Vascular Proliferation and Enhanced Expression of Endothelial Nitric Oxide Synthase in Human Peritoneum Exposed to Long-Term Peritoneal Dialysis

SOPHIE COMBET,*† TOSHIO MIYATA,§ PIERRE MOULIN,‡ DOMINIQUE POUTHIER,* ERIC GOFFIN,* and OLIVIER DEVUYST,* WITH THE TECHNICAL ASSISTANCE OF YVETTE CNOPS*

*Division of Nephrology and †Department of Pathology, Université Catholique de Louvain Medical School, Brussels, Belgium; ‡Division of Cell Biology, Commissariat à l’Energie Atomique, Saclay, France; §Institute of Medical Science and Department of Internal Medicine, Tokai University School of Medicine, Kanagawa, Japan; and 4Division of Nephrology, Centre Hospitalier de Luxembourg, Luxembourg.

Abstract. Long-term peritoneal dialysis (PD) is associated with alterations in peritoneal permeability and loss of ultrafiltration. These changes originate from increased peritoneal surface area, but the morphologic and molecular mechanisms involved remain unknown. The hypothesis that modifications of activity and/or expression of nitric oxide synthase (NOS) isozymes might play a role in these modifications, via enhanced local production of nitric oxide, was tested in this study. NOS activities were measured by the L-citrulline assay in peritoneal biopsies from seven control subjects, eight uremic patients immediately before the onset of PD, and 13 uremic patients on short-term (<18 mo, n = 6) or long-term (>18 mo, n = 7) PD. Peritoneal NOS activity is increased fivefold in long-term PD patients compared with control subjects. In uremic patients, NOS activity is positively correlated with the duration of PD. Increased NOS activity is mediated solely by Ca2+-dependent NOS and, as shown by immunoblotting, an upregulation of endothelial NOS. The biologic relevance of increased NOS in long-term PD was demonstrated by enhanced nitrotyrosine immunoreactivity and a significant increase in vascular density and endothelial area in the peritoneum. Immunoblotting and immunostaining studies demonstrated an upregulation of vascular endothelial growth factor (VEGF) mostly along the endothelium lining peritoneal blood vessels in long-term PD patients. In the latter, VEGF colocalized with the advanced glycation end product pentosidine deposits. These data provide a morphologic (angiogenesis and increased endothelial area) and molecular (enhanced NOS activity and endothelial NOS upregulation) basis for explaining the permeability changes observed in long-term PD. They also support the implication of local advanced glycation end product deposits and liberation of VEGF in that process.

Peritoneal dialysis (PD) is now a standard therapeutic option for chronic renal failure. However, its long-term utilization is limited by progressive alterations of the transport properties of the peritoneal membrane (1,2). Cross-sectional and longitudinal studies have indeed shown that peritoneal solute transport progressively increases with time on PD (3–6). Although associated with an increased removal of uremic solutes, high peritoneal membrane permeability has proven to be a significant risk factor for PD patients, predicting not only technical failure but also patient death (2,7). Potential explanations for this detrimental effect include increased protein loss and glucose reabsorption, leading to malnutrition and atheromatosis, as well as enhanced dissipation of the osmotic gradient, with an attendant fall in ultrafiltration (UF) capacity (7). UF failure has been documented in up to 50% of PD patients treated for more than 6 yr (1,8) and is now second to recurrent peritonitis as the most frequent cause for technical dropout (2). Functional studies have shown that PD progressively increases the effective peritoneal surface area (4–6,9), possibly as the result of vascular proliferation, vasodilation of preexisting vessels, or both (10). The exact nature of these changes, as well as the molecular mechanisms involved, remain to be determined in long-term PD patients.

Nitric oxide (NO) plays a key signaling role in countless biologic processes, including control of vascular tone and permeability (11,12), as well as angiogenesis, via an interaction with vascular endothelial growth factor (VEGF) (13,14). Nitric oxide is synthesized from L-arginine by a family of three NO synthase (NOS) isozymes, named from the tissue in which they were initially cloned: neuronal NOS (nNOS), inducible NOS (iNOS; cloned from macrophages), and endothelial NOS (eNOS) (15). Specific NOS isoforms are expressed in human and rat peritoneum (16,17), and detection of nitrite and nitrate in the dialysate attests to the occurrence of NOS activity in the
peritoneum (18,19). Several lines of evidence suggest that NO is involved in the regulation of peritoneal transport during PD, with or without peritonitis. Addition of the NO donor nitroprusside to the dialysate increases both effective peritoneal surface area and intrinsic permeability of the membrane in animal models (20) or stable PD patients (21). In acute peritonitis, decreased UF is associated with a major increase in peritoneal NOS activity, due to upregulation of both eNOS and iNOS (17). However, it is still unknown whether the production of NO and the expression of the various NOS isoforms in the peritoneum are modified in long-term PD.

