Statistical analyses

Data are expressed as mean ± SD. Parametric comparison was performed by Student's *t* test, and nonparametric comparison was conducted by the Mann–Whitney *U* test to examine statistical significance. Relationships between clinical variables and morphological alterations were analyzed with Spearman's correlation coefficient. Repeated measures analysis of variance (ANOVA) between morphologic changes and the interventions was performed. Multivariate analysis of variance and covariance was used to assess the impact of clinical parameters and interventions on morphological changes. Statistical analysis was performed with StatView version 5.0 software. $p < 0.05$ was considered to be significant.

Table 1 Characteristics of participants

Age (years)	53.1 ± 15.1 (2.4–81)
Gender	
Male: female	42: 19
Causes of CKD (patients)	
DM	19
Non-DM	39
Unknown	3
Duration of PD (months)	37.0 ± 24.9 (2.9–117.2)
Prescription of PD	
CAPD	57
APD	4
Daily UV (patients)	
≥200 mL	18
<200 mL	27
Unknown	16
Use of 2.5 % glucose PDS (patients)	36
Use of 42.5 % glucose PDS (patients)	0
Icodextrin (patients)	22
ARB/ACEI (patients)	38/1
HT Tx unknown (patients)	16
Hybrid Tx (patients)	15
Peritoneal rest (patients)	18
Peritoneal lavage (patients)	27

CKD chronic kidney disease, *UV* urinary volume, *PDS* peritoneal dialysis solution, *ARB* angiotensin receptor blocker, *ACEI* angiotensin-converting enzyme inhibitor, *HT* hypertension, *Tx* treatment, *DM* diabetes mellitus, *CAPD* continuous ambulatory peritoneal dialysis, *APD* automated peritoneal dialysis

Results

Clinical background of participants

The clinical background of the participants is shown in Table 1. Fifty-seven patients were undergoing continuous ambulatory peritoneal dialysis (CAPD) and four patients were on automated peritoneal dialysis (APD). The average PD duration was 33.6 ± 23.1 months. Most of the participants underwent PD catheter removal because of planned transfer from PD to hemodialysis. Among the 61 PD patients, four were regarded as having impaired ultrafiltration capacity (UFC), which was defined by use of more than four hypertonic bags (2.5 % glucose PD solution or icodextrin) in each 24 h period to maintain their solution balance. No PD patient used 4.25 % PD solution in this study. Twenty-seven patients had less than 200 mL urinary volume per day. Fifteen patients received hybrid therapy to manage body fluid balance or a uremic condition. Peritoneal rest and lavage were performed in 18 and 27 patients, respectively, after transfer to hemodialysis simultaneously. As control group, 80 patients using acidic PD solution participated. As for the cause of chronic kidney disease (CKD), 45 patients were with chronic glomerulonephritis (CGN), 9 patients with diabetes (DM), and remaining 26 with other disease or unknown. The average age was 47.3 ± 15.5 years, and the duration of PD was 62.5 ± 43.3 months.

Peritoneal fibrosis of PD peritoneum

In the studied PD patient using neutral PD solution alone, the average peritoneal thickness was 296.7 ± 132.5 μm ranging from 93.2 to 722.6 μm. The sampling site from the PD catheter insertion did not aggravate the SMC thickness (the near site, 287.8 ± 136.5 μm, $n = 22$; the opposite site, 376.7 ± 40.9 μm, $n = 9$, respectively). Peritoneal thickness was not significantly related to the duration of PD treatment (Fig. 2a). In the PD patient using acidic PD solution, peritoneal thicknesses ranged from 45.5 to 777.9 μm. The average SMC thickness was 266.2 ± 159.9 μm ($n = 40$). The SMC thickness significantly increased with PD duration (Fig. 2b). There was no significant difference in SMC between patients using

neutral PD solution and using acidic PD solution for at least 60 months (Fig. 2c).

Peritoneal vasculopathy of PD peritoneum

In PD patients using neutral PD solution, the average *L/V* at PCV was 0.801 ± 0.075, ranging from 0.596 to 0.906 ($n = 39$). The sampling site from the PD catheter insertion was not aggravated by vascular stenosis (the near site, 0.80 ± 0.08, $n = 15$; the opposite site, 0.80 ± 0.12, $n = 7$). The *L/V* decreased with the PD duration in the PD patients using neutral PD solution (Fig. 3a). The *L/V* in the PD patients using acidic PD solution also decreased with PD duration (Fig. 3b). The *L/V* ranged from 0 to 0.869, and the average *L/V* at PCV was 0.494 ± 0.296 in PD patients using acidic PD solution ($n = 66$). The attenuation slope of *L/V* in the acidic PD solution was greater compared with that in the neutral PD solution (Fig. 3b). A decrease in *L/V* of the patient who used acid PD solution for at least 60 months was markedly rapid compare to that of the patients with neutral PD solution (Fig. 3c).

Effect of neutral PD solution on surface coverage with mesothelial cells

Mesothelial cells were presented in 24 out of 43 specimens. The duration of PD was no significantly different between grade 0 and grade 1, 2 and 3 (38.2 ± 21.5 and 33.7 ± 30.4 months, respectively). Mesothelial cells were found in four patients who had undergone PD for more than 5 years. Cubical mesothelial cells was found in 22 specimens, and stratified mesothelium was found in 16 patients.

Impacts of interventions for peritoneal morphologic changes

Sixty-one patients whose specimens were evaluable were divided into four groups according to interventions such as

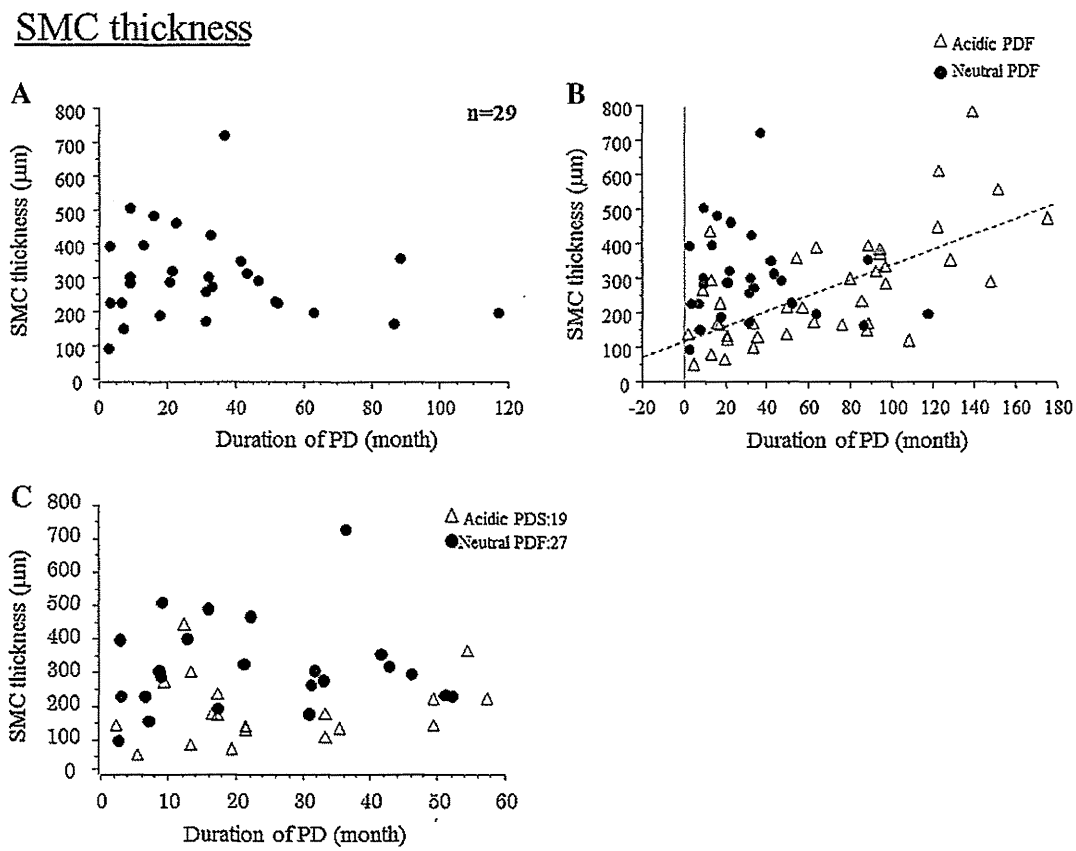

Fig. 2 Average peritoneal thickness (APT) by five-point measurement method. The peritoneal thicknesses at randomly selected five points were measured, and then the APT was calculated (**a**). The relationship between SMC and PD duration in patients using neutral and acidic PD solution (**b**). The relationship between SMC and PD duration in patients using neutral PD solution and patients using acidic PD solution for at least 60 months (**c**). Patients using acidic PD solution show in *open triangle*. Patients using neutral PD solution show in *filled circle*

