Interstitial Fibrosis Restricts Osmotic Water Transport in Encapsulating Peritoneal Sclerosis

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Supplemental data

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COMPLETE METHODS

EPS patients and peritoneal transport analysis

In agreement with EPS diagnosis criteria (1-3), EPS cases were defined as PD patients who presented with recurrent, partial or total, intestinal occlusion, with a definitive EPS diagnosis confirmed by CT-scan and/or laparotomy.

Peritoneal function was assessed yearly, with a modified 3.86% glucose-based peritoneal equilibration test (PET) (4,5), which has the advantages over the conventional 2.27% glucose-based PET (6) to assess sodium sieving (4) and allow the diagnosis of UF failure (net UF < 400ml at the end of the 4h dwell with 3.86% glucose, as defined by the International Society for Peritoneal Dialysis (7)). Before instillation of the test solution, the peritoneal cavity was rinsed with 2 liters of 1.36% glucose dialysate and immediately drained after inflow completion. A 2 liters 3.86% test bag was then infused. Ten milliliters dialysate samples were collected from the test bag before inflow and 0, 30, 60, 120 and 240 min after its intraperitoneal instillation and blood samples were drawn after 120 min of dwell time. Net UF, i.e. the net difference between dialysate volume effluent and volume infused, was recorded. Measurements of serum urea, creatinine, glucose, and sodium, were performed using routine laboratory techniques on an LX 20 analyser (Beckman-Coulter, Fullerton, CA, USA). The Jaffe´ method was used for creatinine determinations and the results were corrected for the interference with high glucose levels. The mass transfer area coefficient (MTAC) of creatinine was calculated according to Waniewski et al. (8). Sodium sieving (ΔD/P sodium) was defined as the ratio of the difference between sodium concentrate dialysate at the beginning of the PET and at 1 h, on serum sodium with a correction for sodium diffusion using MTAC creatinine. The PET was part of the normal procedure of care for PD patients.

Peritoneal sampling and processing

Para-umbilical biopsy samples of human parietal peritoneum were obtained from uremic patients at the time of catheter insertion, and PD patients at the time of catheter removal because of renal transplantation or transfer to hemodialysis as part of a routine protocol in our center, and processed as previously described (9). The time between last PET and peritoneal biopsy was similar in EPS and control patients (9.1 ± 2.1 versus 7.5 ± 1.6 months in EPS and controls; P=0.61). Samples were folded inside-out to avoid abrasion of the mesothelium, washed in ice-cold PBS, fixed in 4% paraformaldehyde in PBS (pH 7.4) and further embedded in paraffin. Whenever possible, a part of the sample was also snapfrozen in liquid nitrogen to perform protein extraction as described previously (9). The suspension was further homogenized with an Ultra-Turrax (Labortechnik, Staufen, Germany) and then briefly sonicated (Branson Sonifier B12, Danbury, CT). The resulting homogenate was centrifuged at 6000 x 3 *g* (Sigma 113 Centrifuge, Osterode am Harz, Germany) for 10 min at 4°C. After determination of protein concentration with the Bradford method (Bio-Rad), the post-nuclear supernatant (total protein extract) was kept at -80°C. None of the patients suffered from peritonitis at the time of biopsy. Informed consent was obtained from all subjects. The use of human biopsy samples has been approved by the Ethical Review Board of the Cliniques universitaires Saint-Luc.

Immunoblot

SDS-PAGE and immunoblotting were performed as previously described (9). The extracts were solubilized by heating (60°C or 95°C, for 2 min) in sample buffer (1.5% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 6.8, 0.6% dithiothreitol, and 6% [vol/vol] glycerol). Proteins were separated by electrophoresis through 7.5% or 12% acrylamide slabs and transferred to nitrocellulose. After Ponceau Red (Sigma, Saint-Louis, MO) staining to check transfer efficiency, destained membranes were blocked for 30 min at room temperature in blocking

buffer (50 mM sodium phosphate buffer, 150 mM NaCl, 0.05% Tween 20, pH 7.4) comprising 5% nonfat dry milk, followed by incubation with the primary antibody (diluted in blocking buffer with 2% bovine serum albumin) at 4°C for 16 to 18 h. The membranes were then washed, incubated for 1 h at room temperature with the appropriate peroxidase-labeled antibody (1:5000 dilution), washed again, and visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL). Quantification was performed using the gel analysis option of ImageJ (1.47 v) software.