We have thus tested the hypothesis that modifications of activity and/or expression of NOS isoforms play a role in the increased effective peritoneal surface area observed in long-term PD patients. Using the L-citrulline assay, we determined the specific activities of NOS isoforms in a series of peritoneal biopsies obtained from control subjects and uremic patients treated with PD. These data were correlated with: (1) morphometric quantification of vascular density and endothelial area; (2) immunoblotting studies for NOS isoforms and VEGF; and (3) immunostaining for eNOS, VEGF, and the advanced glycation end product (AGE) pentosidine.

Materials and Methods

Patients and Peritoneal Samples

Para-umbilical biopsy samples of human parietal peritoneum were obtained from 13 PD patients (44 ± 4 yr old, 39% males; PD duration: 23 ± 4 mo) at the time of renal transplantation (n = 11) or catheter removal before transfer to hemodialysis for personal choice or lack of compliance (n = 2). The 13 patients were divided in a “short-term” (12 ± 1 mo, n = 6) and a “long-term” (32 ± 4 mo, n = 7) group according to their PD durations (<18 mo or >18 mo, respectively). End-stage renal disease in these patients was due to IgA nephropathy (n = 2), interstitial nephritis (n = 2), autosomal dominant polycystic kidney disease (n = 1), thrombotic microangiopathy (n = 1), lupus nephritis (n = 1), and Goodpasture syndrome (n = 1). None of the patients suffered from peritonitis at the time of biopsy; the interval between the last episode of peritonitis and biopsy was 9 ± 2 mo. Previous peritonitis episodes (one episode per 27 patient-months) had been treated by oral and/or intravenous antibiotics; no patient had received intraperitoneal antibiotics. No PD patient had sclerosing peritonitis. Progressive alterations in transport properties of the peritoneum were documented in PD patients with longitudinal peritoneal equilibration tests (22). These tests showed that the permeability for both urea and glucose increased significantly over time in long-term PD patients (mass transfer area coefficient [MTAC] for urea: +23 ± 17% over a mean of 18 mo; MTAC for glucose: +18 ± 15% over a mean of 20 mo). Peritoneal biopsies obtained from eight uremic patients (58 ± 4 yr old, 38% males) at the time of PD catheter insertion were used for determination of NOS activity immediately before the onset of PD. Control peritoneal biopsies were also obtained from seven healthy subjects (33 ± 10 yr old, 71% males) at the time of nephrectomy (living donors, n = 2) or laparotomy for benign surgery (n = 5). Patients and control subjects were followed either at Cliniques Universitaires St.-

Figure 1. Total nitric oxide synthase (NOS) activity in the peritoneum of control subjects, and uremic and peritoneal dialysis (PD) patients. Total NOS activity (in pmol citrulline/mg protein per min) was assayed by the conversion of L-[3H]-arginine to L-[3H]-citrulline in extracts from parietal peritoneum obtained from control subjects (□, n = 7); uremic patients immediately before starting PD (△, n = 4 individual samples; ▲, n = 2 pooled samples); and short-term (■, n = 5) and long-term (●, n = 7) PD patients. Total NOS activity is significantly increased in long-term PD patients versus control subjects (approximately fivefold), and versus short-term PD and uremic patients (approximately threefold) (ANOVA: P < 0.0001; *P = 0.0002, long-term PD versus control subjects; □P < 0.005, long-term PD versus short-term PD and long-term PD versus uremic). Median values (in pmol citrulline/mg protein per min) are 0.016 (control subjects); 0.021 (uremic patients); 0.025 (short-term PD patients); and 0.132 (long-term PD patients). (Inset) Relationship between total NOS activity and PD duration in uremic patients. The linear regression made on the 18 points is y = 1.8 × 10⁻⁵x + 0.03. Uremic patients just before the onset of PD were considered as having a null PD duration.
Luc (Brussels, Belgium), Center Hospitalier de Luxembourg (Luxembourg), or Clinique Ste.-Elisabeth (Namur, Belgium). Informed oral consent was obtained from all subjects. The use of human biopsy samples has been approved by the University Ethical Review Board. Given the small size of some biopsies, priority was given to tissue extraction for determination of NOS enzymatic activity.

