Cyclooxygenase-2 Mediates Dialysate-Induced Alterations of the Peritoneal Membrane


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ABSTRACT

During peritoneal dialysis (PD), exposure of the peritoneal membrane to nonphysiologic solutions causes inflammation, ultimately leading to altered structure and function. Myofibroblasts, one of the cell types that contribute to dysfunction of the peritoneal membrane, can originate from mesothelial cells (MCs) by epithelial-to-mesenchymal transition (EMT), a process that has been associated with an increased rate of peritoneal transport. Because cyclooxygenase-2 (COX-2) is induced by inflammation, we studied the role of COX-2 in the deterioration of the peritoneal membrane. We observed that nonepithelioid MCs found in peritoneal effluent expressed higher levels of COX-2 than epithelioid MCs. The mass transfer coefficient for creatinine correlated with MC phenotype and with COX-2 levels. Although COX-2 was upregulated during EMT of MCs in vitro, COX-2 inhibition did not prevent EMT. In a mouse model of PD, however, COX-2 inhibition with Celecoxib resulted in reduced fibrosis and in partial recovery of ultrafiltration, outcomes that were associated with a reduction of inflammatory cells. Furthermore, PD fluid with a low content of glucose degradation products did not induce EMT or COX-2; the peritoneal membranes of mice treated with this fluid showed less worsening than mice exposed to standard fluid. In conclusion, upregulation of COX-2 during EMT may mediate peritoneal inflammation, suggesting COX-2 inhibition as a potential strategy to ameliorate peritoneal deterioration in PD patients.

Peritoneal dialysis (PD) is a therapeutic option for the treatment of ESRD and is based on the use of the peritoneum as a semipermeable membrane across which ultrafiltration and diffusion take place.1,2 Continuous exposure to nonphysiologic PD solutions and episodes of peritonitis or hemoperitoneum cause inflammation and injury to the peritoneal membrane (PM), which undergoes fibrosis, angiogenesis, and hyalinizing vasculopathy.3 These morphologic alterations seem to be associated with increased small-solute transport rate and with ultrafiltration dysfunction of the PM.2,3 Resident myofibroblasts and infiltrating inflammatory cells

Received February 22, 2008. Accepted October 27, 2008.
Published online ahead of print. Publication date available at www.jasn.org.

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have been considered the main entities responsible of the structural and functional alterations of the peritoneum. More recently, however, it has been shown that mesothelial cells (MCs) may also play an active role in PM alteration. It has been demonstrated that during PD-induced inflammatory and repair responses, MCs show a progressive loss of epithelial phenotype and acquire myofibroblast-like characteristics by an epithelial-mesenchymal transition (EMT). The myofibroblastic conversion of MCs has been confirmed in an animal model based on intraperitoneal overexpression of TGF-β1. The EMT of MCs is a complex process that is characterized by the increased expression of the transcriptional repressor Snail, which negatively regulates the expression of E-cadherin and other adhesion molecules, resulting in disruption of intercellular junctions. Then, MCs adopt a front–back polarity and acquire increased capacity to invade the submesothelial compact zone, where they contribute to inflammatory responses, fibrosis, and angiogenesis that ultimately lead to PM failure.

Cyclooxygenases (COX) are the rate-limiting enzymes that are involved in the synthesis of prostaglandins by oxidation of arachidonic acid. Whereas COX-1 is constitutively expressed and is involved in homeostatic functions, COX-2 expression is inducible and is involved in a number of pathologic processes, including inflammation, angiogenesis, and tumor growth. In regard to the role of COX-2 in fibrosis, both profibrotic and antifibrotic functions have been described for this enzyme. Whether COX-2 is involved in PM deterioration during PD has not been explored in depth. It has been shown that prostaglandins are locally produced in the peritoneal cavity of PD patients and that their synthesis increases during peritonitis, which results in enhanced peritoneal permeability to macro-