Immunohistochemistry and immunofluorescence

Immunoperoxidase staining on human peritoneum sections was performed as described previously (9,10). Peritoneal sections (5 μm thick) were cut from paraffin blocks, deparaffinized, and rehydrated progressively. After inhibition of endogenous peroxidase by incubation in 0.3% H_2O_2 for 30 min, the slides were blocked with 10% normal goat or horse serum in PBS for 20 min at room temperature. All subsequent antibody incubations were carried out for 45 min at room temperature in a humidified chamber. Sections were incubated with the primary antibody diluted in PBS containing 2% bovine serum albumin, washed, incubated with biotinylated goat anti-rabbit or horse anti-mouse IgG (Vector, Burlingame, CA), washed, and then incubated for 45 min with the avidin-biotin peroxidase complex (Vector, Burlingame, CA). After washing, antibody localizations were visualized using aminoethylcarbazole. Sections were scanned using a slide scanner Leica SCN400 (Leica, Heerbrugg, Switzerland).

Immunofluorescence staining was performed on paraffin-embedded sections of human peritoneal biopsies using a sequential staining protocol as described previously (11). Deparaffinized sections were incubated with 10% normal goat serum for 20 min before adding the first primary antibody diluted in phosphate-buffered saline containing 2% bovine serum albumin for 1 h. After washing, sections were incubated with AlexaFluor 633-labelled

secondary anti-IgG antibodies (Invitrogen, Carlsbad, Belgium) for 45 min. The co-staining was performed by adding the second primary antibody which was then revealed with AlexaFluor 488-labeled secondary anti-IgG antibodies before mounting in Prolong Gold Anti-fade reagent (Invitrogen, Carlsbad, Belgium). Sections were viewed under a Zeiss LSM510Meta Confocal microscope (Carl Zeiss, Zaventem, Belgium), using x20/0.8 Plan-Apochromat and x63/1.4 Plan-Apochromat oil-immersion objectives (Zeiss, Wetzlar, Germany). Five pictures of peritoneal vessels were recorded for each patient, with parameters kept constant during the whole acquisition. Vascular densities of AQP1 and vWF were estimated by the mean fluorescence intensities in positive areas for each picture using ImageJ software (1.47v); the mean value for each patient was used for analysis.

Antibodies

We used the following antibodies: mouse monoclonal anti-human eNOS (Transduction Laboratories, Lexington, KY); purified rabbit anti-von Willebrand factor (DAKO, Glostrup, Denmark), monoclonal anti-human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA); polyclonal rabbit anti-human AQP1 (Chemicon International, Temecula, CA).

Picrosirius red staining

5 µm sections were deparaffinized, rehydrated, treated with 1% phosphomolybdic acid and incubated in saturated picric acid solution containing 1% sirius red for 2h at room temperature before washing in 0.01N hydrochloric acid and mounting. Samples were scanned using a SCN400 slide scanner (Leica, Heerbrugg, Switzerland) and also processed using an Axioskop 40 microscope (Zeiss, Wetzlar, Germany) equipped with filters to provide circularly polarized illumination, using a 20x objective.

Image analysis

The adequacy of each specimen was evaluated according to well-established criteria (12) and inadequate specimens were not included in this study.

Peritoneal fibrosis. The extent of peritoneal fibrosis required the presence of mesothelial, submesothelial, and adipose tissue layers. Submesothelial thickness was defined by the thickness of submesothelial compact zone between basal border of surface mesothelial cells and upper border of peritoneal adipose tissue. Five portions were randomly selected for the measurement of submesothelial thickness using TissueIA software (SlidePath), and the average was used for analysis.