**Tissue Processing**

Peritoneal biopsies were obtained at surgery after careful dissection with scissors, with minimal mechanical contact and no electrocoagulation. This technique allows for the preservation of an excellent integrity of the tissue (16). Samples were folded inside-out to avoid abrasion of the mesothelium and washed in ice-cold phosphate-buffered saline (PBS). If size was sufficient, a small part of the samples was fixed in 4% paraformaldehyde in PBS (pH 7.4) and further Figure 2. Ca$^{2+}$-dependent and Ca$^{2+}$-independent NOS activities in the peritoneum of control subjects and PD patients. i-Citrulline assays were performed with or without Ca$^{2+}$, to distinguish between Ca$^{2+}$-dependent (A) and Ca$^{2+}$-independent (B) NOS activities in the peritoneum of control subjects (C, n = 5) and short-term PD (N, n = 5) and long-term PD (●, n = 6) patients. The Ca$^{2+}$-dependent NOS activity is significantly increased in long-term PD patients versus control subjects and short-term PD patients (ANOVA: $P = 0.001$; *$P < 0.003$, long-term PD versus control subjects; **$P = 0.01$, long-term PD versus short-term PD), whereas Ca$^{2+}$-independent NOS is similar in the three groups. There are no significant differences between short-term PD patients and control subjects.

Figure 3. Expression of Ca$^{2+}$-dependent NOS isoforms (endothelial NOS [eNOS] and neuronal NOS [nNOS]) in the peritoneum of control subjects and PD patients. (A) Samples from the parietal peritoneum (40 μg protein/lane) of control subjects (n = 7), short-term PD patients (n = 2), and long-term PD patients (n = 4) were run on 7.5% polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and probed with monoclonal antibodies against eNOS or nNOS (1:1000 dilution). A positive control (lane C) was included for each immunoblot. A single band at 140 kD, similar in size to that identified in bovine aortic endothelial cells (lane C) and corresponding to eNOS, is detected with variable intensity in samples from control subjects (lanes 1 to 7) and short-term PD patients (lanes 1 and 2). A more intense signal is consistently detected in samples from long-term (lanes 3 to 6) PD patients. The 155-kD band corresponding to nNOS, as shown in the pituitary gland lysate (lane C), is not detected in the same peritoneal extracts from control subjects and PD patients. The faint band detected in lane 5 (PD patients) is lower than the expected size of nNOS and probably corresponds to cross-reactivity with the abundant eNOS in that sample. A representative immunoblot of two different experiments is shown. The film was exposed for 2 min (eNOS) or 10 min (nNOS). (B) Densitometry analysis of eNOS expression in peritoneum samples from control subjects and PD patients. The signal intensity for eNOS in long-term PD patients (n = 5) is significantly higher than in control subjects (n = 7) and short-term PD patients (n = 3) (4551 ± 669 versus 1224 ± 315 and 1117 ± 325 arbitrary units, respectively). *$P < 0.0001$ versus control subjects and **$P = 0.002$ versus short-term PD patients. Determinations were done in duplicate from two different experiments.
Figure 4. Immunohistochemical localization of Ca\(^{2+}\)-dependent NOS isoforms (eNOS and nNOS) and nitrotyrosine in the peritoneum of control subjects and long-term PD patients. Representative sections from the parietal peritoneum of control subjects (A, C, F, and H) and long-term PD patients (B, D, E, G, and I) stained for eNOS (A through D), nNOS (F and G), and nitrotyrosine (H and I). (A through D) The staining for eNOS (1:50 dilution) is located in the endothelium lining all types of peritoneal blood vessels. When compared with control subjects (A and C), the staining intensity for eNOS is increased in long-term PD patients (B and D).
embedded in paraffin (16). The major part of the samples was snap-frozen in liquid nitrogen to perform protein extraction as described previously (16,17). Briefly, samples were grounded in liquid nitrogen and suspended in 2 ml/g ice-cold homogenization buffer (50 mM Tris, pH 7.4, containing 0.1 mM ethyleneglycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetra-acetic acid [EGTA], 0.1 mM ethylenediaminetetra-acetic acid [EDTA], 2 mM β-mercaptoethanol, 5 μM leupeptin, and 4 μM pepstatin). 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 × 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.