Figure 1. COX-2 and Snail expression in omentum- and PD effluent-derived MCs. (A) Phase-contrast microscopy shows different morphologic characteristics of MCs. (Left) Typical phenotype of human peritoneal MCs. (Middle and Right) MCs with epithelioid and nonepithelioid phenotypes. (B) Differences in COX-2 mRNA expression in MCs with different phenotypes. (C) Differences in Snail mRNA expression in MCs with different phenotypes. (D) Statistically significant linear correlation between COX-2 and Snail mRNA expression in effluent-derived MC. (E) Differences in Snail mRNA expression in MCs from patients treated with standard PD fluids with high content of GDP (High GDPs) or biocompatible PD fluids with low content of GDPs (Low GDPs) or receiving one exchange per day with icodextrin-containing solution (Icodextrin). (F) Differences in COX-2 mRNA expression in MCs from patients treated with different PD fluids. Bars represent means ± SE. Symbols show statistical differences between groups.
molecules. The most possible sources of prostaglandins released into the PD dialysate are macrophages and MCs. Experiments in vitro have demonstrated that exposure of monocytes to advanced glycation end products results in increased COX-2 expression and prostaglandin E2 (PGE$_2$) secretion and that high glucose concentration increases PGE$_2$ synthesis in MCs.

Herein, we demonstrate that COX-2 is upregulated during the EMT of MC and that peritoneal transport rate correlates with COX-2 expression in vitro. We also show that COX-2 inhibition does not prevent EMT in vitro but ameliorates PM worsening in vivo in a mouse model of PD fluid exposure. The data point to COX-2 as a key player in the setting and maintenance of peritoneal inflammation and reveal anti-inflammatory therapy as a strategy to preserve PM integrity in PD patients.

**RESULTS**

**Upregulation of COX-2 Expression during the EMT of MCs in PD Patients**

We analyzed the possible association of COX-2 upregulation with the EMT of MCs, a key process in PM dysfunction. Effluent-derived MC from 23 clinically stable PD patients were grouped in epithelioid and nonepithelioid phenotypes according to their morphology at confluence (Figure 1A) and expression patterns of epithelial or mesenchymal markers. Omentum-derived MC from five nonuremic donors were used as a control. The baseline characteristics of the patients and the differences between the subgroups according to the phenotype of effluent MCs are shown in Table 1. Quantitative reverse transcription–PCR analysis showed a progressive and significant upregulation of COX-2 mRNA expression ex vivo as EMT proceeded, with maximum increase in nonepithelioid MCs (Figure 1B). The expression of the EMT marker Snail also showed a progressive increase along the transdifferentiation process (Figure 1C). The expression of COX-2 and Snail mRNAs presented a significant correlation, indicating that COX-2 expression was augmented in cells that underwent an EMT (Figure 1D).

Interestingly, the phenotype of effluent MCs was associated with the PD fluids used in patients. The distribution of patients according to PD fluids is shown in Supplemental Table S1. All of the patients (seven of seven) treated with standard PD fluids, with high content of glucose degradation products (GDPs), contained nonepithelioid MCs in their effluents. In contrast, patients treated with PD fluids containing low GDP concentration and patients treated with standard fluids and receiving one exchange per day with icodextrin-containing solution showed nonepithelioid MCs in 33% (three of nine) and 0% (zero of seven) of the cases, respectively (two-tail Fisher test, high GDPs versus low GDPs, $P = 0.01$; high GDPs versus icodextrin, $P = 0.001$). Furthermore, the expression of Snail showed correlation with the type of PD fluid, its expression being significantly higher in patients treated with standard PD fluids when compared with patients treated with low GDP solutions and patients receiving one icodextrin exchange (Figure 1E). The expression of COX-2 was also significantly higher in the standard PD fluids group when compared with the icodextrin group, but it did not reach statistical significance when compared with the low GDP group (Figure 1F).

**Upregulation of COX-2 Expression during the EMT of MC In Vitro**

The induction of COX-2 during EMT was confirmed in vitro using various stimuli. As shown in Figure 2A, omentum MC stimulated with TGF-β1 plus IL-1β showed a rapid and transient induction of COX-2 mRNA, which paralleled the expression pattern of Snail mRNA. As mentioned, the expression of COX-2 and Snail mRNAs showed a significant correlation.
The treatment with the cytokines also resulted in increased COX-2 protein expression in parallel with the mesenchymal marker fibronectin (Figure 2C). The upregulation of COX-2 during MC transdifferentiation was further verified in wound-healing experiments in which EMT was triggered by mechanical injury of confluent omentum-derived cell monolayers (Figure 2D).