Vasculopathy. Vasculopathy was assessed using two different methods. The first graded the degree of vasculopathy from 1 to 4, as previously described (13): a grade of 1 or 2 indicating subendothelial hyaline material <7 or >7 μm in thickness, respectively; a grade of 3, luminal distortion or narrowing; a grade of 4: luminal obliteration. The second method assessed vasculopathy in post-capillary venules (external diameter of 25 to 50 μm) by calculating the ratio of luminal diameter to vessel external diameter (L/V), which represents an extent of patency of the blood vessel (12). When different vessels showed different severities of the vasculopathy, the most severely affected vessel of each specimen was chosen for the measurement. The average of two L/V measured by two examiners was taken as the representative value of that case. Inter-observer agreement for both vasculopathy grade (unweighed K=0.88) and L/V ratio (Pearson r = 0.87, P<0.0001) was excellent.

Vascular proliferation. Vascular proliferation was assessed by the density vascular structures stained with anti-vWF antibodies (n vessels/field, with a field area of 0.4 mm² at a magnification of 20x). Five measures of vascular density were performed in randomly selected areas of each specimen, by two independent scientists, and the mean of these measures was used for analysis. Correlation between observers was excellent (Pearson r = 0.93; P<0.0001).

Collagen volume fraction and collagen structure. Collagen volume fraction and collagen structure were assessed using picrosirius red polarization microscopy and image analysis using ImageJ (1.47v). Collagen volume fraction was calculated as previously described (14), as the sum of stained tissue divided by the sum of submesothelial area, a measure that closely correlates with hydroxyproline concentration in the tissue (15).

Statistics

Data are presented as mean ± SEM. Comparisons between the means from different groups were performed using unpaired t-tests, Fisher's exact test, or one-way ANOVA, followed by Bonferroni's multiple comparison tests, as appropriated. Trends were assessed by linear ordinary least square regression. Multivariate analysis was performed using a logistic regression random-effects model and included potential risk factors for EPS (age at PD start, PD duration, peritonitis rate, beta blocker use, residual renal function, glucose exposure) in a first model; in a second model, the mean value of sodium sieving during the last 2 years of PD was added to these variables. Probabilities of EPS in function of sodium sieving and UF levels were estimated by multivariate logistic regression. All analyses were performed by GraphPad Prism (6.01 v) or Stata (12 v) software. Significance level is indicated in each figure (*p<0.05, **p<0.01, ***p<0.001).

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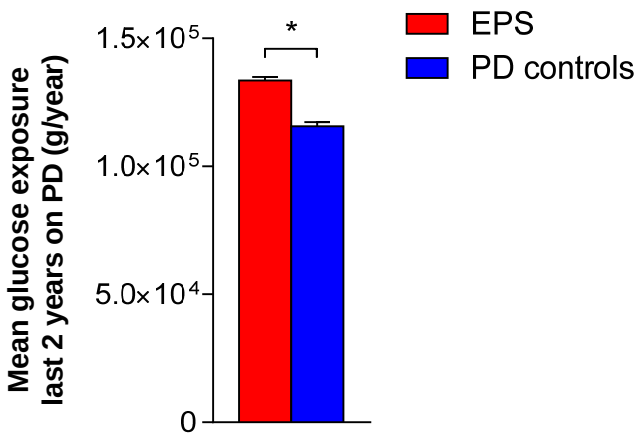
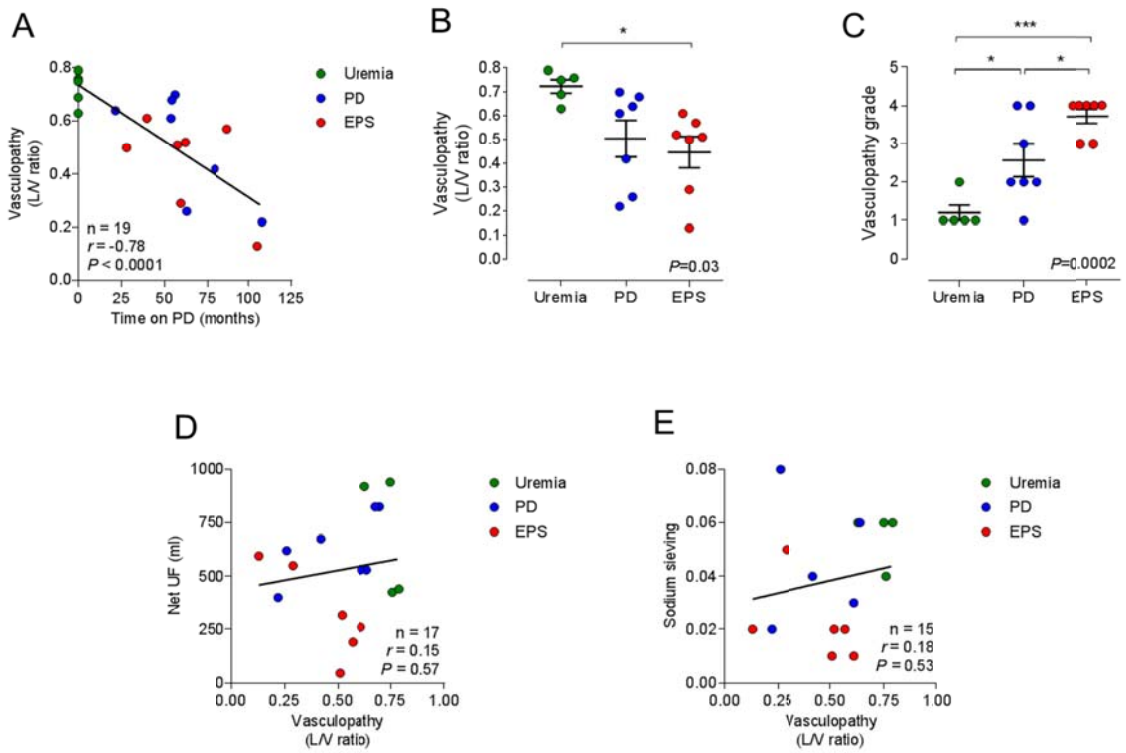
SUPPLEMENTAL FIGURES