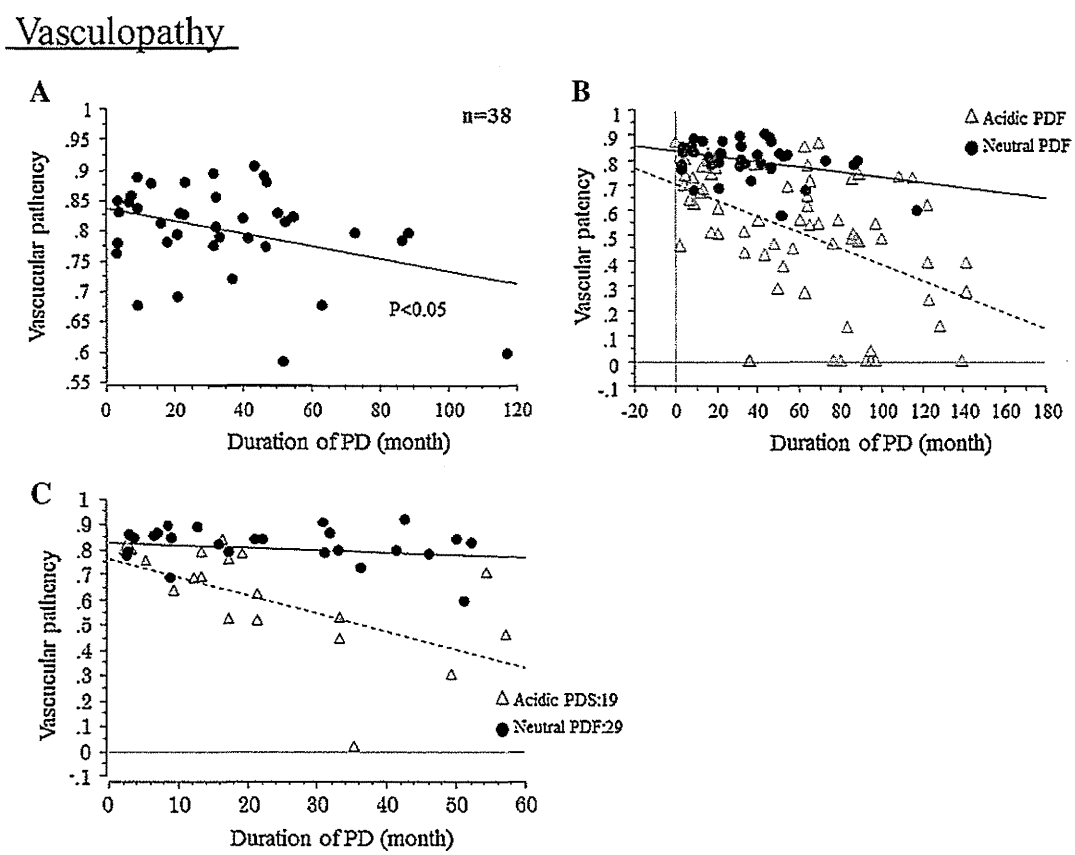

Fig. 3 Quantitative evaluation of vasculopathy at post-capillary venules (PCVs). For evaluation of the severity of luminal narrowing, the ratio of lumen-to- vessel diameter (*L/V*) was determined, representing the extent of patency of the blood vessel. The *L/V* decreased with PD duration in patients using neutral PD solution (a). The relationship between *L/V* and PD duration in patients using neutral and acidic PD solution (b). The relationship between *L/V* and PD duration in patients using neutral PD solution and patients using acidic PD solution for at least 60 months (c). Patients using acidic PD solution show in *open triangle*. Patients using neutral PD solution show in *filled circle*

hybrid therapy and peritoneal rest and lavage (Table 2). The duration of PD in patients who had undergone peritoneal lavage was significantly longer than in patients who had undergone PD alone ($p < 0.05$). The degree of surface coverage with mesothelial cells in the patients who had undergone hybrid therapy was lower than in the patients without hybrid therapy. The use of icodextrin had no influence on the prevention of morphologic changes.

Impact of icodextrin for peritoneal morphologic changes

We analyzed the independent impact of clinical parameters such as PD duration, residual renal function (RRF), icodextrin, usage of 2.5 % glucose PDS, hybrid therapy and peritoneal rest/lavage on peritoneal morphological changes in the multivariate analysis (Table 3). PD duration was an independent coefficient factor for vascular patency.

Discussion

Peritoneal dialysis (PD), one of the established renal replacement treatments worldwide, has advantages over hemodialysis (HD) in the preservation of residual renal function and enablement of high quality of life. The limited timeframe of PD treatment is a matter of concern, since progression of the functional and morphological alterations and increase of encapsulating peritoneal sclerosis (EPS) incidence occur with lengthening PD duration. Bioincompatible PD solution, containing high concentration of glucose and lactate and low pH is a major deteriorating factor of the peritoneal injury that accompanies PD. The conventional acidic PD solution including high concentration of glucose, lactate, and GDP induced an increased state of carbonyl stress on the peritoneum and promoted the development of peritoneal sclerosis [12, 13].

Table 2 Impacts of interventions in peritoneal morphological changes

	Hybrid therapy		PD alone	
	Peritoneal lavage/rest ($n = 10$)	None ($n = 5$)	Peritoneal lavage/rest ($n = 11$)	None ($n = 14$)
Duration of PD (month)	44.2 ± 20.4*	34.9 ± 21.6*	43.5 ± 34.4*	19.1 ± 16.9
SMC thickness (μm)	233.8 ± 72.7	330.0 ± 227.3	302.1 ± 90.2	306.0 ± 141.9
Vascular patency	0.80 ± 0.10	0.76 ± 0.07	0.79 ± 0.09	0.82 ± 0.04
Grade of mesothelial preservation	0.3 ± 0.5#	0.8 ± 0.5	1.8 ± 1.3	1.3 ± 1.2

* $p < 0.05$, vs PD alone, # $p < 0.02$ vs peritoneal lavage/rest alone

UV urinary volume, *PDS* peritoneal dialysis solution, *SMC* submesothelial compact zone

Table 3 Multivariable analysis of selected clinical parameters for pathological changes

Variables	Coefficient	CI	*p* value
SMC thickness			
Grade of RRF	−23.8	−301.6 to 254.0	0.851
Vascular patency			
PD duration	−0.001	−0.002 to 0.000	0.027
Grade of RRF	0.023	−0.069 to 0.114	0.633
Hybrid therapy	−0.041	−0.128 to 0.046	0.411
Icodextrin	0.007	−0.095 to 0.108	0.892
Time of 2.5 % PDS	0.006	−0.038 to 0.049	0.787

SMC submesothelial compact zone, *CI* confidential interval, *RRF* residual renal function, *PDS* peritoneal dialysis solution

Recently, peritoneal morphologic changes in patients using neutral PD solution have been reported by Ayuzawa and Kawanishi [14, 15]. However, these authors indicated that the neutral PD solution was able to minimize the functional and morphological peritoneal damage compared with the amount of damage reported in previous studies. In present study, we compared the impact of acidic and neutral PD solution on peritoneal morphological changes using quantitative assessment in a multicenter study. The usefulness of neutral PD solution in preventing peritoneal morphological injury was clarified, especially pertaining to vasculopathy in PD patients.

