Isolation of interstitial fluid and demonstration of local proinflammatory cytokine production and increased absorptive gradient in chronic peritoneal dialysis

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Rosengren BI, Sagstad SJ, Karlsen TV, Wiig H. Isolation of interstitial fluid and demonstration of local proinflammatory cytokine production and increased absorptive gradient in chronic peritoneal dialysis. Am J Physiol Renal Physiol 304: F198–F206, 2013. First published November 14, 2012; doi:10.1152/ajprenal.00293.2012.—In peritoneal dialysis (PD) patients, the frequent exposure to “unphysiological” dialysis fluids elicits a chronic state of a low-grade peritoneal inflammation leading to interstitial matrix remodeling and angiogenesis. Proinflammatory cytokines are important regulators involved in this inflammatory process that ultimately leads to dysfunction of the peritoneum as a dialysis membrane. We aimed to measure the local concentrations of proinflammatory cytokines in the peritoneal interstitial fluid (IF). Furthermore, we wanted to assess how the driving forces for fluid and solute exchanges are affected in a remodeled interstitial matrix and thus measured the colloid osmotic pressure (COP) gradient in rats that were exposed to chronic PD. After 8 wk of peritoneal dialysis, IF from peritoneum was isolated using a centrifugation method, and was analyzed for cytokine content and COP along with plasma. For several of the proinflammatory cytokines there were gradients from IF to plasma, showing local production. For some cytokines, the concentration in IF was increased severalfold, whereas IL-18 was increased systemically due to PD. Furthermore, the presence of the catheter per se seemed to increase cytokine levels. COP in IF was significantly decreased in the PD group, while collagen and hyaluronan content was increased. Collectively, our data suggest that the increased levels of proinflammatory cytokines after PD may be an integral component of the development of fibrosis and angiogenesis commonly seen in PD patients, and the decreased COP in IF after chronic PD may shift the Starling equilibrium across peritoneal capillaries to an absorptive state.

interstitial fluid; fluid reabsorption; colloid osmotic pressure; cytokines; inflammation

There are many data published on cytokine levels in plasma and in dialysate (for review, see e.g., 8, 20). Flessner et al. have measured cytokine levels in peritoneal tissues in response to PD semiquantitatively (4, 5), but cytokine levels in the peritoneal interstitial fluid, i.e., the cellular microenvironment, which is likely to be fundamental to local inflammatory processes, have not been measured.

The reabsorption of isotonic fluid from the peritoneal cavity (PC) is considered to occur by direct capillary absorption, and to a smaller extent by lymphatic absorption (14). The capillary absorption is driven by the Starling forces, i.e., the balance between the hydrostatic pressure gradient (DP) and the effective colloid osmotic pressure gradient (σΔΠ) prevailing over the capillary wall according to: $J_v = L_pS(ΔP - σΔΠ)$, where $L_pS$ represents the product of hydraulic conductivity and surface area available for exchange, and $σ$ represents the reflection coefficient. In a previous study we showed that the colloid osmotic pressure (COP) gradient across peritoneal capillaries was reduced by 55% after a single PD dwell in rats (19). Thus the Starling equilibrium was shifted to an absorptive state, giving rise to enough driving force for capillary fluid reabsorption after peak time during a PD dwell. The increased COP gradient may be due to interstitial volume expansion, but some involvement of macromolecular wash-out may have been involved. It was speculated that in chronic PD, the latter may be more important giving rise to even higher COP gradients. The clinical implication of such a reset Starling equilibrium across the peritoneal capillaries would be a decreased net fluid removal.

On this background we hypothesized that the cytokine levels in the peritoneal microenvironment, i.e., in the peritoneal interstitial fluid (IF), are different from those previously measured in spent dialysis fluid and in plasma. Furthermore, we hypothesized that the COP gradient in the chronic PD state is further increased compared with the acute state due to washout of colloids. To get access to the peritoneal microenvironment to increase our knowledge on the inflammatory processes and the transcapillary fluid balance we needed to develop and validate a new method to isolate IF. Thus we modified a centrifugation method previously used on bone marrow and tumor tissue (23, 24).

Here we show that the local interstitial cytokine concentrations are significantly different from those in plasma (and spent dialysate), indicating local production during chronic PD, and moreover that there is an increase in the peritoneal COP gradient after chronic PD, thus shifting the Starling equilibrium toward an absorptive state. This represents the first data on cytokine concentrations in peritoneal IF and on COP in peritoneal tissues after chronic PD.
MATERIALS AND METHODS

Experiments were performed on 82 female Wistar-Møller rats having an average body weight of 312 ± 6 g. The rats had free access to food and water until the day of experiment. Blood samples were collected by cardiac puncture and the rats were killed by intracardiac injection of saturated KCl. All experiments were performed in accordance with the recommendations given by the Norwegian State Commission for Laboratory Animals and were approved by the local ethical committee.

