Chronic Infusion of Sterile Peritoneal Dialysis Solution Abrogates Enhanced Peritoneal Gene Expression Responses to Chronic Peritoneal Catheter Presence

Chronic exposure to sterile peritoneal dialysis (PD) solutions is associated with microvascular and interstitial changes within the blood–peritoneal barrier (peritoneum). These changes are commonly linked to loss of peritoneal function over time, presumably because of angiogenesis-related increased vascular area. However, the effects on peritoneal microvascular function of chronic peritoneal exposure to PD solutions are unknown. The present study examined peritoneal microvascular function after chronic exposure to sterile PD solution.

Six rats underwent permanent catheter insertion under anesthesia. Three rats were treated with approximately 16 mL conventional PD solution daily for 6 weeks; catheter insertion controls received 1 mL saline daily. At 6 weeks, visceral peritoneal microvascular function was assessed in vivo using intravital microscopy. Endothelial cell functions were assessed using messenger RNA (mRNA) gene microarray analysis.

In both groups, significant angiogenesis was seen, predominantly in the base of the mesentery. Sensitivity and reactivity of the intestinal visceral peritoneal pre-capillary arterioles (A3 arterioles, 8 – 15μm in diameter) were decreased in the catheter controls, but not in the chronic PD infusion rats. Chronic catheter presence increased the expression of 18 genes in the controls as compared with 12 genes in the chronic infusion rats. In both groups, expression of fibronectin, integrin-β, integrin-α5, collagen type XVIII-α1, and matrix metalloproteinase was enhanced. Endothelial expression of proinflammatory genes (interleukin-1β, tissue pathway inhibitor, chemokine ligand 2) was enhanced by chronic catheter insertion, but not after chronic PD fluid infusion.

Increased expression of genes encoding proteins involved in inflammation and tissue remodeling results from peritoneal catheter-related endothelial cell activation. Chronic exposure of the nonuremic peritoneum to sterile PD solutions overrides the catheter-related endothelial cell proinflammatory phenotype to restore peritoneal microvascular function.

Key words
Vascular endothelium, dialysis solution, vascular reactivity, gene expression, microarray

Introduction
The blood–peritoneal barrier is simply described as the “peritoneal membrane.” A dialysis solution dwell in the peritoneal cavity allows for solute exchange between circulating blood in tissue capillaries and the peritoneal solution. Within the complex peritoneal membrane structure, the number of perfused capillaries (that is, the effective available capillary surface area) and the basic capillary permeability determine the magnitude of solute removal and of ultrafiltration. Chronic infusion of peritoneal dialysis (PD) solutions is associated with structural changes in the blood–peritoneal barrier. Commonly described changes are thickening of the submesothelial compact zone, mesothelial cell transformation, and angiogenesis (1–5). Although these changes are probably related to the duration of infusion and are potentially determined by some components of the PD solution, the molecular mechanisms responsible for these changes are not fully characterized.

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Apart from angiogenesis, little is known about the status of the peritoneal microcirculation after chronic PD infusion. Under conditions of ongoing tissue remodeling related to the continued physical presence of solution within the peritoneal cavity, the local microvasculature might express multiple abnormalities. Peritoneal microvessels might express unique proteins and have bizarre morphologic features, including arteriole–capillary–venule hierarchy loss, tortuosity, variable diameter, defective endothelial monolayer, and leakiness. No data are available to define the integrity, microvascular control mechanisms, and basal permeability of the new microvessels. Molecules that are active during tissue and vascular remodeling, such as vascular endothelial growth factor and other growth factors, are known to increase permeability by promoting fenestration, opening gaps, and clustering fusion of caveolae within the vascular endothelium (6–8).