Measurement of NOS Activity

NOS activity was determined by the conversion of L-[3H]arginine to L-[3H]-citrulline as described previously (17,23). Briefly, 25 μl of tissue extract (approximately 250 to 400 μg of total protein) containing 20 mM 3-[(cholamidopropyl)dimethylamino]-1-propane sulfonate was added to 200 μl of Tris buffer (50 mM, pH 7.4) containing 10 mM dithiothreitol, 10 μM tetrahydrobiopterin, 10 μg/ml calmodulin, 1 mM NADPH, 4 μM flavin adenine dinucleotide, 4 μM flavin mononucleotide, 2 μM L-arginine, and 10−7 M L-arginine. Assays were performed for 30 min at 37°C and terminated with 2 ml of ice-cold stop buffer (20 mM CH3 COONa, pH 5.5, containing 2 mM EDTA, 0.2 mM EGTA, and 1 mM L-citrulline). L-[3H]-Citrulline was separated from the incubation mixture by cation exchange chromatography, using Dowex AG 50W-X8 resin (Bio-Rad) and, after elution with water, quantified by liquid scintillation counting (SL3000; Intertechnique, Plaisir, France). The NOS activity (in pmol citrullination with water, quantified by liquid scintillation counting) was performed with Ca2+; NOS. When a sufficient amount of sample was available, assays were performed with Ca2+ (1 mM CaCl2) or without Ca2+ (0 mM CaCl2, 2 mM EGTA, and 2 mM EDTA) to measure total versus Ca2+-independent NOS activities, respectively. The Ca2+-dependent NOS activity was obtained by subtracting Ca2+-independent NOS activity from total NOS activity. Results were normalized for protein content. Determination of NOS enzymatic activity as a function of protein concentration, temperature, and time verified that the assay was made in the linear part of the curves (23). The determinations were performed in duplicate.

Antibodies

NOS isoforms were detected with mouse monoclonal antibodies raised against human eNOS and nNOS, and mouse iNOS (Transduction Laboratories, Lexington, KY). Specificity of these antibodies against NOS isoforms has been demonstrated (17). Other antibodies included a purified rabbit anti-human factor VIII IgG (Dakopatts, Glostrup, Denmark), a rabbit polyclonal antiserum against nitrotyrosine (Upstate Biotechnology, Lake Placid, NY) (24), a monoclonal antibody against human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) (25), and an affinity-purified rabbit antibody against pentosidine (26).

Other Reagents and Supplies

 Peroxidase-labeled goat anti-rabbit IgG and goat anti-mouse IgG were from Dako (Glostrup, Denmark); rabbit and mouse IgG avidin-biotin peroxidase complex (ABC) kits were from Vector Laboratories (Burlington, CA). Electrophoresis reagents were from Bio-Rad (Melville, NY), and enhanced chemiluminescence was from Amersham (Arlington Heights, IL). L-[3H]-Arginine was from Amersham (Buckinghamshire, United Kingdom) and liquid scintillation reagent was from Lumac (Groningen, The Netherlands). Other reagents and supplies were from Sigma Chemical Co. (St. Louis, MO), J. T. Baker (Phillipsburg, NJ), National Diagnostics (Atlanta, GA), Boehringer (Mannheim, Germany), Polysciences (Warrington, PA), and Pierce (Rockford, IL).

Immunoblot Analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as described earlier (16,17). The extracts were solubilized by heating (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 (40 μg/lane) were separated by electrophoresis through 0.1 × 9 × 6 cm 7.5% or 12% acrylamide slabs and transferred to nitrocellulose. After Ponceau Red (Sigma) 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). The specificity of the immunoreaction was determined by comparison with the signal observed with positive controls and incubation with nonimmune rabbit or mouse IgG (Vector). Densitometry analysis was performed with a StudioStar Scanner (Agfa-Gevaert, Mortsel, Belgium), using the NIH-Image V1.57 software. Optical densities (given in arbitrary densitometry units) were obtained in duplicate from two different gels.

Immunohistochemistry

Immunoperoxidase staining on human peritoneum sections was performed as described previously (16,17). Six-micrometer sections were cut from paraffin blocks, dewaxed, and rehydrated in graded ethanolys. After incubation of endogenous peroxidase by incubation in 0.3% H2O2 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,

Figure 4 (cont.) (E) No specific staining is observed when a similar peritoneal section is incubated with control, nonimmune mouse IgG. (F and G) Absence of specific staining for nNOS (1:50 dilution) in the parietal peritoneum from both control subjects (F) and long-term PD patients (G). (H and I) A diffuse, weak staining for nitrotyrosine (1:50 dilution) is observed in the peritoneum of control subjects (H). The staining is markedly increased in long-term PD patients, particularly along the capillary endothelium (I). Magnification: ×300 in A and B and E through I; ×400 in C and D.
washed 3 × 5 min, incubated with biotinylated goat anti-rabbit or horse anti-mouse IgG (Vector), washed 3 × 5 min, and then incubated for 45 min with the avidin-biotin peroxidase complex (Vector). After washing for 3 × 5 min, antibody localizations were visualized using aminoethylcarbazole. For pentosidine staining, rehydrated sections were incubated in a buffer (0.05 M Tris-HCl, pH 7.2, 0.1 M NaCl) containing 0.5 mg/ml Pronase (Dako) for 15 min at room temperature, washed in PBS, blocked in 4% milk for 2 h, and subsequently incubated with the anti-pentosidine rabbit IgG (26) overnight at 4°C. After washing, sections were incubated with a peroxidase-conjugated antibody, and detection was performed with 3,3′-diaminobenzidine containing 0.003% H₂O₂. Sections were viewed under a Leica DMR coupled to a Leica MPS60 photomicrographic system (Leica, Heerbrugg, Switzerland). The specificity of the immunolabeling was confirmed by: (1) incubation without primary or secondary antibody; and (2) incubation with nonimmune rabbit or mouse IgG (Vector).