Peritoneal morphological alterations in PD patients in the era of acidic PD solution are characterized by mesothelial injury, SMC thickening and vasculopathy in small vessels. Flat mesothelial cells are transformed into cubical cells, and then detached from the peritoneal surface. Vascular stenosis and obliteration caused by hyalinized thickening of vascular walls in PCVs and capillaries and angiogenesis are found as vasculopathy in PD patients. Finally long exposure to PD solution results to be the denudate thickens peritoneum [2, 7]. Willians [2] and Honda [7] reported that SMC thickness progressively increased with PD duration. In particular, vascular patency decreased with increasing PD duration [7, 11]. Mateijsen [16] examined the relationship between morphological changes and PD duration in 15 uremic patients and 25 PD patients including 11 peritoneal sclerosis patients. They revealed that neoangiogenesis and vasodilatation of the capillaries can be found in long-term CAPD patients. Participants were transferred to hemodialysis by planned discontinuation according to the guidelines for peritoneal dialysis (PD) of the Japanese Society for Dialysis Treatment 2009 [17] excluding one patient with ultrafiltration failure. The SMC thickness increased with the use of neutral PD solution (average SMC thickness; 296.7 ± 132.5 μm), however, there was no relationship between the SMC thickness and PD duration in the present study. Vascular patency decreased with lengthening PD duration in this observation. Conventional acidic PD solution strongly accelerated the progression of vasculopathy compared with neutral PD solution. Vascular patency in PCVs is related to peritoneal permeability and the dialysate-to-plasma ratio of creatinine (D/P creatinine) in the peritoneal equilibration test (PET) [11]. Previous neutral PD fluid study [9] indicated that the use of a neutral pH, low GDP fluid, is accompanied by a significant improvement in effluent markers of peritoneal membrane integrity. Therefore, the improvement in ultrafiltration and peritoneal transport might be ascribed to the sustained vascular patency in patients receiving neutral PD solution.

Half of the participants were not administered hyperosmotic PD solution. Twenty-four out of 57 patients received icodextrin for adequate ultrafiltration. Additionally, hybrid therapy, peritoneal rest, and lavage were performed in many participants as strategies of preventing peritoneal injury. The reasons of these interventions were variable depending on the individual patients' clinical backgrounds and their physicians' opinions concerning dialysis modality. The duration of PD was not different between the patients who did and did not receive interventions. There were no differences in SMC and vascular patency between the patients with and without intervention. The possibility that peritoneal lavage promoted a cover by mesothelial

cells was suggested in this study. Previous studies [9, 10] reported the elevation of Cancer Antigen 125 (CA-125) in the effluent of patients using neutral PD solution. In a cohort study of 247 PD patients, Yamamoto [18] pointed out that the overall incident of EPS was significantly lower in the lavage group than in the non-lavage group. Since mesothelial cells play a central role in the peritoneal repair after acute injury, such as bacterial infection or operative procedures, the preservation of mesothelial cells might be related to the peritoneal repair after PD withdrawal. Therefore, the use of neutral PD solution together with combined interventions can play a favorable role on preventing or improving peritoneal morphological alterations. An additional large clinical study is needed to establish a consensus on the usefulness of the different interventions in PD patients.

The peritoneal specimens in PD patients using acidic PDS, obtained for assessment of basic morphologic findings in Japan, were utilized as control [7]. According to the small number of participants and lack of baseline clinical data in the participants using acidic PDS, we could not provide the relationship between the morphological and the functional improvement and the impact of peritoneal lavage and rest, hybrid therapy and use of icodextrin on peritoneal injury in this study. Only neutral PDS has been available in Japan since 2004. Further studies are needed to determine whether these beneficial impacts of the neutral PD solution could improve the long-term clinical outcome of the PD patients.

In conclusions, neutral PD solution is more biocompatible with respect to preventing morphological injury than acidic PD solution for PD.

Conflict of interest The authors have no conflicts of interest to declare expect Dr. Yasuhiko Ito. He is a professor in endowed chair supported by Baxter Japan.

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The efficacy of tolvaptan as a diuretic for chronic kidney disease patients

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Background Tolvaptan selectively binds to the vasopressin V2 receptor and inhibits reabsorption of free water. Although its efficacy for heart failure has been proven, its efficacy for chronic kidney disease (CKD) patients has not been assessed in detail.

Methods We examined 20 CKD patients (13 men and 7 women) who presented with volume overload and who were administered tolvaptan. We assessed urine volume (UV) and blood biochemistry before administration (d0), 1 day after administration (d1), and 7 to 14 days after administration (d7-14).

Results The mean age was 74.0 ± 13.1 years. Besides CKD, there were 9, 8, and 5 patients with heart failure, liver failure or liver cirrhosis, and severe oedema, respectively. UV significantly increased from 959.0 ± 503.8 mL/day at d0 to 1605.4 ± 964.0 mL/day at d7-14 ($P < 0.01$). Serum creatinine levels were not exacerbated (3.89 ± 3.43 mg/dL at d0 and 3.66 ± 3.02 mg/dL at d7-14). Serum albumin (ALB) levels and urinary protein creatinine ratio (uPCR) did not correlate with UV change. Estimated glomerular filtration rate (eGFR) correlated with UV change from d0 to d1 ($r = 0.6619$, $P < 0.01$). Serum sodium elevation correlated with increased UV ($r = 0.4951$, $P < 0.05$).

Conclusion Tolvaptan is useful to reduce volume overload without exacerbation of the renal function; its effect does not depend on ALB or uPCR. The eGFR correlated with the efficacy of tolvaptan. If UV increases drastically after tolvaptan administration, serum Na levels should be carefully monitored.

Keywords *Chronic kidney disease – CKD – tolvaptan – diuretic.*

INTRODUCTION

Tolvaptan selectively binds to the vasopressin V2 receptor and inhibits the movement of aquaporin 2 into the luminal side of cortical collecting duct cells. Thus, tolvaptan inhibits the reabsorption of free water[1-3]. As a diuretic, tolvaptan has been proven to be effective for the treatment of congestive heart failure; its efficacy for the treatment of hepatic oedema in liver cirrhosis patients has also been reported. Furthermore, tolvaptan does not reduce renal blood flow and is a promising diuretic for patients with impaired renal function. Recently, the efficacy of tolvaptan for autosomal dominant polycystic kidney disease (ADPKD) was proven[4]. However, few reports have examined the efficacy of tolvaptan for the treatment of water overload in chronic kidney disease (CKD) patients. Therefore, we examined the efficacy of tolvaptan for reducing water overload in CKD patients.

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PATIENTS AND METHODS

This study was performed according to the principles of the Declaration of Helsinki, the Japanese National Ethical Guidelines, and the institutional review boards of Nagoya University Hospital (approval number; 2013-0272).

The study population was selected from patients who were hospitalized in or referred to our department because of volume overload between 2010 and 2013. Since it was difficult to reduce volume overload with furosemide only, patients were also administered tolvaptan. We assessed all patients who were administered tolvaptan, retrospectively. Body weight (BW), urine volume (UV), heart rate (HR), systolic blood pressure (sBP), diastolic blood pressure (dBP), blood cell count, and biochemistry were examined before tolvaptan administration (d0), 1 day after administration (d1), and 7 to 14 days after administration (d7-14).

Values are expressed as means ± standard deviations (SD), unless otherwise stated. We evaluated the differences in d0, d1, and d7-14 by analysis of variance followed by Fisher's protected least significant difference test as a multiple comparison. We examined the Pearson correlation coefficient for univariate correlations between UV change and several parameters.

RESULTS

Baseline characteristics

Twenty patients were included in the study. Table 1 shows the patients' baseline characteristics. There were 13 men and 7 women. The mean age was 74.0 ± 13.1 years. In addition to CKD, there were 9, 8, and 5 patients with heart failure, liver failure, and severe oedema caused by renal factors, respectively. Four of the patients with the above-mentioned comorbidities (No. 1, 2, 6, and 8) had started receiving dialysis in these events just before tolvaptan administration. Tolvaptan was administered because the urine volume of these 4 patients was maintained and promising to increase despite initiation of dialysis. Sample collecting points of these 4 patients at d0 was just before dialysis session. One patient (No. 1) was administered furosemide and tolvaptan simultaneously because of severe volume overload, and correction of that volume overload was considered difficult by using furosemide only.