Experiments with acute or chronic exposure of the peritoneum to PD solutions in nonuremic rats show that hypertonicity of the PD solution inhibits the inflammatory reaction during peritoneal dialysis (9–12). Rippe and Wieslander (13) showed that long-term PD with new, more-biocompatible solutions in nonuremic rats, enhanced effluent markers of peritoneal membrane integrity such as cancer antigen 125 and procollagen peptides (procollagen I C-terminal propeptide, procollagen III N-terminal peptide) and also reduce hyaluronan. They also found no measurable effects on peritoneal exchange parameters, including net ultrafiltration. More recent studies suggest that chronic intraperitoneal fluid infusion results in significant changes in the structure of the blood–peritoneal barrier regardless of the composition of the fluid. The changes were more prominent with conventional glucose-based, lactate-buffered PD solutions (14–18) and were associated with a significant decrease in net ultrafiltration, which was explained by increased peritoneal lymph flow (15,19,20).

Reconciliation of data addressing the structural–functional relationship in chronic infusion models as a surrogate for the clinical scenario of peritoneal dysfunction is complicated by many factors:

- the different techniques of intraperitoneal fluid administration;
- the different methods of measuring peritoneal transport; and
- more importantly, the gap in current knowledge about microvascular control mechanisms and permeability properties of the new vessels formed after chronic exposure to sterile PD solutions.

The present study was conducted to define peritoneal microvascular functions after chronic PD infusion.

Materials and methods

Animal care and surgery

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, U.S.A.) were housed in Association with Assessment and Accreditation of Laboratory Animal Care–approved facilities and were maintained on standard rat diet and water *ad libitum* for at least 1 week before use. All animal care and experimental procedures conformed to the *Principles of Laboratory Animal Care* (21) and the *Guide for the Care and Use of Laboratory Animals* (22) and were approved in advance by the Institutional Animal Care and Use committee of the University of Louisville and the Louisville Veterans Administration Hospital.

Catheter insertion and chronic infusion

Sprague–Dawley rats (n = 6, approximately 100 g each) were anesthetized with intramuscular injections of pentobarbital sodium (60 mg/kg). Using aseptic techniques, an incision was made in the skin of the posterior neck and in the skin overlying the linea alba in the abdominal wall; blunt dissection of each area was performed. A Silastic catheter connected to a small subcutaneous chamber (Rat-O-Port CP6–9S: Access Technologies, Norfolk, VA, U.S.A.) was tunneled from the neck under the skin to the abdomen. A trocar was used to penetrate the abdominal wall, and the catheter tip was inserted into the peritoneal cavity, strictly following the protocol described by Miller *et al.* (23), and secured with a purse stitch in the abdominal wall. The skin overlying the abdomen was closed with wound clips. The chamber in the neck was secured to the underlying subcutaneous tissue with absorbable sutures. Before closure of the skin over the chamber, 1 mL of isotonic Krebs–Ringer bicarbonate containing ceftazidime 125 mg/L, gentamicin 8 mg/L, and ciprofloxacin 25 mg/L was administered via the chamber and the catheter (24), followed by 1 mL containing heparin 10 U. The animal was returned to its cage and carefully monitored until recovery. Over the
subsequent week, each animal was examined for infection, as evidenced by wound dehiscence, weight loss, or abnormal or lack of movement. Three weeks after catheter insertion, 3 rats underwent daily 15- to 20-mL injections of PD fluid [Delflex: Fresenius U.S.A., Ogden, UT, U.S.A. (containing 5.67 g/L sodium chloride, 3.92 g/L sodium lactate, 0.257 g/L calcium chloride, and 0.152 g/L magnesium chloride at a pH of 5 – 6 and an osmolality of 398 mOsm/L)] via the port. The instilled volume was scaled by body weight [(x kg/70 kg)0.7 × 2000 mL (25)] to produce intraperitoneal pressures equivalent to those observed in humans (26,27). Other than the initial dose given at catheter placement, prophylactic antibiotics were not administered. Wound dehiscence near the port with an active infection, failure to thrive, catheter obstruction, or visceral-to-parietal peritoneal adhesions at laparotomy were preset criteria for removing an animal from the study.