Quantification of Vascular Immunoreactivity for Factor VIII

Quantification of factor VIII reactivity was performed by image analysis (27). After staining for factor VIII, sections of human peritoneum were systematically examined through a Zeiss microscope using a KS-400 image analysis system (Kontron, Munich, Germany) coupled to a DAGE-MTI charge-coupled device 72 camera (Michigan City, IN). The stained peritoneum areas were digitized to allow automated detection of stained structures and selection of the objects corresponding to vessel walls. Analyses included determination of: (1) the density of stained vascular structures (in n vessels/field); (2) the mean vessel area, defined as the mean area of the lumen of the blood vessels stained for factor VIII (in μm²); and (3) the cumulative vascular area and the cumulative endothelial area (both in % of the fields examined). All slides were coded and analyzed on a single-blind basis by the same operator.

Quantification of Pentosidine Levels

Pentosidine content in human peritoneum was assessed by HPLC assay as described previously (28). Briefly, the peritoneal sample was lyophilized and then hydrolyzed in 50 μl of 6N HCl for 16 h at 110°C under nitrogen, followed by neutralization with 50 μl of 5N NaOH and 100 μl of 0.5 M phosphate buffer (pH 7.4), then filtered through a 0.5-μm filter and diluted with PBS. A sample (corresponding to approximately 20 μg of protein) was injected into an HPLC system and fractionated on a C18 reversed-phase column (Waters, Tokyo, Japan). The effluent was monitored at an excitation-emission wavelength of 335/385 nm using a fluorescence detector (RF-10A, Shimadzu, Japan). Synthetic pentosidine (29) was used to obtain a standard curve. The identity of the substance in the specimens, determined as pentosidine by fast-atom bombardment-mass spectrometry (379.4 Daltons). Limits of detection were 0.1 pmol of pentosidine per milligram of protein.

Statistical Analyses

Data are presented as mean ± SEM. Statistical significance of the differences between groups was assessed using t test (two means) or ANOVA (three or more means), as appropriate.

Results

NOS Enzymatic Activity

The l-citrulline assay detected a significant level of total NOS activity in all tested human peritoneum samples. (Due to a low yield of protein extraction, four samples in the uremic group were pooled by two, and one sample in the short-term PD group was not assayed.) Total NOS activity in the peritoneum of control subjects was within the range detected in normal rat peritoneum or dog heart (17,30). As shown in Figure 1, total NOS activity was significantly increased in long-term PD patients compared with control subjects (0.11 ± 0.01 versus 0.02 ± 0.01 pmol citrulline/mg protein per min), uremic patients (0.03 ± 0.01 pmol citrulline/mg protein per min), and short-term PD patients (0.04 ± 0.01 pmol citrulline/mg protein per min). The total NOS activity in short-term PD patients was slightly higher than, but not statistically different from, that observed in uremic patients or control subjects. However, when all uremic patients were considered, there was a significant relationship between PD duration and the magnitude of total NOS activity (Figure 1, inset). It must be noted that analyses involving the uremic group give similar statistical conclusions regardless of whether the two pooled points are included.

The l-citrulline assay was performed in parallel with and without Ca²⁺ to determine which NOS isoform (Ca²⁺-dependent and/or Ca²⁺-independent) was involved. This analysis was performed in five control subjects and in five short-term and six long-term PD patients in whom sufficient peritoneal extract was available. As shown in Figure 2, the increase in total NOS activity observed in long-term PD patients was solely due to an increase in Ca²⁺-dependent NOS activity, which averaged 0.10 ± 0.02 pmol citrulline/mg protein per min in long-term PD patients, compared to 0.015 ± 0.01 in control subjects and 0.03 ± 0.01 in short-term PD patients (Figure 2A). In contrast, the Ca²⁺-independent NOS activity (Figure B) was similar in long-term PD patients, short-term PD patients, and control subjects (0.03 ± 0.01, 0.02 ± 0.01, and 0.015 ± 0.01 pmol citrulline/mg protein per min, respectively).