Change of parameters before and after tolvaptan administration

Figure 1 shows the time course change of the parameters. The amount of UV increased along with the time course. BW decreased significantly at d7-14, reflecting an increase in UV. The level of creatinine (Cre) was not exacerbated throughout the study period. Other parameters, such as sBP, dBP, and HR, as well as haemoglobin, urea nitrogen, and potassium levels did not change. Serum sodium (Na) levels increased significantly; however, a mean

Table 1 Baseline characteristics of all patients

No.	Age (years)	Sex	Disease	Furosemide Dose At D0 (Mg/Day)	eGFR (mL/min/1.73 m2)	Dialysis
1	52	M	Liver	0	25.8	Yes
2	39	F	Kidney	240	3.3	Yes
3	83	F	Kidney and heart	20	7.8	No
4	80	F	Heart	120	5.5	No
5	66	M	Heart	120	9.6	No
6	71	M	Liver	80	14.1	Yes
7	77	M	Liver	80	19.6	No
8	69	M	Heart	360	3.4	Yes
9	87	F	Heart	320	8.1	No
10	85	M	Liver and heart	60	21.4	No
11	80	M	Liver	40	61.4	No
12	86	F	Kidney	20	85.5	No
13	84	F	Heart	160	16.7	No
14	79	M	Kidney	100	7.3	No
15	79	M	Liver	80	55.6	No
16	78	M	Heart	60	20.3	No
17	54	M	Liver	60	41.4	No
18	71	M	Liver	120	16.0	No
19	70	F	Heart	80	16.2	No
20	90	M	Kidney	80	49.5	No

Liver: liver failure or liver cirrhosis. Kidney: severe oedema caused by renal factors. Heart: heart failure.

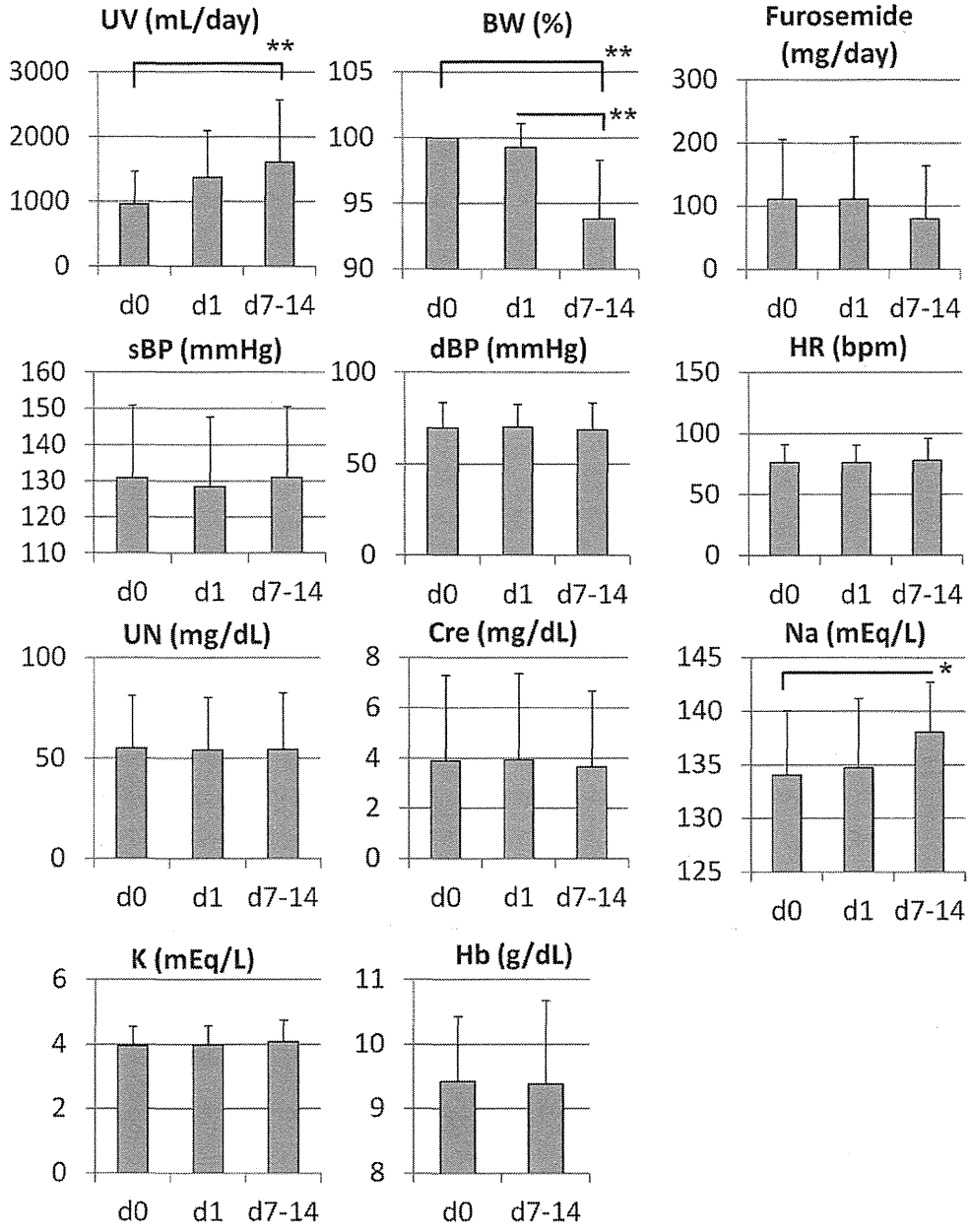


Fig. 1 Time course changes of each parameter. UV: urine volume, BW: body weight, sBP: systolic blood pressure, dBP: diastolic blood pressure, HR: heart rate, UN: urea nitrogen, Cre: creatinine, Na: sodium, K: potassium, Hb: haemoglobin. * $P<0.05$, ** $P<0.01$.

Na level of 138.0±4.7 mEq/L at d7-14 was not considered clinically harmful. Furthermore, the dose of furosemide tended to decrease although it did not show statistical significance. There were no adverse effects such as liver enzyme elevation, and all patients could continue tolvaptan treatment in the period that we considered necessary.

Correlation between UV change and several parameters

We analysed the correlation between UV increase and several parameters (tables 2 and 3). Table 2 shows that UV increase from d0 to d1 correlated significantly with UV, Cre, estimated glomerular filtration rate (eGFR), and urine specific gravity (S.G.) at d0. UV, Cre, and eGFR at d0 are considered to reflect renal function and response to high S.G. is considered to reflect the mechanism of tolvaptan.

Hypoalbuminaemia and proteinuria have been reported to reduce the effect of furosemide. However, the level of serum albumin (ALB) did not show a significant correlation with UV increase from d0 to d1. Furthermore, urinary protein creatinine ratio (uPCR) at d0 also did not show any significant correlation with UV increase. Since data were missing in one patient, uPCR was analysed in 19 patients. Hypoalbuminaemia or proteinuria did not affect the efficacy of tolvaptan.

Table 2 Correlation between UV increase (mL/day) from d0 to d1 and various parameters at d0

Parameter	r	*P* value
n = 20		
Age (years)	0.2880	0.2181
BW (kg)	− 0.1497	0.5288
UV at d0 (mL/day)	0.4629	0.0398 *
UN (mg/dL)	− 0.4201	0.0651
UA (mg/dL)	− 0.4193	0.0658
Cre (mg/dL)	− 0.4523	0.0452 *
ALB (g/dL)	− 0.4431	0.0504
Na (mEq/L)	0.1315	0.5806
K (mEq/L)	− 0.0104	0.9652
AST (IU/L)	0.0650	0.7853
ALT (IU/L)	0.1140	0.6323
Hb (g/dL)	− 0.2841	0.2247
Furosemide (mg/day)	− 0.3568	0.1225
eGFR (mL/min/1.73 m²)	0.6619	0.0015 **
uPCR (g/gCr) (n = 19)	0.0934	0.6952
S.G.	0.4942	0.0268 *

BW: body weight, UV: urine volume, UN: urea nitrogen, UA: uric acid, Cre: creatinine, ALB: albumin, Na: sodium, K: potassium, AST: aspartate aminotransferase, ALT: alanine aminotransferase, Hb: haemoglobin, eGFR: estimate glomerular filtration rate, uPCR: urinary protein creatinine ratio, S.G.: urine specific gravity. * $P < 0.05$, ** $P < 0.01$.