In vivo peritoneal microvascular function
Six weeks after catheter insertion or PD fluid infusion, each animal underwent in vivo intravital microscopy as detailed elsewhere (28). Briefly, the peritoneal cavity was exposed through a midline abdominal incision of approximately 1.5 cm, and a 2 – 3 cm segment of distal ileum was gently withdrawn from the peritoneal cavity with its neurovascular supply intact. The ileum was opened along the antimesenteric border by electrocautery. Enteric contents and mucus were gently removed from the mucosal surface, and the animal was positioned on a specially designed polyurethane board. The opened ileum was suspended serosal side up by 4-0 silk sutures over a viewing port while submerged in Krebs solution in a tissue bath. The bathing solution was maintained at 37°C, bubbled with nitrogen and carbon dioxide to maintain the pH at 7.4 throughout the surgical preparation and equilibration times. Isoproterenol was added to the bathing solution in a very dilute concentration (1 × 10^-8 g/mL) to retard peristalsis. That isoproterenol dose is lower than the threshold that alters vascular smooth muscle tone (29). The animal on the board was positioned on the stage of a trinocular microscope for direct in vivo intravital microscopy. Microvascular images were transmitted through the microscope to a photodiode array in an optical Doppler velocimeter (Microcirculation Research Institute, Temple, TX, U.S.A.) to measure centerline red blood cell velocity for the calculation of intestinal A1 microvascular blood flow. The microvascular image was then transmitted to a digital camera (Hitachi Denshi, model K-P-D51: Hitachi, Tokyo, Japan) project to a high-resolution computer monitor. The microvascular digital images were stored as streamed video in the computer hard drive for offline measurement of microvascular diameter with calipers. Criteria for an acceptable preparation of intestine for intravital microscopy included a baseline mean arterial pressure >90 mmHg, a red blood cell velocity in a first-order arteriole of >20 mm/s, and active vasomotion in the arteriolar system.

Tissue harvest and gene profiling
At the conclusion of intravital microscopy, while the animal was still under anesthesia, a midline incision was made to fully open the peritoneal cavity. Tissues of each animal were removed in a set order: the left hemidiaphragm, the right middle abdominal muscle, the caudate lobe of the liver, and the small intestine. Tissues were further sectioned for preservation in RNALater (Ambion, Foster City, CA, U.S.A.).

RNA extraction and processing
Tissues stored in RNALater at −20°C were warmed to room temperature. Tissues were then removed from RNA Later and placed in TRIzol (Gibco BRL, Rockville, MD, U.S.A.) for RNA isolation. The RNA was further purified for each individual animal according to the manufacturer’s instructions (SuperArray RNA Isolation Kit: SuperArray, Frederick, MD, U.S.A.), quantitated, and quality-assured using a full-spectrum ultraviolet and visible spectrophotometer (NanoDrop ND-1000: Thermo Fisher Scientific, Waltham, MA, U.S.A.). The RNA samples from individual rats in each group were pooled through extraction of 1 μg/μL per sample and combined in a common tube. For each group, 5 μL (5 μg) of well-mixed solution were removed for amplification and biotinylation. The RNA was converted to complementary DNA (cDNA) according to the manufacturer’s instructions (SuperArray) before final concentration measurements were made using the ND-1000.

Microarray analysis
Equal concentrations of cDNA samples were loaded into Hyb-tubes (SuperArray) that contained the microarrays for overnight hybridization. The microarrays (OligoGEArray Rat Endothelial Cell Biology Microarray: SuperArray) were washed appropriately and tagged.
with alkaline phosphatase–streptavidin to bind the chemiluminescent media. Experimental cDNA was utilized to map the endothelial gene expression changes between the catheter and the chronic infusion groups. The Oligo GEArray Rat Endothelial Cell Biology Microarray is designed to analyze the expression of 113 genes associated with endothelial cell biology. This array includes genes involved in permissibility and vessel tone, angiogenesis, endothelial cell activation, and endothelial cell injury.

**Data analysis and statistics**

Microvascular diameters were normalized as percentage change from baseline and are presented as mean ± standard error of the mean. Microvascular diameter changes from baseline or between the two groups were assessed using a one-way analysis of variance with Bonferroni posttest. Statistical significance was determined *a priori* at *p* < 0.05.