Isolation of interstitial fluid. Interstitial fluid was isolated using a modified centrifugation method (23). This method is based on centrifugation of tissue samples placed on a nylon mesh at an optimal G-force. The abdomen and the back of the rats were shaved, and the rats were immediately transferred to an infant incubator kept at room temperature (20–22°C) and 100% relative humidity. A small hole was cut in the skin of the abdominal wall, and the hole was enlarged using blunt dissection. A 1.5 × 1.5 cm piece of the abdominal wall was carefully excised from the medial portion, rinsed in saline, and gently blotted with tissue paper. The tissue sample was placed in a centrifugation tube with the mesothelial side facing the nylon mesh, and the tube was immediately capped. In the same manner, a 2 × 2 cm piece of back skin was excised and placed with subcutis facing the nylon mesh in a centrifugation tube, which was immediately capped.

The preweighed centrifuge tubes were provided with a basket of nylon mesh (pore size ≈ 1 μm) designed to keep the sample up from the bottom of the tube (1). All tubes were reweighed and spun in an Eppendorff 5417 R centrifuge (kept at 4°C), and immediately brought back to the incubator. The basket containing the tissue was removed, and the tubes were capped and reweighed to determine isolated fluid volume, and returned to the incubator.

Validation of interstitial fluid isolation method. Under isoflurane anesthesia, a catheter was inserted in the tail artery in six rats, and a bolus dose of pentobarbital was delivered through the catheter, after anesthesia, a catheter was inserted in the tail artery in six rats, and a bolus dose of 125I-labeled albumin was given through the jugular vein during the equilibration period, blood samples were collected, and the rats were killed. Interstitial fluid was isolated as described above, and the plasma and IF were analyzed in a LKB gamma counter (model 1282, Compugamma, Turku, Finland) using window settings of 240–400 keV for 51Cr and 15–80 keV for 125I. Standards were counted in each assay, and appropriate corrections for background and spillover were made. The concentrations of 51Cr-EDTA and of 125I-albumin in IF were then compared with those in plasma.

Additional IF was collected from rats in the same manner as described above for analysis by HPLC using a Superox 12 size exclusion column (PharmaCia-Biotech, Uppsala, Sweden). Furthermore, peritoneal tissue samples were taken and transferred, intact or homogenized, to vials containing HPLC-buffer and left overnight for elution. A volume of 100 μl of eluent was injected onto the HPLC system using a Gilson 234 autoinjector (200 μl loop). A constant flow of 1 ml/min was obtained by a SpectraSystem P2000 pump (Thermo separations for products), and protein was detected by UV at 220 nm (Spectra- trasys UV100). The UV signal was digitalized, sampled at 2 Hz, and computer analyzed using ChromoQuest (version 2.51, ThermoQuest). For comparison with our previous study (19), we sampled peritoneal IF using the wing technique in rats, and analyzed the isolated IF as described above on HPLC.

Optimization of centrifugation protocol. To optimize the sampling conditions, and to minimize the risk of cell compression, we systematically varied centrifugal force, starting at a low speed of 27 g [500 revolutions per minute (rpm)] for 10 min and increasing the rpm in steps of 100 rpm until a sample appeared in the bottom of the tube. At 68 g (800 rpm) a small sample volume appeared in all samples, and this was chosen as the minimal G-force to sample peritoneal IF. In additional experiments, centrifugation speeds of 106 g (1,000 rpm), 424 g (2,000 rpm), 1,700 g (4,000 rpm), and 6,800 g (8,000 rpm) were used to evaluate the effect of cellular compression. With increasing G-force, a higher amount of erythrocytes accumulated in the centrifuge. At centrifugation speeds of 1,700 g (4,000 rpm) and above, massive hemolysis and blurring of the centrifuge occurred, and accordingly these samples were discarded. Back skin was sampled and treated as described in previous publications (23).

Implantation of catheters. Titanium injection ports (Soloport MINA-CBAS-C50, Instech Solomon, Plymouth Meeting, PA) with catheters were implanted under isoflurane anesthesia mixed with O2 and N2O, delivered through a mask designed for small animals. All surgical procedures were performed under sterile conditions. Body temperature was kept at 36.5–37.5°C during surgery on a heating plate with a rectal probe. Incisions were made in the skin of neck and of the left flank of the abdominal wall. Using a scalpel, the abdominal wall was penetrated, and a blunt syringe was carefully inserted into the peritoneal cavity (PC). The catheter was inserted through the syringe that was then removed, leaving the catheter in the PC. The catheter was secured to the abdominal wall with a minute volume of tissue glue (Histoacryl, B Melsungen, Germany). Using the syringe, the catheter was then carefully tunneled subcutaneously to the neck incision. The neck incision was enlarged using blunt dissection to house the port chamber. The catheter was cut to a length appropriate for the individual rat and attached to the chamber. The skin over the abdomen and neck was closed using absorbable sutures. The animals were carefully observed until recovery after anesthesia and returned to their cages. They were allowed to recover 1 wk after surgery under careful observation for any signs of infections or poor healing. No postoperative medication or antibiotics were used, and no infusions were made through the ports during recovery.