Table 3 Correlation between UV increase (mL/day) from d0 to d7-14 and various parameters at d0

Parameter	r	*P* value
n = 20		
Age (years)	0.2633	0.2621
BW (kg)	0.1679	0.4792
UV at d0 (mL/day)	− 0.3332	0.7158
UN (mg/dL)	− 0.4103	0.0724
UA (mg/dL)	− 0.0129	0.9571
Cre (mg/dL)	− 0.1563	0.5104
ALB (g/dL)	− 0.2009	0.3957
Na (mEq/L)	− 0.1310	0.5819
K (mEq/L)	− 0.1649	0.4871
AST (IU/L)	0.3825	0.0961
ALT (IU/L)	− 0.0300	0.8999
Hb (g/dL)	− 0.2237	0.3431
Furosemide (mg/day)	− 0.1079	0.6507
eGFR (mL/min/1.73 m²)	0.2153	0.3619
uPCR (g/gCr) (n = 19)	− 0.3692	0.1092
S.G.	0.1290	0.5877

BW: body weight, UV: urine volume, UN: urea nitrogen, UA: uric acid, Cre: creatinine, ALB: albumin, Na: sodium, K: potassium, AST: aspartate aminotransferase, ALT: alanine aminotransferase, Hb: haemoglobin, eGFR: estimate glomerular filtration rate, uPCR: urinary protein creatinine ratio, S.G.: urine specific gravity.

Table 3 shows the correlation between UV increase from d0 to d7-14 and various parameters. No parameter showed a significant correlation. We considered that various factors were involved through the relatively long period.

Difference in response between patients with and without dialysis

Table 2 shows the correlation between renal function and the effect of tolvaptan. During the study period, a difference of UV change was noted between patients who were and were not undergoing dialysis. Hence, we assessed the data by separating the patients into 2 groups. Sixteen patients (10 men and 6 women) were not receiving dialysis. Their mean age was 78.1 ± 9.1 years. Besides CKD, there were 8, 6, and 4 patients with heart failure, liver failure, and severe oedema caused by renal factors, respectively, among these patients. The number of patients undergoing dialysis was 4 (3 men and 1 woman). Their mean age was 57.8 ± 15.1 years; 1, 2, and 1 patient had heart failure, liver failure, and severe oedema, respectively.

Figure 2 and supplementary figure 1 show the time course changes in parameters of the patients who were

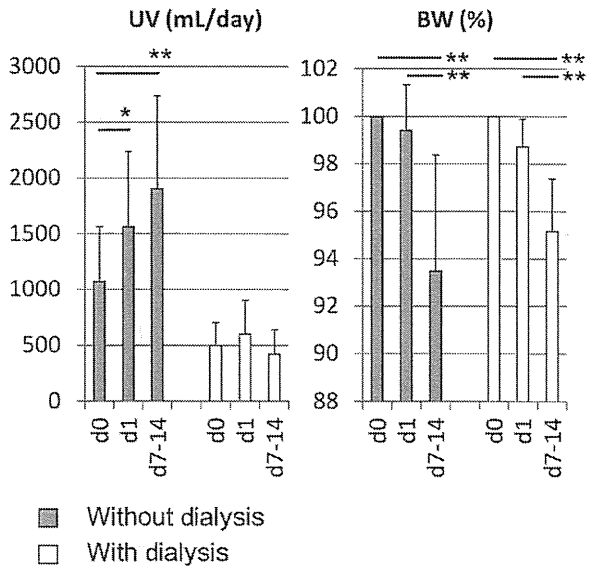
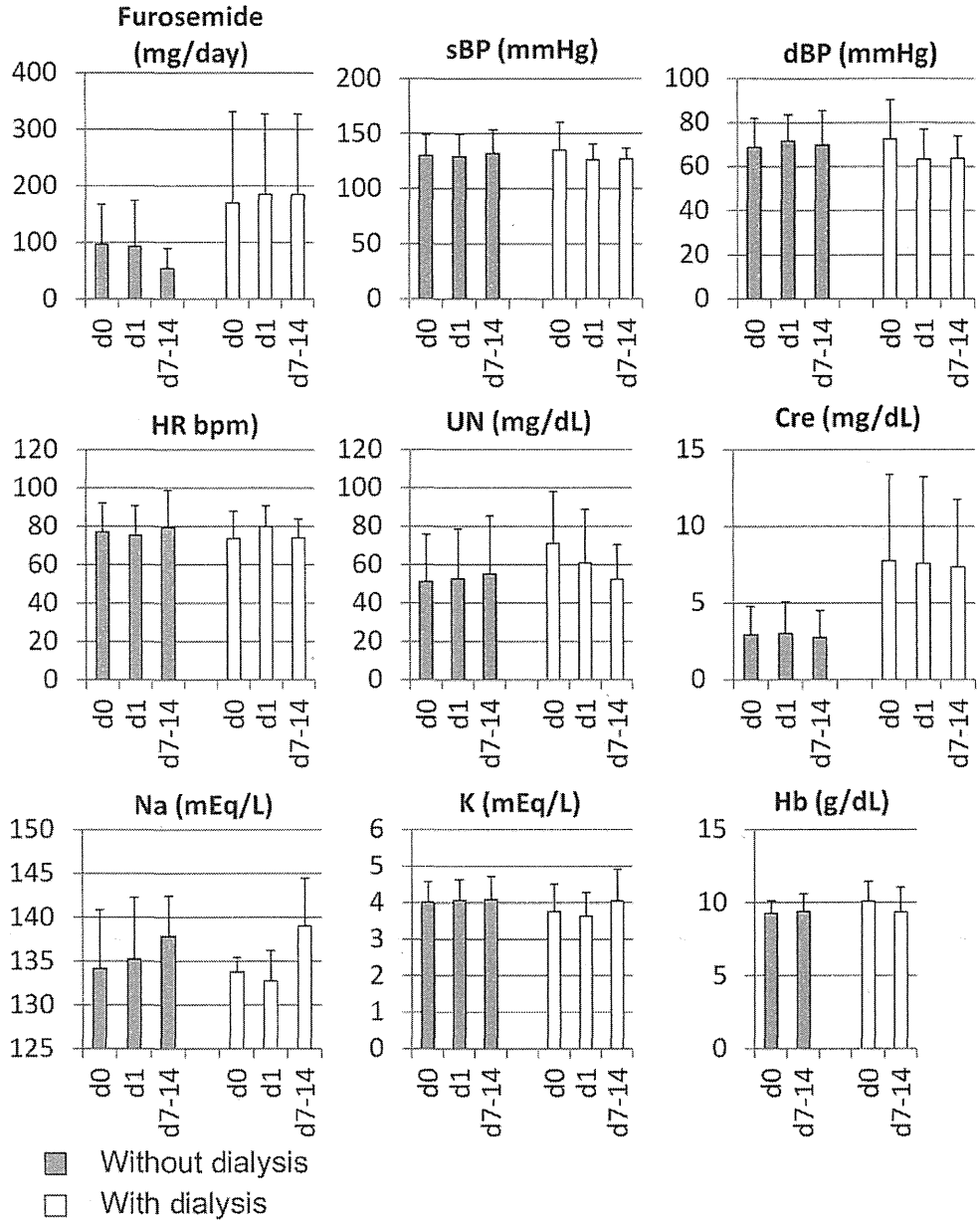


Fig. 2 Time course changes of each parameter in patients who were and were not undergoing dialysis. White bars show patients who were maintained on dialysis. Gray bars show patients who did not undergo dialysis. UV: urine volume, BW: body weight. * $P < 0.05$, ** $P < 0.01$.



Supplementary fig. 1 Time course changes of each parameter in patients who were and were not undergoing dialysis. White bars show patients who were maintained on dialysis. Gray bars show patients who did not undergo dialysis. sBP: systolic blood pressure, dBP: diastolic blood pressure, HR: heart rate, UN: urea nitrogen, Cre: creatinine, Na: sodium, K: potassium, Hb: haemoglobin.

and were not undergoing dialysis. UV response was significantly better in patients without dialysis. Body weight also decreased in patients who did not receive dialysis. The dose of furosemide in patients not receiving dialysis tended to decrease along the time course; however, it did not reach statistical significance. Patients who were not undergoing dialysis showed better UV response to tolvaptan. On the other hand, patients who were undergoing dialysis required a high dose of furosemide, and we were not able to reduce the dose over time. In summary, patients whose renal function was maintained without dialysis had a better response to tolvaptan, as indicated by the increasing UV and the ability to reduce their dose of furosemide.