Expression of NOS Isoforms in Human Peritoneum: Immunoblot Analysis

Immunoblot analysis was performed to identify the isoform, eNOS or nNOS, implicated in the increased Ca²⁺-dependent NOS activity observed in long-term PD patients. Extracts from bovine aortic endothelial cells and rat pituitary gland were used as positive controls for eNOS (140 kD) and nNOS (155 kD), respectively. As demonstrated on the representative immunoblot shown in Figure 3A, a weak band corresponding to eNOS was detected with a variable intensity in control subjects and short-term PD patients; that band was significantly upregulated in the blot shown in Figure 3A, a weak band corresponding to eNOS was detected with a variable intensity in control subjects and short-term PD patients; that band was significantly upregulated in the peritoneal samples examined, even with longer film exposure (Figure 3A). No signal was detected when immunoblots were probed with nonimmune, control mouse IgG, or with a monoclonal anti-iNOS at the same dilution (data not shown).
Immunolocalization of eNOS and Nitrotyrosine in the Peritoneum of Control Subjects and PD Patients

A relatively faint immunostaining for eNOS was detected in the parietal peritoneum from both control subjects and long-term PD patients (Figure 4, A through D). As described previously (16), the signal for eNOS was located in the endothelium lining peritoneal blood vessels. Compared with control subjects (Figure 4, A and C), the staining was more intense in the peritoneum of long-term PD patients (Figure 4, B and D). No staining was detected in sections incubated with nonimmune, control mouse IgG at the same dilution (Figure 4E). No specific staining for nNOS could be observed in the peritoneum of both control subjects and PD patients (Figure 4, F and G). The immunoreactivity for nitrotyrosine was tested to investigate whether the increased NOS activity in long-term PD patients was associated with peroxynitrite formation and nitration of tyrosine residues. The peritoneal staining for nitrotyrosine was consistently increased in long-term PD patients, particularly along peritoneal capillaries (Figure 4, compare H and I). No specific staining was observed when the same sections were incubated with control rabbit IgG (data not shown).

Figure 5. Staining for factor VIII in the peritoneum of control subjects and long-term PD patients. Low-power micrographs of representative sections from the parietal peritoneum of control subjects (A) and long-term PD patients (B) stained for factor VIII. The positive signal (dark staining), located in the endothelium lining blood vessels, venules, and capillaries, is clearly more disseminated in long-term PD patients. Note the thickened submesothelial fibrous band in the long-term PD patient. m, mesothelium. Magnification, ×100.
Quantitative of Vascular Immunoreactivity for Factor VIII

Quantitative studies investigated whether the increased expression of eNOS in the peritoneum of long-term PD patients correlated with a proliferation of blood vessels within the peritoneum. Immunostaining for factor VIII (31), performed in representative peritoneal sections from control subjects and long-term PD patients, showed a net increase in the density of stained structures in the latter (Figure 5). Quantification of factor VIII immunoreactivity (Table 1) showed a significant, 2.5-fold increase in the density of stained vessels in long-term PD patients compared with control subjects. The mean area of the stained vessels (the area included in the labeling, i.e., the lumen plus the subendothelial area) was also slightly increased in PD patients. As a result, there was a significant increase of both the cumulative endothelial area and the cumulative vascular area in long-term PD patients. This morphometry analysis has been validated on serial sections of the peritoneum, with a mean interassay variability of 16 ± 8% (density parameter).

Expression of VEGF and Pentosidine

Expression studies for VEGF were conducted to address the putative role of that growth factor in the vascular proliferation observed in the peritoneum of long-term PD patients. Immunoblot analysis (Figure 6) showed a strong signal for VEGF in most short-term and long-term PD patients, whereas no signal was detected in control subjects. Immunostaining for VEGF in the peritoneum confirmed a marked increase of the labeling, mostly along capillary endothelium, in long-term PD patients versus control subjects (Figure 7, A and B). A signal for VEGF was also detected in the mesothelium (Figure 7, A and B). No staining was observed when sections were incubated with nonimmune mouse IgG (Figure 7C).

To substantiate a putative link between carbonyl stress, AGE deposition in the peritoneum, and VEGF secretion by peritoneal cells (32,33), we determined the level of pentosidine in peritoneal biopsies obtained in control subjects and PD patients. The pentosidine level was below the detection limit (<0.1 pmol/mg protein) for all control subjects tested (n = 5), while a mean level of 11 ± 8 pmol/mg protein (range, 0.2 to 36.1 pmol/mg protein) was detected in the peritoneum of tested long-term PD patients (n = 4). This difference in pentosidine levels was reflected by immunostaining for pentosidine in representative sections of the peritoneum (Figure 8). A weak but significant staining for pentosidine was indeed detected in the peritoneum of long-term PD patients, whereas it was absent in control subjects (Figure 8, A and B). Staining for pentosidine in the peritoneum of PD patients was located both in the endothelium and mesothelium. The use of serial sections from a long-term PD patient demonstrated that staining for pentosidine (Figure 8C) colocalized with staining for VEGF (Figure 8D) in the endothelium.