Peritoneal dialysis procedure. In this study, PD was performed by injecting 20 ml of 3.9% Gambrosol trio (Gambro Lundia AB, Lund, Sweden), which essentially is a lactated Ringer solution containing glucose as osmototic agent, into the peritoneal cavity. The injections were made twice daily with 12 h between injections by perforating the skin and injecting the fluid into the port chamber. The rats were awake during injections and gently immobilized wrapped in a surgical cloth to minimize stress. Before the injections the skin over the port chamber was cleaned with chlorhexidine (Fresenius, Kabi, Haukeland University Hospital, Bergen, Norway). The dwell time for the termination of the experiments was set to 4 h, which according to previous acute PD experiments (e.g., 16) results in a net fluid removal of 1.5 ml and a positive intraperitoneal pressure (IPP) of less than 2 mmHg (6, 29). Catheter control (CC) and negative control (NC) rats were not dialyzed.

Colloid osmotic pressure measurements. Colloid osmotic pressure of IF and plasma was measured on a colloid osmometer constructed for submicroliter samples (25), and interstitial fluid COP was compared with that of plasma. COP was measured directly after isolating the IF.

Cytokine analysis. A multiplex fluorescent bead immunoassay kit (Linco Research, St. Charles, MI) was used for the simultaneous quantification of the following 24 cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IFN-γ, TNF-α, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), regulated upon activation normal T-cell expressed and secreted (RANTES), TNF-α, and VEGF. Samples were diluted in serum diluents according to the specifications supplied by the manufacturer. A mixture of fluorescent polystyrene beads conjugated with specific anticytokine primary antibodies was added to the
samples, resulting in binding of the cytokines to the beads with the corresponding antibody. Biotinylated anti-cytokine secondary antibodies were then added and allowed to bind to the cytokine-bead complex, followed by the addition of fluorescent phycoerythrin-conjugated streptavidin. Total surface fluorescence was then measured with a flow-based dual laser system (LumineX100, LumineX, Austin, TX) for the detection of different color-coded beads and quantitation of cytokines. The concentration of cytokines was calculated with reference to a standard curve based on a broad range of standards (4.8–20,000 pg/ml), providing the lower and upper detection levels for all the assayed cytokines.

Transforming growth factor-β (TGF-β) was assayed in a separate analysis using an ELISA-kit (Rat TGF-β1 BMS623, Bender Medsystems, Vienna, Austria). Plasma and IF samples were prepared and diluted as specified by the manufacturer, and a standard curve supplied with the kit was used for calculation of TGF-β concentrations.

**Peritoneal extracellular matrix elements.** The concentration of hyaluronic acid (HA) from peritoneal tissue samples was analyzed on a sandwich protein binding assay (HA test kit 029–001, Corgenix). Diluted samples and HA reference solutions were incubated in HA-binding protein (HABP)-coated microwells, allowing the HA present in the samples to bind to the immobilized HABP. HABP conjugated with horseradish peroxidase (HRP) was then added to the wells, forming complexes with bound HA. A chromogenic substrate (TMB/H2O2) was added to develop a colored reaction. HA concentrations were calculated by comparing the absorbance of the sample against a reference curve prepared from the reagent blank and five HA reference solutions (50, 100, 200, 500, and 800 ng/ml) included in the kit.

The collagen content of peritoneal tissue was determined according to the Woesner method (27) based on the determination of hydroxyproline content, assuming the conversion factor of 6.94 μg hydroxyproline/μg collagen (7).