Elevation of serum Na correlated with UV increase

Table 4 shows the correlation between Na level elevation and several parameters. UV increase from d0 to d1 showed a significant correlation with serum Na

Table 4 Correlation between Na increase (mEq/L) from d0 to d1 and various parameters at d0

Parameter	r	P value
n = 20		
Age (years)	0.3579	0.1213
BW (kg)	−0.0294	0.9022
UV at d0 (mL/day)	0.2431	0.3018
UV increase (mL/day)	0.4951	0.0261 *
UN (mg/dL)	−0.3684	0.1100
UA (mg/dL)	−0.3081	0.1864
Cre (mg/dL)	−0.1360	0.5675
ALB (g/dL)	−0.0638	0.7893
Na (mEq/L)	−0.0010	0.9965
K (mEq/L)	−0.0492	0.8369
AST (IU/L)	−0.3794	0.0990
ALT (IU/L)	−0.3943	0.0853
Hb (g/dL)	−0.2502	0.2873
Furosemide (mg/day)	−0.0709	0.7666
eGFR (mL/min/1.73 m²)	0.4411	0.0516
uPCR (g/gCr) (n = 19)	−0.3225	0.1655
S.G.	0.1152	0.6287

BW: body weight, UV: urine volume, UV increase:UV increase from d0 to d1, UN: urea nitrogen, UA: uric acid, Cre: creatinine, ALB: albumin, Na: sodium, K: potassium, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, Hb: haemoglobin, eGFR: estimate glomerular filtration rate, uPCR: urinary protein creatinine ratio, S.G.: urine specific gravity. *; $P < 0.05$, **; $P < 0.01$.

level elevation. Patients with higher eGFR tended to show higher serum Na levels. However, no significant correlation was found between these two parameters. These results indicate that serum Na level elevation and UV increase should be carefully monitored after tolvaptan administration in patients with good renal function.

DISCUSSION

The efficacy of tolvaptan in the treatment of congestive heart failure patients has already been proven[5,6]. Furthermore, its efficacy for the treatment of hepatic oedema in liver cirrhosis patients has also been reported[7,8]. However, few reports have examined in detail the efficacy of tolvaptan for the treatment of water overload in CKD patients[9]. Furosemide, a frequently used diuretic for volume overload, reduces kidney blood flow and may worsen renal function and prognosis[10,11]. Hence, administration of high-dose furosemide is difficult in CKD patients. On the other hand, tolvaptan does not reduce renal blood flow and tends to maintain renal function[12,13].

In this study, we administered tolvaptan in CKD patients, including those with severe renal failure, and succeeded in increasing UV and reducing BW. There was no adverse event and all patients were able to continue tolvaptan treatment. Although serum Na levels increased significantly, we did not consider it clinically harmful. On the contrary, tolvaptan has been reported to be useful for the correction of hyponatraemia[14]. Hence, tolvaptan may be effective for serum Na level correction, with appropriate selection of hyponatraemic patients. Moreover, we could resolve volume overload without exacerbation of renal function. The fact that CKD patients with impaired renal function could be treated without renal function exacerbation is promising. Recently, the efficacy of tolvaptan for ADPKD was proven[4]. In this study, tolvaptan was useful for ADPKD patients with well-maintained renal function. Although the dose of tolvaptan is much lower and the aim is resolving volume overload, administration of tolvaptan was useful in patients with various degrees of CKD.

Moreover, tolvaptan treatment may reduce the need for high-dose furosemide. Although the dose of furosemide should be fixed for the examination of a rigorous effect of tolvaptan, our results suggest the efficacy of tolvaptan in resolving water overload, which was difficult to improve by furosemide only. Although furosemide impairs efficacy in the presence of hypoalbuminuria and proteinuria[15,16], tolvaptan did not show this tendency. These results indicate some of the advantages of tolvaptan, as a diuretic, over furosemide.

From the mechanism of tolvaptan, urinary osmolality is considered to correlate with the effect of tolvaptan. Regrettably, we did not measure urinary osmolality in all patients. However, urine high S.G. at d0 correlated with UV increase (table 2) and may be useful to predict the effect of tolvaptan.

Our results indicate that tolvaptan may be a good treatment option for patients with already impaired renal function, progressive renal failure caused by furosemide, and high-dose furosemide treatment. Furthermore, since tolvaptan is not affected by the presence of hypoalbuminuria and proteinuria, which are often found in CKD, it may be useful in these patients.

However, the efficacy of tolvaptan may be limited in cases of extremely impaired renal function. Hence, we should consider dialysis initiation in patients showing poor response to tolvaptan. Moreover, Na level elevation should be carefully monitored in patients who respond well to tolvaptan, for example, in those with a significant UV increase. Because the increase of UV correlated with eGFR, serum Na level should be carefully monitored after tolvaptan administration in patients with well-maintained renal function.

CONCLUSION

Tolvaptan is useful to reduce volume overload without exacerbation of renal function. The effect is not affected by hypoalbuminaemia or urinary protein levels. The eGFR correlated with the efficacy of tolvaptan. Serum Na level elevation should be carefully monitored if an increased UV is estimated.

POTENTIAL FINANCIAL CONFLICTS OF INTEREST

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Vascular endothelial growth factor receptor-3 is a novel target to improve net ultrafiltration in methylglyoxal-induced peritoneal injury

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Appropriate fluid balance is important for good clinical outcomes and survival in patients on peritoneal dialysis. We recently reported that lymphangiogenesis associated with fibrosis developed in the peritoneal cavity via the transforming growth factor-β1-vascular endothelial growth factor-C (VEGF-C) pathway. We investigated whether VEGF receptor-3 (VEGFR-3), the receptor for VEGF-C and -D, might be a new target to improve net ultrafiltration by using adenovirus-expressing soluble VEGFR-3 (Adeno-sVEGFR-3) in rodent models of peritoneal injury induced by methylglyoxal (MGO). We demonstrated that lymphangiogenesis developed in these MGO models, especially in the diaphragm, indicating that lymphangiogenesis is a common feature in the peritoneal cavity with inflammation and fibrosis. In MGO models, VEGF-D was significantly increased in the diaphragm; however, VEGF-C was not significantly upregulated. Adeno-sVEGFR-3, which was detected on day 50 after administration via tail vein injections, successfully suppressed lymphangiogenesis in the diaphragm and parietal peritoneum in mouse MGO models without significant effects on fibrosis, inflammation, or neoangiogenesis. Drained volume in the peritoneal equilibration test using a 7.5% icodextrin peritoneal dialysis solution (the 7.5% icodextrin peritoneal equilibration test) was improved by Adeno-sVEGFR-3 on day 22 ($P<0.05$) and day 50 after reduction of inflammation ($P<0.01$), indicating that the 7.5% icodextrin peritoneal equilibration test identifies changes in lymphangiogenesis. The solute transport rate was not affected by suppression of lymphangiogenesis. In human peritoneal dialysis patients, the dialysate to plasma ratio of creatinine positively correlated with the dialysate VEGF-D concentration ($P<0.001$). VEGF-D mRNA was significantly higher in the peritoneal membranes of patients with ultrafiltration failure, indicating that VEGF-D is involved in the development of lymphangiogenesis in peritoneal dialysis patients. These results indicate that VEGFR-3 is a new target to improve net ultrafiltration by suppressing lymphatic absorption and that the 7.5% icodextrin peritoneal equilibration test is useful for estimation of lymphatic absorption.
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Maintenance of optimal fluid balance is important for good clinical outcomes and survival in patients who undergo peritoneal dialysis. Overhydration states, which are often associated with higher peritoneal transport rates, are reported to be a major cause for dialysis discontinuation.[1,2] The pathological features of peritoneal membrane injury in peritoneal dialysis patients with high solute transport rates are submesothelial fibrosis, accumulation of extracellular matrix, and neoangiogenesis.[3,4]

We recently showed that lymphangiogenesis and vascular endothelial growth factor-C (VEGF-C) expression, one of the key mediators of lymphangiogenesis, were associated with fibrosis using human tissues, peritoneal dialysis effluent samples, cultured cells derived from peritoneal dialysis effluent, and rat chlorhexidine gluconate -induced peritoneal injury models.[5] In those studies, we found that the VEGF-C content in the peritoneal dialysis effluent correlated with the peritoneal membrane transport rate and transforming growth

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factor-beta (TGF-β) concentration, and that expression of VEGF-C and markers of lymphatics was higher in ultrafiltration failure-peritoneum.[5] In cultured mesothelial cells and macrophages, TGF-β–induced VEGF-C expression was blocked by a TGF-β type I receptor (TGFβRI) inhibitor.[5] Furthermore, VEGF-C expression and lymphangiogenesis were suppressed by the TGFβRI inhibitor or by the cyclooxygenase-2 (COX-2) inhibitor, celecoxib, in chlorhexidine gluconate-induced peritoneal injury models.[5] Thus, we proposed that lymphangiogenesis in the peritoneal membrane, similar to renal fibrosis is linked with the fibrotic process via the TGF-β–VEGF-C pathway.[6–8] The lymphatic absorption rate, which is measured by the rate at which intraperitoneally administered radioactive serum albumin or the macromolecule dextran 70 disappears, is significantly higher in patients with ultrafiltration failure, and lymphatic absorption is considered to be one of the causes of the decrease in net ultrafiltration.[9–12] However, the results from these clinical approaches have been controversial,[13,14] and there are no other methods available to assess lymphatic function.