Discussion

The present study demonstrates for the first time that PD is associated with a progressive increase of NOS activity within the peritoneum. This increase is solely mediated by Ca2+-dependent NOS. Immunostaining and immunoblotting experiments demonstrated that, among the NOS isoforms, only eNOS is upregulated. The biologic relevance of this phenomenon is eventually demonstrated by its association with enhanced nitrotyrosine formation and with an approximately 2.5-fold increase in vascular density and endothelial area in the peritoneum of long-term PD patients. These findings cast new light on the molecular mechanisms involved in the progressive deterioration of peritoneal membrane permeability observed during long-term PD.

As pointed out in the introduction, the most frequent trans-

---

Table 1. Quantification of vascular immunoreactivity for factor VIII

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Subjects (n = 4)</th>
<th>Long-Term PD Patients (n = 4)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density of stained vessels (n/field)</td>
<td>6.4 ± 0.8</td>
<td>16 ± 5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cumulative endothelial area (%)</td>
<td>0.59 ± 0.03</td>
<td>2.2 ± 0.2</td>
<td>0.0003</td>
</tr>
<tr>
<td>Cumulative vascular area (%)</td>
<td>1.3 ± 0.5</td>
<td>3.5 ± 0.7</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean vessel area (µm²)</td>
<td>665 ± 175</td>
<td>1000 ± 430</td>
<td>NS</td>
</tr>
</tbody>
</table>

A mean of eight fields (field area: 0.4 mm²) was examined for each sample. The number of stained vessels per sample averaged 40 ± 10 (control subjects) and 142 ± 43 (long-term PD patients). The mean vessel area corresponds to the mean lumen area of the factor VIII-stained vessels within each group. PD, peritoneal dialysis.
port abnormality developing during long-term PD is UF failure (2,33), a complication that eventually leads to the transfer of the patient to another modality of renal replacement therapy (33). The pathophysiology and the molecular mechanisms involved in the UF failure associated with long-term PD are unclear. Recurrent episodes of peritonitis, with associated inflammatory changes, may contribute to peritoneal damage over time (10), but are not a prerequisite for development of UF failure (9,33). Functional studies have demonstrated that the loss of UF is associated with an increased transport of small solutes such as urea and glucose, and an attendant enhanced dissipation of the osmotic gradient across the peritoneal membrane (5,6,34). Modeling studies have suggested that the increased solute transport is mediated by an augmented peritoneal surface area (22). Because the endothelium lining peritoneal blood vessels represents the most important barrier to small solute transport (22), the expanded peritoneal surface must correspond to an increased vascular area (33). Indeed, preliminary reports have documented an increased vascular density in the peritoneum of long-term PD patients (35).

The present results provide a morphologic and molecular basis for this hypothesis. A significant fivefold increase in NOS activity is measured in the peritoneum of long-term PD patients compared with control subjects. Furthermore, in uremic patients, NOS activity is positively correlated with the duration of PD (Figure 1). The results of the global L-citrulline assay in the peritoneum are entirely due to a Ca$^{2+}$-dependent NOS isozyme (Figure 2), which has been further characterized as eNOS by immunoblotting and immunostaining (Figures 3 and 4). The increased NOS activity in the peritoneum is highly specific, and these results differ from those obtained during acute peritonitis, a condition associated with upregulation of both eNOS and iNOS (17). No clinical and biologic signs of acute peritonitis were present in our patients, and absence of peritonitis is further attested by the lack of variation in Ca$^{2+}$-independent NOS activity and the absence of signal for iNOS in the samples tested.

The combined increase in NOS activity and eNOS expression suggests that enhanced NO production might contribute to the progressive loss of peritoneal UF, via a vasodilation-induced rise in peritoneal vascular area and a subsequent dissipation of the osmotic gradient. Several lines of evidence support that hypothesis. In physiologic conditions, eNOS is the prevailing form of NOS in the vascular system, and it is indeed significantly expressed in the peritoneum vasculature (16). Our studies in human and rat peritoneum have shown that eNOS is markedly upregulated in peritoneal inflammation (16) or acute peritonitis (17). In the latter condition, increased NOS activity is negatively correlated with UF capacity (17,36). Furthermore, the NO donor nitroprusside increases effective peritoneal surface area and intrinsic permeability (20,21), whereas NOS inhibitors such as $\text{NG}-\text{nitro-L-arginine methyl ester}$ decrease these parameters (37).