**Histology and immunohistochemistry.** Frozen sections (15 μm) were prepared for visualization of leukocytes using immunohistochemistry. The sections were first fixed in 100% acetone for 10 min before washing with PBS. Nonspecific immunolabeling was initially blocked by incubation in 5% normal goat serum (Vector S 2000; Vector Laboratories, Burlingame, CA) diluted in PBS for 30 min at room temperature. Primary antibody for CD45 (1:400, Ab10558, AbCam) diluted in 2.5% goat serum in 0.5% PBS containing Triton-X and 1% BSA was incubated for 72 h at 4°C. Secondary antibody goat anti-rabbit in PBS (1 μl/ml) was added after thoroughly washing with PBS-TX and antigen-antibody complexes were detected by the avidin-biotin peroxidase (ABC) method, using a commercially available kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), and visualized by 3,3′-diaminobenzidine (DAB; Sigma-Aldrich Chemie, Steinheim, Germany) in the presence of 0.2% (NH4)2Ni(SO4)6H2O to enhance the immunostaining. Finally, the sections were counterstained with methylene blue/azure II in 1% sodium borate and distilled water, called the Richardson’s staining. The sections were then dehydrated in a graded alcohol series, cleared in xylene, and coverslipped using Eukitt (O. Kindler, Freiburg, Germany). The sections were then evaluated using a Nikon photomicroscope (Nikon Eclipse E600; Nikon Instruments, Kanagawa, Japan) connected to a digital camera using Lucia imaging software (Lucia v. 480; Laboratory Imaging, Hostivař, Czech Republic).

**Calculations and statistical analysis.** All data are expressed as means ± SE. Statistics were obtained using the Mann-Whitney test and for multiple comparisons Kruskal-Wallis test. All statistical calculations were made using the computer program SPSS (SPSS, Chicago, IL).

**RESULTS**

Rats were checked daily for any irregularities, and all rats increased in weight similarly throughout the experiments in sham and PD group. We checked for possible peritoneal inflammation at the end of the 8-wk period by counting white blood cells in spent dialysate after the final 4 h PD dwell, and in all rats they were below the arbitrary level (<2,500 cells/mm3) indicating inflammation (22). In total there were six dropouts; five due to catheter obstruction, and one rat had a suspected peritonitis and was taken out of the study during ongoing dialysis.

**Evaluation of a centrifugation method for IF isolation.** To evaluate the level of fluid with intracellular origin, which may dilute the isolated interstitial fluid, as well as assessing the possibility of sample evaporation, separate experiments were performed administering 51Cr-EDTA as an extracellular marker. The partitioning of the marker should be equal in extracellular fluid and plasma after equilibration; otherwise dilution of IF by cellular water may have occurred, or, in the case of an increased ratio, evaporation of samples. The relative concentration of 51Cr-EDTA in IF isolated by centrifugation at 68 g relative to the concentration in plasma was on average 0.95 ± 0.06 (n = 6, not significantly different from 1.0), indicating that the fluid isolated by tissue centrifugation was not contaminated by cellular water, nor were there signs of sample evaporation. To evaluate the level of direct leakage of proteins from plasma to IF, 125I-labeled albumin was used as an intravascular marker. The relative concentration of 125I-albumin in IF isolated at 68 g compared with plasma concentration was on average 0.008 ± 0.003 (n = 6), indicating very low leakage of plasma proteins from capillaries to centrifugate.

To evaluate the composition of the isolated IF, the fluid was analyzed by size-exclusion chromatography to separate molecules based on their hydrodynamic radius. On HPLC analysis we could observe the presence of molecules larger than albumin (e.g., IgG, IgM) in plasma (Fig. 1A), while there were very low levels of molecules smaller than albumin. Interstitial fluid from peritoneum isolated at 68 g showed lower amounts of molecules larger than albumin compared with plasma, and higher levels of smaller molecules compared with plasma (Fig. 1B). The level of smaller molecules in peritoneal IF increased with increasing G-force, indicating increased degree of cellular compression and cell damage, and thus leakage of intracellular molecules, with higher G-force (Fig. 1C). Peritoneal IF isolated by the wink technique (Fig. 1D), sampled for comparison with our previous study (19), had a correlative HPLC pattern with the present technique of IF isolation. HPLC of peritoneal tissue eluted overnight in buffer showed a similarity in the high molecular weight range with the fluid isolated by centrifugation (Fig. 1F). There were however, higher levels of molecules smaller than albumin compared with fluid isolated by centrifugation, suggesting addition of intracellular substances. In HPLC of fluid from homogenized peritoneal tissue eluted overnight in buffer (Fig. 1E), however, we noted a high prevalence of molecules not found in plasma, likely of intracellular origin. Thus the HPLC analyses indicated that the fluid isolated by centrifugation had a molecular composition resembling that of plasma, without contaminations from the vascular compartment, or from the intracellular compartment at low G-forces. With increasing G-forces, however, the isolated IF was displaying HPLC patterns indicating contamination from compartments other than the IF. Thus a G-force of 68 g was chosen for peritoneal tissue and 106 g for back skin.