In the present study, we investigated whether VEGF receptor-3 (VEGFR-3), the receptor for VEGF-C and -D, might be a new target to increase net ultrafiltration by suppression of lymphangiogenesis using an adenovirus-expressing soluble VEGFR-3 (Adeno-sVEGFR-3) fused with human IgG (Supplementary Figure 1) in models of murine peritoneal injury induced by methylglyoxal (MGO).[15–17] MGO is a precursor of advanced glycation end products, which accumulate in dialysis patients.[18] In addition, we proposed a new method for the peritoneal equilibration test by using a 7.5% icodextrin peritoneal dialysis solution (7.5% icodextrin peritoneal equilibration test) to assess lymphatic absorption (Supplementary Figure 1). Finally, we studied the expression of VEGF-D in lymphangiogenesis in human samples.

MATERIALS AND METHODS

MGO-Induced Peritoneal Injury Model

All animal studies were carried out in accordance with the Animal Experimentation Guidelines of Nagoya University Graduate School of Medicine (Nagoya, Japan). Ten-week-old male C57BL/6J mice (Japan SLC, Hamamatsu, Japan) initially weighing 24–29 g were used throughout the study. The animals were maintained under conventional laboratory conditions and had free access to food and water. The mice received an intraperitoneal injection of 100 ml/kg body weight of peritoneal dialysis fluid (Dianeal-N PD-4-2.5, 2.27% glucose, Baxter, Tokyo, Japan) containing 20 mmol/l MGO (MP Biomedicals LLC, Illkirch, France) for 3 weeks, 5 consecutive days per week as described previously[17] (Experiment 1, Supplementary Figure 2a). Control mice received the same dosage of peritoneal dialysis fluid without MGO. This peritoneal dialysis fluid was prepared by purification through a 0.2-μm pore-size filter and by adjustment to pH 5.0 immediately before injection every day. The mice were killed on day 22, and parietal peritoneal and diaphragmatic samples were procured. The harvested samples were used for immunohistochemical analysis of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), VEGFR-3, CD31, type III collagen, CD68, and VEGF-D, and for analysis of the mRNA expression of VEGF-D, VEGFR-3, LYVE-1, and CD31.

MGO Model Mice and Studies of Lymphangiogenesis Inhibition using an Adenovirus Vector

The experimental design and protocols are as follows (Supplementary Figure 2):

(a) Experiment 1: development of a MGO-induced peritoneal injury model in mice. The mice received peritoneal dialysis fluid (100 ml/kg) with MGO (20 mM) intraperitoneally from day 1 until day 22 (Supplementary Figure 2a).
(b) Experiment 2: inhibition studies using an adenovirus-expressing sVEGFR-3 and a control adenovirus-expressing Lac Z in a MGO-induced peritoneal injury model. Recombinant Adeno-sVEGFR-3 or β-galactosidase (Adeno-LacZ) were kindly provided by K Alitalo (the University of Helsinki, Helsinki, Finland) and were amplified and purified for use as described previously.[19–21] On day 0, prior to establishment of MGO-induced peritoneal injury as in **a**, the mice were first administered 1.0×10^9 p.f.u. of one of the adenoviral vectors intravenously through the tail vein. On day 22, the mice were assessed using a conventional peritoneal equilibration test with a 4.25% glucose-based peritoneal dialysis fluid and a peritoneal equilibration test with 7.5% icodextrin (Adeno-LacZ Day 22 group and Adeno-sVEGFR-3 Day 22 groups; Supplementary Figure 2b).
(c) Experiment 3: development of a MGO-induced peritoneal injury model with peritoneal rest from day 22 to day 50. The mice were treated as in **a** until day 22. From day 22 until their sacrifice on day 50 no dialysate was infused into the peritoneal cavity (peritoneal rest) (Supplementary Figure 2c).
(d) Experiment 4: inhibition studies using adenovirus-expressing sVEGFR-3 and control adenovirus-expressing Lac Z in a MGO model as in **b** except that the mice further underwent peritoneal lavage using a 1.5% peritoneal dialysis fluid from day 22 to day 50. For this treatment, the mice received an intraperitoneal injection of 100 ml/kg body weight of a low concentration peritoneal dialysis fluid (Dianeal-N PD-4 1.5, 1.36% glucose) for 20 days, 5 consecutive days per week. This procedure is similar to a previously reported method.[22] In these mice, peritoneal functions were assessed on day 50 using a conventional peritoneal equilibration test with a 4.25% glucose-based peritoneal dialysis fluid and a peritoneal equilibration test with a 7.5% icodextrin peritoneal dialysis fluid (Adeno-LacZ Day 50 group and Adeno-sVEGFR-3 Day 50 group; Supplementary Figure 2d).

Tissue and blood samples were also obtained from the mice for further analysis.

Histology and Immunohistochemistry

Routine histological and immunohistological analyses of animal and human tissues were performed and assessed as we described previously.[5–7,23,24] The antibodies used are listed in Supplementary Table 1. Mast cells were evaluated using sections stained with 0.2% toluidine blue.

RNA Preparation from Peritoneal and Diaphragm Tissues, and PCR Analysis

Animal peritoneal and diaphragm tissues were immersed in RNAlater (Ambion, Austin, TX, USA) for >1 day. RNA preparation and the synthesis of first-strand cDNA were performed as described previously.[5–7,23,24] Total RNA (1 μg) was then reverse transcribed. Quantitative real-time PCR (qPCR) analysis was performed with an Applied Biosystems (South San Francisco, CA, USA) Prism 7500HT sequence detection system using TaqMan gene expression assays as described previously.[24] The TaqMan Gene Expression Assays (Applied Biosystems) used are described in Supplementary Table 2. 18S ribosomal RNA was used as an endogenous control.[5,7,23,24]

Assessment of Lymphatic Absorption

To assess absorption via the lymphatic vessels, animals that received 2000 μl of 7.5% icodextrin peritoneal dialysis solution into the peritoneal cavity were killed at 4 h after infusion. An accurate drained volume was measured.

Enzyme-Linked Immunosorbent Assays (ELISAs)

The concentration of sVEGFR-3-Ig fusion protein in the serum was determined using an ELISA kit for human IgG1 (Cayman Chemical Company, Ann Arbor, MI, USA) as described previously.[25] Levels of VEGF-D protein and prostaglandin E2 (PGE2) in the peritoneal dialysis effluent were measured using the ELISA kits for human VEGF-D (R&D Systems, Minneapolis, MN, USA) and PGE2 (Cayman Chemical Company), respectively, according to the manufacturers' instructions. The samples were frozen at the time of collection and were stored at −80 °C. The samples were not subjected to freeze–thaw cycles.

Human Patient Studies

All of the studies were approved by the Ethics Committee for Human Research of the Faculty of Medicine, Nagoya University (Approval #298 and #299). All patients provided informed consent prior to participation in this study.