Expression intensity values were extracted from the raw image files captured with a photon scanner using a robust online Image Data Extraction Applet [IDEA: SuperArray (geasuite.superarray.com)]. Quantile normalization was performed to normalize the distribution of probe intensities for all the arrays in a given set. The genes were median centered and log 2 transformed, before the data were automatically imported into the online software suite for analysis. The data were depicted in a scatter plot that displays the relative difference in the expression levels of genes in the two groups (catheter control group assigned to the *x* axis, and chronic infusion group assigned to the *y* axis). If the relative increase is greater than boundary value, the gene is represented as a red plus sign located above the upper line. The further the sign from the upper line, the greater the relative difference. If the relative difference is less than boundary value in either direction, the gene is represented as a black plus sign. Black plus signs mean that the relative change is nonsignificant. If the relative decrease is greater than boundary value, the gene is represented as a green plus sign located below the lower line. The further the plus sign from the lower line, the greater the relative decrease.

**Results**

**Gross appearance**

The control and chronic infusion groups both expressed distinct changes from the normal appearance of the intestine and mesentery of naïve rats. In both groups, the mesenteric arteries and arterioles are tortuous and distinctly prominent as compared with those in naïve rats. This difference was associated with new vessel formation, particularly in the base of the mesentery. These data suggest that the presence of a catheter (foreign body) in the peritoneal cavity results in structural changes in the gross appearance of the tissue.

Visceral peritoneal microvascular reactivity

Intravital microscopy was performed 6 weeks after catheter insertion or chronic PD infusion in each group. Figure 1 presents the results of the microvascular reactivity. As the figure shows, instillation of PD solution in the tissue bath produced a rapid and sustained microvascular response from baseline at all arteriolar levels (6 vessel types or groups, *p* < 0.05). However, in the catheter control group as compared with the PD chronic infusion group, the magnitude of the response in the pre-capillary arterioles was remarkably lower (*p* < 0.05), suggesting a decreased sensitivity and reactivity of these arterioles because of the physical presence of a peritoneal catheter.

**Gene expression profiles**

Of the 113 genes of the endothelium microarray, catheter insertion alone produced significant expression...
FIGURE 2 Performance outline of the anterior abdominal muscle. Genes depicted in parentheses (–) are considered absent (not expressed). Genes highlighted in bold are considered present. A spot within the microarray grid is considered present if its stretched average is greater than its stretched local background, and the average density of the spot is greater than 1.5 times the mean value of the local backgrounds of the lower 75th percentile of all non bleeding spots. All other spots are considered absent.

(A) With catheter insertion. (B) With catheter insertion and chronic peritoneal dialysis infusion.

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FIGURE 2 Performance outline of the anterior abdominal muscle. Genes depicted in parentheses (–) are considered absent (not expressed). Genes highlighted in bold are considered present. A spot within the microarray grid is considered present if its stretched average is greater than its stretched local background, and the average density of the spot is greater than 1.5 times the mean value of the local backgrounds of the lower 75th percentile of all non bleeding spots. All other spots are considered absent. (A) With catheter insertion. (B) With catheter insertion and chronic peritoneal dialysis infusion.
of 26 genes involved in various aspects of endothelial cell function. In contrast, in the chronic infusion group, only 19 genes were expressed (Figures 2 and 3). These data demonstrate that much of the endothelial cell activation and local inflammatory response can be attributed to the peritoneal catheter as a foreign body and not to the dialysis solution. Chronic infusion of dialysis solution exerted an overall anti-inflammatory function by reducing the expression of proinflammatory genes such as interleukin-1β and other chemokines (Figure 3).

The persistent expression of fibronectin, integrin-β1, integrin-α5, collagen type XVIII-α1, and matrix metalloproteinase 14 membrane-inserted concomitantly with genes encoding proteins of vasoactive mediators such as nitric oxide synthase 3 (endothelial cell constitutive NO synthase) and angiotensin 1 converting-enzyme suggest the importance of ongoing tissue remodeling in the peritoneal microvascular tone–control mechanisms under the conditions of the present experiment.