**Colloid osmotic pressures and matrix remodeling.** To assess the driving forces responsible for transcapsillary fluid balance,
we measured the COP of the isolated IF and of plasma. The absolute values of COPs are presented in Fig. 2A. The chronic PD group rats had a significantly lower COP in peritoneal IF (8.4 mmHg) than the catheter control group (11.7 mmHg; \( P = 0.040 \)). There were no differences between the groups in absolute values of COPs are presented in Fig. 2B. The COP values of IF isolated at 68 \( g \) (Fig. 2B) are compared with plasma COP. Again, there was a significant decrease in the ratio of COP for peritoneal IF to plasma for the chronic PD rats compared with the catheter control group \( (P = 0.014) \), while that for back skin IF was unchanged \( (P = 0.189) \).

The chronic changes of the peritoneal interstitial matrix induced by PD were assessed by measuring two major matrix components, collagen and hyaluronan. The collagen content in peritoneal tissue per tissue dry weight (Fig. 3A) was significantly increased for both catheter control rats and chronic PD rats \( (P = 0.03 \) and \( P = 0.005 \), respectively). The hyaluronan content of peritoneal tissue was increased \( (P = 0.018) \) in chronic PD rats, while there was a trend toward increased HA content in the CC group \( (P = 0.072) \) compared with the NC rats (Fig. 3B). These data showed that interstitial matrix remodeling had occurred due to PD and to some degree due to the presence of the catheter per se.

**Local and systemic cytokine concentrations.** We wanted to measure the local concentrations of inflammatory mediators, in the peritoneal cellular microenvironment, and thus we analyzed a spectrum of cytokines in the isolated IF and compared their levels to the plasma concentrations. The measured cytokine concentrations in plasma and in peritoneal IF for NC, CC, and PD group are summarized in Table 1, and in Fig. 4. Data from inconclusive measurements are not shown, and missing values are due to numbers below detection level. The local cytokine concentrations in IF were significantly higher than in plasma \( (P < 0.05) \) in the PD group for all cytokines except for IFN-\( \gamma \), and for VEGF where the value in IF was below detection level. The same general pattern could be seen in the CC and NC groups. IL-1\( \alpha \) and IL-1\( \beta \) were significantly higher in the PD group compared with CC \( (P = 0.002) \). There was a trend toward increasing IL-6 concentration in peritoneal IF of the PD group \( (P = 0.119 \) vs. sham). IFN-\( \gamma \) concentration
in peritoneal IF was increased in PD group compared with CC
\((P = 0.027)\), and there was a trend toward increased IFN-\(\gamma\)
concentration in PD compared with NC \((P = 0.063)\). IFN-\(\gamma\)
concentration in plasma was increased in the PD group compared
with both NC \((P = 0.004)\) and the CC group \((P = 0.001)\). TNF-\(\alpha\) was significantly increased in the peritoneal IF
of PD rats compared with both CC and NC \((P = 0.012, and
\(P = 0.005, \text{respectively})\). IL-18 was markedly increased in the
PD group \((P < 0.001 \text{ vs. NC}, \text{and } P = 0.001 \text{ vs. CC})\), and was
also systemically increased \((P = 0.012 \text{ vs. NC})\). Furthermore,
there was a trend toward increased concentrations of monocyte
chemotactic protein-1 (MCP-1) in the chronic PD group \((P = 0.075 \text{ vs. sham})\). VEGF was not affected and was generally low
in plasma and peritoneal IF. TGF-\(\beta\), however, was markedly
increased in peritoneal IF after chronic PD \((P = 0.009)\) and
in catheter control group CC \((P = 0.009)\) compared with the
NC group (Fig. 4A). The plasma concentration of TGF-\(\beta\) also
increased significantly for both catheter containing groups (CC
\(P = 0.009, PD \ P = 0.009)\) compared with NC (Fig. 4B),
indicating a systemic effect on TGF-\(\beta\) concentration due to the
presence of the catheter.

**Histology and immunohistochemistry.** Sections of peritoneum
showed a significant increase in CD45\(^+\) leukocytes in the PD
group compared with CC (Fig. 5; \(P = 0.0159\)). Thus, in PD there
were 63.2 cells/mm compared with 9.6 cells/mm in CC sections,
confirming that chronic peritoneal dialysis resulted in an inflam-
atory response.

**DISCUSSION**

In the present study we have described and validated a
method to sample peritoneal interstitial fluid in rats. We used
this method to demonstrate that chronic PD markedly alters the
COP in the peritoneal tissue, thereby contributing to a shift in the
Starling equilibrium from a filtrative to an absorptive state.
The effect was restricted to the peritoneal membrane, since the
COP in skin was unchanged. We speculate that the increase in
peritoneal hydrostatic pressure following frequent exposure to
dialysis fluid during PD causes an increase in the interstitial
tissue volume, with washout of colloids. There was also matrix
remodeling occurring after chronic PD as seen by increased
collagen and hyaluronan content in the peritoneal tissues.
Previous data on cytokine concentrations in chronic PD models
are from plasma and spent dialysis fluid. We were able to
measure the actual concentrations in the microenvironment of
the peritoneal tissue after chronic PD, and could show that a
range of cytokines were generally much higher in the perito-
eal tissue than in peritoneal effluent or plasma, showing local
production in the peritoneal interstitium.