It must be emphasized that another potentially detrimental consequence of increased NO levels within the peritoneum might arise from biochemical modifications, including covalent modifications of proteins. As a radical, NO reacts with oxygen, superoxide anions, and transition metals, leading to S-nitrosylation of critical cysteine or tyrosine residues (38). These modifications are well illustrated by the increased staining for nitrotyrosine that characterizes the peritoneum of long-term PD patients (Figure 4). It is tempting to speculate that these modifications might play a role in the structural changes of the peritoneum (such as interstitial fibrosis or nitrosylation of endothelial proteins) that occur with time on PD (33,39).

Several factors might explain the increased expression of
eNOS in long-term PD patients. Specific immunohistochemistry shows that eNOS is located in the endothelium lining peritoneal blood vessels. The vascular density is clearly augmented in long-term PD patients, as reflected by quantitative morphometry after staining for factor VIII (Figure 5 and Table 1). These results thus confirm a preliminary study (35) and suggest that eNOS upregulation might reflect vascular proliferation and increased endothelial surface area. Alternatively, it is conceivable that eNOS expression in the peritoneum might be modulated by changes in immune defense systems induced by long-term PD, e.g., local cytokine generation (40). The observations that eNOS is upregulated in the inflammatory peritoneum (16) or upon virus-induced activation of interferon-γ and tumor necrosis factor-α (41) support the latter hypothesis.

The relationship between eNOS regulation and angiogenesis deserves further consideration. Of course, as mentioned earlier, increased eNOS may be a simple consequence of angiogenesis and increased endothelial area. However, increased NO levels might also be at the origin of angiogenesis within the peritoneum. Recent studies have shown that: (1) eNOS expression and angiogenesis are correlated during embryogenesis (42); (2) NO can induce angiogenesis in vivo (43); and (3) NO is necessary for the biologic activity of VEGF (13,14). We now document that peritoneal angiogenesis is associated with an augmented expression of VEGF—a hitherto unreported characteristic of long-term PD. Immunostaining for VEGF showed a marked increase of the labeling, mostly along capillary endothelium, compared with control samples. These morphologic findings are confirmed by immunoblot analysis of peritoneal samples. Thus, enhanced VEGF expression together with an augmented NO release might play a determining role in the neoangiogenesis and its subsequent increase in peritoneal vascular area.

In an effort to identify the cause of the enhanced VEGF expression, we investigated the relationship between the latter phenomenon and the well-known AGE accumulation in the peritoneum during long-term PD (44–46). Through an inter-

Figure 8. Immunohistochemical staining for pentosidine and VEGF in the peritoneum of control subjects and long-term PD patients. Representative sections from the peritoneum of a long-term PD patient (A, C, and D) and a control subject (panel B) stained for pentosidine (A through C) and VEGF (D). A weak but significant staining for pentosidine is observed in the peritoneum of a long-term PD patient, located both in the endothelium and the mesothelium (A), whereas it is not detected in a control subject (B). Staining for pentosidine (C) and VEGF (D) on serial sections from a long-term PD patient demonstrated colocalization between the two antigens at the endothelium level. Magnification, ×40.
action with their receptors, AGE induce the release of cytokines and growth factors (47). Furthermore, glucose degradation products such as methylglyoxal promote the transcription of VEGF by endothelial and mesothelial cells (32). We concentrated on one AGE structure, pentosidine, and document significantly higher levels of pentosidine in peritoneal extracts obtained from long-term PD patients than in control subjects. Furthermore, immunohistochemistry reveals a marked increase in pentosidine at both the endothelial and mesothelial levels of the peritoneum in long-term PD patients, as well as a colocalization with VEGF at the endothelial level (Figure 8). It is thus tempting to speculate that progressive AGE deposits on endothelial and mesothelial cells within the peritoneum might promote liberation of VEGF, the latter stimulating angiogenesis in association with eNOS (13,14,48).

In conclusion, our data provide a morphologic (vascular proliferation and increased endothelial area) and molecular (eNOS upregulation and increased NOS activity) basis for explaining the increased peritoneal transport and surface area associated with long-term PD. Additional studies should focus on the implication of AGE and VEGF in that detrimental process.

Acknowledgments

These studies were supported in part by the Fonds National de la Recherche Scientifique (crédit 9.4540.96), the Fonds de la Recherche Scientifique Médicale (convention 3.4566.97), and grants from Baxter. We thank Ch. van Ypersele, J.-L. Balligand, N. Lameire, and J.-M. Pochet for providing invaluable suggestions and critiques, as well as J.-P. Squifflet, J. Malaise, and M. Mourad for help in providing peritoneal biopsies. In particular, we thank the many patients and nurses without whom this study would not have been possible. The expert technical assistance of M. de Rudder, S. Rutten, and L. Wendericks is highly appreciated.

References


Access to UpToDate on-line is available for additional clinical information at http://www.lww.com/JASN.