VEGF-D Concentration in the Peritoneal Effluent of Peritoneal Dialysis Patients

The VEGF-D concentrations in dialysates were measured in overnight-dwelled (8.95 ± 1.63 h) samples collected from 83 peritoneal dialysis patients (27 women and 56 men) who were treated between July 2005 and April 2008 in the Department of Nephrology and Renal Replacement Therapy of Nagoya University Hospital (Nagoya, Japan) and at affiliated hospitals.[5] This is the same cohort in which VEGF-C was measured in recent studies.[5] The mean age of all patients was 55.9 ± 13.5 (range, 28 to 89) years, and the mean duration of peritoneal dialysis treatment was 31.9 ± 32.0 (range, 1 to 132) months.[5] Diabetic nephropathy was the cause of end-stage renal disease in 27 peritoneal dialysis patients (32.5%). All patients were free from peritonitis for at least 1 month prior to the study, and patients with other diseases, such as liver or lung diseases and malignancy, were excluded. Patients undergoing combination therapy (hemodialysis+peritoneal dialysis) were not included in this study. Peritoneal membrane transport was assessed based on the dialysate to plasma ratio of creatinine, and the average value was 0.67 ± 0.14 (range, 0.28 to 0.96).[5] A fast peritoneal equilibration test was performed using a 2.27% glucose-based dialysis solution (Dianeal-N PD-4) as described by Twardowski *et al* (Supplementary Information 1).[26] The correlation between VEGF-D concentration in the peritoneal dialysis effluent and the dialysate to plasma ratio of creatinine was analyzed. In addition, we measured the VEGF-D concentration in peritoneal effluent samples at 4 h of the peritoneal equilibration test. These samples were collected from 40 peritoneal dialysis patients (13 women and 27 men) treated between November 2008 and June 2009 at the Handa Municipal Hospital and the Nagoya University Hospital. The mean age of all patients was 52.9 ± 10.9 (range, 30 to 70) years, and the mean duration of peritoneal dialysis treatment was 26.1 ± 24.6 (range, 1 to 103) months.[5,23]

VEGF-D mRNA Expression in the Human Peritoneum

Fifty-four peritoneal tissue samples were obtained from 29 peritoneal dialysis patients and 25 pre-dialysis chronic renal failure patients at the time of peritoneal dialysis catheter insertion. Among the 29 peritoneal dialysis patients, 7 were regarded as having impaired ultrafiltration capacity, which was defined as described previously.[5,23,27] Twenty-two patients (incident) had their catheters removed because of transplantation, severe exit site infection, mental disorders, or difficulty in performing the bag exchanges (Table 1).

VEGF-D mRNA Expression in the Cultured Mesothelial Cells

Reverse Transcription–PCR (RT–PCR) was performed using the HotStarTaq PCR kit (Qiagen, Tokyo, Japan)[7,28] to examine whether VEGF-D mRNA was expressed in three kinds of mesothelial cells: the human mesothelial cell line Met5A,[5,23] mesothelial cells from the peritoneal dialysis effluent of the patients on peritoneal dialysis,[5,23] and mesothelial cells derived from the omentum.[29,30] The primers according to the reported sequences of human VEGF-D and GAPDH were 5′-GTATGGACTCTCGCTCAGCAT-3′ (sense) and 5′-AGGCTCTCTTCATTGCAACAG-3′ (antisense, PCR products 225 bp);[31] and 5′-ATCATCCCTGCCTCTACTGG-3′ (sense) and 5′-CCCTCCGACGCCTGCTTCAC-3′ (antisense, PCR products 188 bp),[28] respectively.

Statistical Analyses

The Shapiro–Wilk test was applied to test normal distributions. Values are expressed as mean ± s.d. Differences between two groups were analyzed by the unpaired *t*-test or by the Mann–Whitney *U*-test. Comparisons among groups were performed by one-way analysis of variance with the Tukey's test, the Games–Howell test or the Dunnett's test, or by the Kruskal–Wallis multiple comparison test. Spearman's correlation coefficient was used to analyze correlations. Differences were considered to be statistically significant if $P<0.05$. All analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

Table 1 Profiles of peritoneal biopsy cases evaluated for VEGF-D mRNA expression

	Pre-dialysis uremia	Incident	UFF
N	25	22	7
Male	16	13	4
Female	9	9	3
Age, years	63.2 ± 10.4	58.5 ± 14.1	60.4 ± 10.0
Duration of treatment, years	0	3.7 ± 3.0	10.1 ± 5.1
Average thickness of peritoneum, μm	131.3 ± 38.9	148.1 ± 90.5	317.1 ± 118.0

RESULTS

Lymphangiogenesis Developed in a MGO-Induced Peritoneal Injury Model

Twenty-two days after administration of MGO (Experiment 1, Supplementary Figure 2a) LYVE-1- and podoplanin-positive lymphatic vessels were increased and dilated in the diaphragm of MGO model mice compared with controls (Figure 1a,Supplementary Figure 3); however, lymphangiogenesis was not pronounced or significant in the parietal peritoneum wall (Figure 1a). In contrast, as shown in

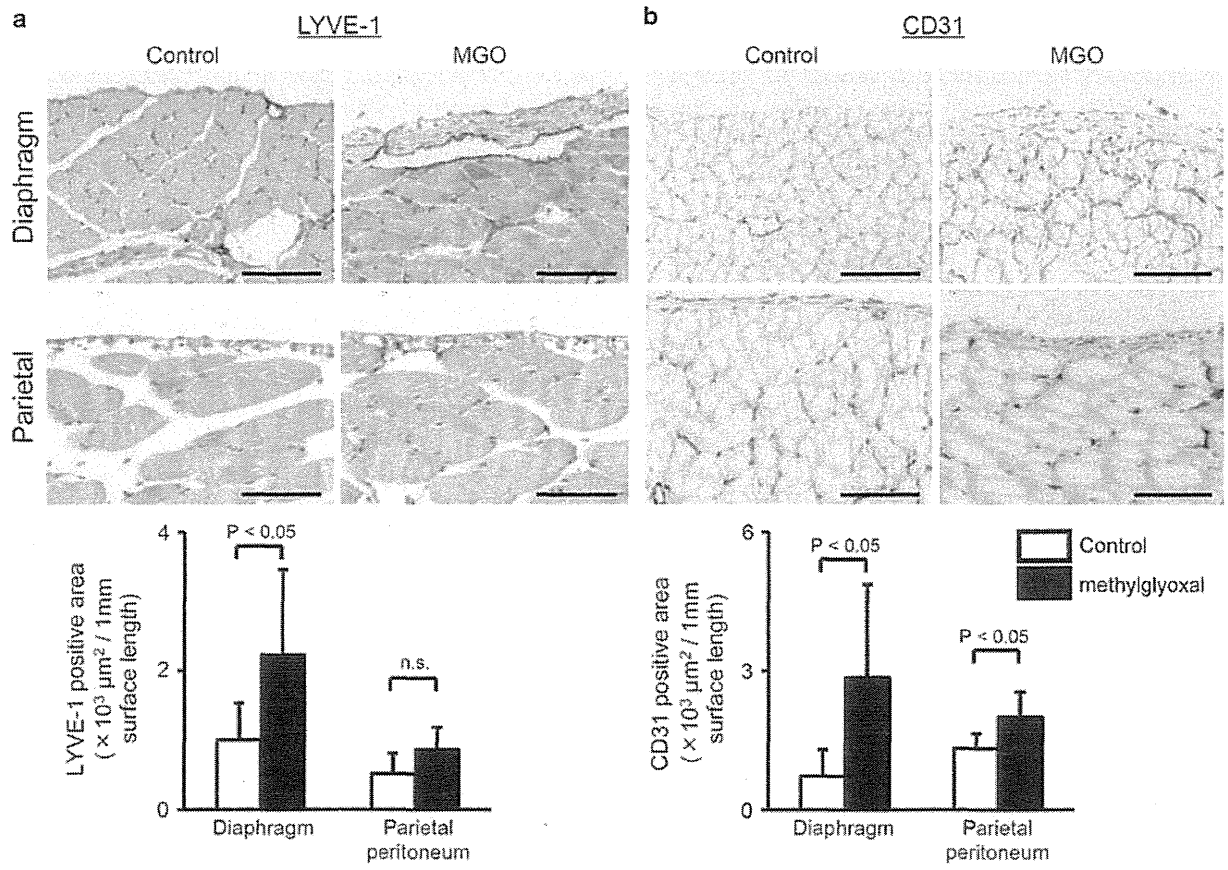

Figure 1 LYVE-1-positive lymphatic vessels were increased and dilated in the diaphragm of methylglyoxal mouse models compared with controls. Expression of lymphatic vessels was predominant in the diaphragm compared with that of control mice. Quantification of immunohistochemical parameters indicated the following: (**a**) LYVE-1-positive lymphatic vessels were significantly increased in the diaphragm, but not in the parietal peritoneal membrane; CD31-positive vessels (**b**), expression of type III collagen (**c**), and CD68-positive macrophages (**d**) were significantly increased in the diaphragm of methylglyoxal model mice compared with normal control mice. (each group, $n=6$). Scale bars, 100 μm. LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; *NS, not significant.*