One of the aims of the present study was to develop a new
method to sample extraperitoneal interstitial fluid using a
modified centrifugation technique. To validate the method,
we allowed an extracellular marker \(^{51}\text{Cr-EDTA}\) to equilibrate
with the interstitium and compared the tracer concentrations in

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**Fig. 2.** A: colloid osmotic pressures (COP) of plasma and of interstitial fluid
(IF) isolated from peritoneum and skin in negative control rats (NC), in
catheter control rats (CC) and in chronic PD rats (PD). There was a significant
reduction in the COP of peritoneal IF after chronic PD \((^{*P = 0.04})\). Data are
means ± SE. B: ratios of interstitial fluid COP (COP\(i\)) to COP in plasma
(COP\(p\)) for NC, CC, and PD rats. There was a significant fall in the COP ratio
for the PD group \((^{*P = 0.014})\). Data are means ± SE.

**Fig. 3.** A: collagen content in peritoneal tissue per tissue dry weight for negative
control rats (NC), catheter control (CC), and chronic PD rats (PD). The peritoneal
tissue collagen content increased significantly in the CC \((^{*P = 0.03})\) as well as in
the PD group \((^{+P = 0.005})\). B: hyaluronan content in peritoneal tissue per tissue
dry weight for negative control rats (NC), catheter control (CC), and chronic PD
rats (PD). The peritoneal tissue hyaluronan content increased significantly in the
PD group \((^{*P = 0.018})\). Data are means ± SE.
Furthermore, we analyzed plasma and centrifugate by HPLC. The contamination of centrifugate by plasma was negligible. Centrifugate was coming directly from plasma, showing that directly from plasma. Less than 1% of the protein found in method we measured the level of protein (albumin) derived plasma and centrifugate (ratio 0.95). To further validate the method we found almost identical concentrations of $^{51}$Cr-EDTA in plasma and centrifugate, implying that cellular water has contaminated the centrifugate (lower concentration of $^{51}$Cr-EDTA in centrifugate), which could implicate that cellular water has contaminated the centrifugate. However, a higher concentration of the tracer in centrifugate would imply that dehydration of isolated fluid has occurred. However, while a higher concentration of the tracer in centrifugate would indicate that dehydration of isolated fluid has occurred, the method also responded to perturbations (PD) as discussed below and to varying the G-force during centrifugation as might be expected. Thus we conclude that fluid isolated from peritoneum by the described centrifugation method is representative for interstitial fluid.

The chronic PD model was modified from previous studies using similar technologies. We had few drop-outs, and the reason for not completing the 8 wk on PD was catheter obstructions, two cases of catheter getting disconnected from the subcutaneous chamber, and one rat had a suspected peritonitis and was taken out of the study. The chronic PD model applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the applied in the present paper has been used in several previous studies. The setting is nonuremic and as such it differs from the clinical situation. We wanted to study the inflammatory responses to chronic PD per se, and thus exclude the inflammatory contributions caused by uremia. Furthermore, in contrast to the present animal model, in the clinical situation the.

### Table 1. Cytokine concentrations in plasma and in peritoneal interstitial fluid (IF)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Plasma Peritoneal IF</th>
<th>Plasma Peritoneal IF</th>
<th>Plasma Peritoneal IF</th>
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<tbody>
<tr>
<td>IL-1α</td>
<td>76.4 ± 30</td>
<td>351.4 ± 67</td>
<td>54.9 ± 14</td>
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<tr>
<td>IL-1β</td>
<td>23.4 ± 6</td>
<td>268.3 ± 29</td>
<td>293.4 ± 62</td>
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<tr>
<td>IL-6</td>
<td>264.9 ± 128</td>
<td>530.2 ± 136</td>
<td>642.4 ± 97</td>
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<td>IFN-γ</td>
<td>164.7 ± 49</td>
<td>146.2 ± 26</td>
<td>66.7 ± 7</td>
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<tr>
<td>TNF-α</td>
<td>283.1 ± 32</td>
<td>271.3 ± 53</td>
<td>1,424.8 ± 350</td>
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<tr>
<td>IL-18</td>
<td>111.5 ± 27</td>
<td>7.818 ± 1.344</td>
<td>181.0 ± 106</td>
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<td>MCP-1</td>
<td>236.6 ± 26</td>
<td>380.2 ± 48</td>
<td>110.1 ± 17</td>
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<tr>
<td>VEGF</td>
<td>66.4 ± 6</td>
<td>145.9 ± 8</td>
<td>36.5 ± 10</td>
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</table>