**Discussion**

Acute exposure of the peritoneal microvasculature results in rapid microvascular vasodilation (30–35). This PD solution–induced vasodilation can influence the effective capillary surface area by functional recruitment of capillaries. In contrast, an increase in vascular surface area in long-term PD adds physical vascular area by neovascularization and angiogenesis. The former was suggested as the cause of enhancement of the permeability surface area product (PS for small solutes) during the initial phase of a dwell (36), but the latter was proposed as the mechanistic explanation for the increased peritoneal transport rate for small solutes seen in long-term PD (37). However, the permeability properties and pattern of vascular reactivity of the newly formed microvessels after chronic dialysis remain to be determined.

The present study closes the gap in knowledge about peritoneal microvascular function after long-term exposure to sterile PD solution. Long-term exposure to sterile PD solution is associated with significant peritoneal microvascular endothelial dysfunction. This cellular dysfunction is exclusively the result of the physical presence of a peritoneal catheter (foreign body) that prompts a proinflammatory endothelial cell phenotype. It appears that the structural changes within the blood–peritoneal barrier are caused mainly by stressors other than the traditionally accepted “bioincompatibility” of the PD solution. The catheter, as a “foreign body” within the peritoneal cavity, initiates a sterile local inflammatory response that stimulates tissue and vascular remodeling. Remodeling is expressed histologically in the interstitium by the deposition of the interstitial ground matrix (fibrosis and thickening) and in the microvasculature by new vessel formation (angiogenesis).

Peritoneal dysfunction (ultrafiltration loss and increased solute clearance) after long-term clinical use is widely assumed to result from the structural changes within the blood–peritoneal barrier that are thought to occur because of the bioincompatibility of the solution. However, the structural–functional relationship has not been fully elucidated (38–41). Although these changes are well described, neither the factors responsible nor the time during which they develop has been
identified (42,43). Because of the recovery of proinflammatory mediators from effluents of dialysis patients, other investigators have introduced the concept that the peritoneum in long-term PD is a chronically inflamed organ (44). Levels of dialysate proinflammatory mediators such as interleukin-6 (IL-6) increased over time in patients treated with conventional PD solutions. The IL-6 levels correlated with peritoneal transport rate and with markers of oxidative stress and angiogenesis (45). Peritoneal dysfunction has also been found to correlate with the number of peritonitis episodes during the treatment course (41,46,47). Biopsy studies in PD patients and in animal models of chronic infusion with PD solutions, described similar structural changes within the blood–peritoneal barrier. Histologic examination of peritoneal membranes from pre-dialysis uremic patients and hemodialysis patients revealed a significantly thicker submesothelial compact zone in these patient cohorts (48), suggesting that uremia as a systemic inflammatory condition contributes to these peritoneal changes (49).

Because the microvascular wall within the composite blood–peritoneal barrier is the major barrier to both ultrafiltration and solute transport during PD, investigators have focused on the microvascular changes associated with chronic PD to provide a direct mechanistic explanation for peritoneal dysfunction (50). Honda and coworkers found a correlation between decreased ultrafiltration and the prevalence of vasculopathy and submesothelial fibrosis (51,52); however, the major resistance to small-solute transport across the blood–peritoneal barrier is encountered within the interstitium and not at the wall of capillaries spatially distributed in the interstitium (53,54). Therefore, enhanced small-solute transport as an early manifestation of peritoneal dysfunction should be interpreted according to the prevailing interstitial dynamics and not on the basis of increased surface area resulting from angiogenesis. This interpretation is consistent with recent studies showing no changes in peritoneal transport characteristics after chronic exposure of the nonuremic peritoneum to sterile PD solutions (15).

Conclusions
Increased expression of genes encoding proteins involved in inflammation and tissue remodeling results from peritoneal catheter–related endothelial cell activation. Chronic exposure of the nonuremic peritoneum to sterile PD solutions overrides the catheter-related endothelial cell proinflammatory phenotype to restore peritoneal microvascular function.

Acknowledgments
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