Supplemental figure 1



Mean glucose exposure during the last two years on PD in EPS and PD controls. Glucose exposure was significantly higher during the last 2 years on PD in the EPS group as compared with PD controls (133,606±1332 versus 115,697±1586 g/year in EPS and PD controls, respectively; *P*=0.01).

Supplemental figure 2



Impaired water transport does not correlate with peritoneal vasculopathy. (A) The degree of vasculopathy (lumen-to-vessel ratio, L/V) is inversely correlated with time on PD (Pearson r=-0.78, P<0.0001). (B-C) EPS patients tend to have a higher degree of peritoneal vasculopathy (L/V ratio), and have a higher vasculopathy grade as compared with PD controls. (D-E) There is no correlation between net ultrafiltration or sodium sieving and the degree of vasculopathy in this cohort (Pearson r=0.15, P=0.57 and Pearson r=0.18, P=0.53, respectively). Peritoneal samples are from 5 uremic, 7 long-term PD without EPS, and 7 EPS patients. Circles represent individuals; green circles for uremic, blue for PD controls, and red for EPS patients.

SUPPLEMENTAL TABLE

Supplemental table 1. Clinical and functional characteristics of PD patients in which peritoneal biopsies were analyzed.

	Uremic	**Long-term PD**	**EPS**
	***n* = 5**	***n* = 7**	***n* = 7**
PD duration (months)	-	62.4 ± 9.3	62.8 ± 9.2
Charlson comorbidity index	4.8 ± 1.0	4.0 ± 0.8	4.9 ± 1.0
Diabetes, %	20%	29%	29%
Residual diuresis at PD start, ml/day	1252 ± 224	835 ± 143	1083 ± 159
Total number of peritonitis	-	1.5 ± 0.4	1.4 ± 0.4
Functional parameters at the time of biopsy			
Net UF (ml)	681 ± 124	629 ± 55	326 ± 79*
D/P creatinine 4h	0.75 ± 0.07	0.75 ± 0.02	0.84 ± 0.04
Sodium sieving	0.06 ± 0.00	0.05 ± 0.01	0.02 ± 0.01*
Time between last PET and biopsy (months)	-	7.5 ± 1.6	9.1 ± 2.1

EPS, encapsulating peritoneal sclerosis; PD, peritoneal dialysis; UF, ultrafiltration