Data are means ± SE. Cytokine concentrations in plasma and in peritoneal interstitial fluid (IF) of negative control rats (NC), catheter control rats (CC), and chronic PD rats (PD). *P < 0.05 for IF vs. plasma within group, †P = 0.0002 vs. CC, ‡P = 0.0002 vs. CC, §P = 0.027 vs. CC, ††P = 0.004 vs. NC, and P = 0.001 vs. CC. †P = 0.012 vs. CC, ††P = 0.001 vs. CC, †‡P < 0.001 vs. NC, and P = 0.001 vs. CC, ‡P = 0.012 vs. NC. There was a trend toward increased monocyte chemotactic protein-1 (MCP-1) in the chronic PD group (P = 0.075 vs. CC). Empty cells are data below detection level.
patients drain the PD fluid after each dwell. However, such frequent draining would most likely increase the washout of proteins, and thus we are probably overestimating the IF protein concentration and COP. We might thereby be underestimating the pressure gradients in our model compared with the clinical situation.

The levels of cytokines were generally higher in peritoneal IF than in plasma. Compared with previous studies where cytokine levels have been measured in dialysate, the levels in IF are neither represented well by the levels measured in dialysate due to dilution, nor by levels measured in systemic plasma because of the distance from the local tissue reaction. It could be expected that cytokine concentrations in dialysate are significantly diluted by the dialysis fluid. Importantly, we could assess the absolute concentration in the interstitial fluid and whether substances were produced locally in the microenvironment, based on an interstitial fluid/plasma ratio >1.0. Thus the cytokine concentrations in peritoneal IF of the PD rats compared with sham, implicating a local inflammatory response. This implies local production of the substances in question.

By now it is well known that chronic PD induces angiogenesis (13), and an elevated VEGF in response to PD would be expected in our model. However, we did not find increase in VEGF in the PD group. This may well be due to the fact that we are looking at one time point only, and VEGF may have been increased and involved in angiogenic processes during the 8 wk period of PD before the actual termination of the experiments. Furthermore, TGF-β, which was increased in the chronic PD group (Fig. 4), has angiogenic properties and may contribute to increasing the vascularization of the peritoneum after chronic PD. The increased TGF-β is likely also driving the matrix remodeling seen in the chronic state of PD, here as increased collagen and hyaluronan content (Fig. 3). With the present study design we only have cytokine data from one time point. It would be interesting to see how the concentrations change by time. Flessner et al. have investigated how the inflammatory response in the peritoneum changes over a 20-wk period in a chronic infusion model of rats similar to the present model (5). They measured VEGF and TGF-β semiquantitatively in peritoneal tissues at 0, 4, 8, and 20 wk of exposure to PD. It was found that the strongest inflammatory response was occurring at 4 wk, at 8 wk VEGF-staining decreased but TGF-β-staining was at its maximum, and at 20 wk there was less staining of the two cytokines. The time point chosen in the present study, however, was based on the assumption that 8 wk of PD for a rat corresponds to a patient under long-term PD. Jelicic et al. (9) have shown that PD patients have a positive correlation between time on PD and the levels of IL-6 in the peritoneal effluent as well as in plasma, and a higher concentration in effluent compared with plasma, indicating local inflammation that is also reflected systemically. Furthermore, we found that in rats that were exposed to a chronic catheter, but without PD, the hyaluronan and collagen deposition in the interstitial matrix increased to almost the same magnitude as in the diazylated group. This finding corresponds well with previous studies by Flessner et al. where the same phenomenon was seen (4, 5).

Previous data on COP in the peritoneal tissues are very scarce. In the intermuscular spaces of the abdominal wall of rabbits, Negrini et al. (11) registered values similar to the present study (14 vs. 12 mmHg) in the control animals. However, Negrini and coworkers did not perform any PD in their animals. In a previous study (19), we showed that after a single PD dwell in rats, the COP of peritoneum was decreased from 12 mmHg in control to 7 mmHg after PD. In those two papers, IF was isolated with a different technique than in the present study, i.e., a modified wick technique. Due to the need of higher volumes of IF for the analyses to be performed in the present study, we needed to develop a new technique of isolating interstitial fluid. The gain of IF with the wick technique is usually 1–2 μl, while we usually had a gain of 6–7 μl of IF from peritoneal tissues when using the centrifugation technique. Furthermore, the present technique of IF isolation is technically more simple, and less traumatic compared with the wick technique. In the present study, the chromatographic pattern of IF isolated from peritoneum had a close resemblance to the IF isolated in our previous study using the wick technique. Furthermore, the COPs of IF isolated with the centrifugation technique had values corresponding to the ones in our previous study (COP in the peritoneal IF of control was 11.7 mmHg vs. 12.1 mmHg in the wick paper; PD group COP in peritoneal IF was 8.4 mmHg vs. 6.9 mmHg in the wick paper). The latter result was a bit unexpected, as we speculated in the acute study that in the chronic state we would see more washout of colloids from the peritoneal interstitium, and thus expected a further reduction in COP of peritoneal interstitium. Indeed, here we found a fall in COP of the peritoneal IF of 4 mmHg after a 4 h PD dwell in the chronic state. This fall was not as pronounced as that after an acute PD dwell (19). Other factors involved in the transcapillary fluid balance may, however, be changed after chronic exposure to glucose-containing dialysis fluids, such as hydraulic conductivity (Lp) and capillary surface area available for exchange, and there may be local COP gradients formed after chronic exposure as discussed previously (19). Any such gradients will not be detected with the present technique of IF isolation. The measured reductions of COP in peritoneal IF, however, are sufficient to shift the Starling equilibrium to an absorptive state. Fluid is then transported across the peritoneum directly back into the capillaries, driven by the transcapillary Starling forces, with a minor contribution from lymphatic absorption. This is amplified when the interstitial COP is lowered (10, 14).

In the present paper we have used the standard Starling equation, which considers the balance between the colloid osmotic and hydrostatic pressures, and includes the capillary surface area available for exchange as well as the hydraulic conductivity of the transport barrier. This model does not, however, consider local variations in any of these parameters.
In our model of IF isolation we sample the whole thickness of the peritoneum and will not be able to discern any gradients that may exist. Flessner has previously shown that the main fluid and solute exchange during PD occur within the most superficial 500 μm of the peritoneum (3). Using the wick technique, this part of the peritoneum is accessible and this may partly explain why we did not see a more pronounced fall in the COP gradient during chronic PD compared with our acute study (19). Furthermore, it has been demonstrated that the endothelial glycocalyx is degraded during inflammatory conditions (for review see Ref. 2). It has been shown in many previous chronic animal models, as well as in the clinical setting (26), that substantial angiogenesis is a common feature during chronic PD, and it can be assumed to occur in our model. Inflammation and angiogenesis would certainly affect small solute fluxes during PD. The sum effect of increased capillary area available for exchange and a thinner glycocalyx would be a higher solute flux.

As discussed previously, any local COP gradients would be difficult to detect using the present method for IF isolation. However, Stachowska-Pietka et al. (21) have modeled protein transport within the peritoneum during PD. These authors described bidirectional transport of albumin, and could demonstrate that there is an accumulation of albumin within the deeper layers of the peritoneum, and a washout in the more superficial layers. These data support the present paper, and would explain the unexpected result that the COP gradient in chronic PD was less pronounced than during acute PD. With the centrifugation technique we sample both the superficial and the deep parts of the peritoneal IF.

The main clinical implication of our findings is that the fall in peritoneal COP gives enough driving force to explain how dialysis fluid can be reabsorbed after peak time during a PD dwell. Thus the reabsorption of fluid does not need to involve lymphatic absorption to a large extent. This gives useful information with respect to parameters involved in computer programs designed to calculate peritoneal fluid transport in patients, and to prescribe the correct dialysis dose to patients. A quantitative understanding of the driving forces that govern fluid transport may lead to strategies to minimize fluid loss to the patient’s body and to improve fluid recovery at the end of a PD dwell. This can also assist in our understanding and improvement of intraperitoneal therapies designed to administer macromolecular drugs to intraperitoneal tumors. The local production of proinflammatory mediators may be involved to a large extent in the inflammatory responses seen after long term PD of patients. It remains to elucidate the interactions and time course of these substances to design any pharmacological interventions of the inflammatory responses.

In summary, we were able to develop and validate a method to collect interstitial fluid from the parietal peritoneum. Using this method, we found that after chronic PD, the COP of the peritoneal interstitium fell, shifting the Starling equilibrium to a state that is favoring peritoneal fluid reabsorption. In part, the decreased peritoneal COP may reflect an increased interstitial tissue volume after PD, but a great degree of washout of colloids may also contribute. Furthermore, we were able to measure the concentrations of several cytokines in the peritoneal interstitial fluid, and could show that the local concentrations of many in the peritoneal tissues were higher than the systemic (and the ones previously measured in spent dialysis fluid or after peritoneal lavage), indicating a local production. It remains to understand the role each of these cytokines have in inflammatory processes in the peritoneum that are commonly seen in long term PD, and that leads to the deterioration of the peritoneum as a dialysis membrane.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