Exposure of MCs to standard PD fluid, with high content of GDPs and buffered with lactate, resulted in a progressive downregulation of E-cadherin expression, indicative of EMT, and in a rapid and strong induction of PGE2 secretion and COX-2 expression (Figure 3). When MCs were incubated with a more biocompatible PD fluid containing low GDP concentration and buffered with bicarbonate, the cells did not show repression of E-cadherin; in fact, there was induction of this adhesion molecules at 24 h (Figure 3A), and the synthesis of PGE2 and COX-2 was only slightly induced (Figure 3, B and C). Similar results were obtained with another low-GDP dialysis fluid buffered with lactate (Supplemental Figure S1).

**Correlation between COX-2 Expression Ex Vivo and Peritoneal Transport Rate**

Our data suggested that local upregulation of COX-2 in MCs could play an important role in the PM failure; therefore, we analyzed the correlation between COX-2 expression ex vivo by MCs and the transport characteristics of the PD patients. We observed a significant correlation ($r = 0.6, P < 0.001$) between COX-2 mRNA expression and mass transfer coefficient of creatinine (Cr-MTC; Figure 4A). When PD patients were subdivided into two groups according to their peritoneal transport characteristics—Cr-MTC $\leq$ 11 ml/min (low and low-average transporters) and Cr-MTC $>11$ (high and high-average transporters)—we observed significant higher expression of COX-2 mRNA ex vivo ($P < 0.001$) in this last group (Figure 4B). In agreement with our previous study, Cr-MTC was also associated with effluent MC phenotype. None of the patients with epithelioid MCs in their effluents showed Cr-MTC $>11$, whereas 80% (eight of 10) of patients with nonepithelioid MCs showed Cr-MTC $>11$ (two-tail Fisher test, $P < 0.01$). These results suggested that COX-2 upregulation during the EMT of MCs could play a role in the establishment of inflammatory response leading to PM deterioration in PD patients.

**Inhibition of COX-2 Does Not Affect EMT of MCs In Vitro**

Because COX-2 expression was upregulated during the transdifferentiation of MCs, we investigated the role of COX-2 in the process of EMT. We analyzed the effect of the selective COX-2 inhibitor NS398 on EMT of MCs in vitro. The inhibitor did not prevent the TGF-$\beta$ plus IL-1–induced morphologic changes or E-cadherin downregulation (Figure 5, A and B). As expected, NS398 inhibited the synthesis of the COX-2 metabolite PGE2 (Figure 5C). In contrast, COX-2 inhibitor did not affect the upregulation of EMT-associated molecules, including the matrix components fibronectin (Figure 5D) and collagen I (data not shown) and the proangiogenic factor vascular endothelial growth factor (VEGF; Figure 5E). These results demonstrated that COX-2, despite being induced during EMT, was not involved in the mesenchymal conversion of MCs.

**Inhibition of COX-2 Ameliorates PD-Induced PM Inflammation and Structural Alteration**

Peritoneal inflammation is an early response to PD fluid exposure, which in turn may promote the induction of EMT of MCs, the accumulation of extracellular matrix (ECM), and angiogenesis. Thus, we analyzed whether the anti-inflammatory action of the COX-2 inhibitor Celecoxib might prevent PM worsening in a mouse model of PD fluid exposure. Mice...
were daily instilled via catheters with saline or standard lactate-based PD fluid and treated with Celecoxib or vehicle by oral route for 5 wk. The histologic analysis of parietal peritoneum biopsies showed that PD fluid exposure resulted in increased inflammation, ECM accumulation, and thickness compared with saline-treated groups. The administration of the COX-2 inhibitor significantly reduced all of these morphologic changes. No significant differences were observed between mice that were instilled with saline and treated or not with Celecoxib (Figure 6, A and B). To test the effect of Celecoxib on PD fluid–induced angiogenesis, we stained blood vessels of parietal peritoneum with an anti-CD31 antibody. PD fluid–instilled mice showed a significant increase of vessel number compared with saline-instilled groups. The administration of the COX-2 inhibitor to PD fluid–instilled mice resulted only in a slight tendency to reduce angiogenesis, but it did not reach statistical significance (Figure 6, C and D). To analyze the functional relevance of the observed morphologic changes of the peritoneum, we performed a 90-min peritoneal equilibrium test in each group of mice at the last day of treatment. As shown in Figure 6E, the volumes recovered from animals treated with PD were lower than those from saline-treated mice. A partial increase of recovered volumes was obtained in PD fluid–exposed mice treated with Celecoxib.

To evaluate the effect of COX-2 inhibition on the early inflammatory response of the peritoneum, we exposed mice to PD fluid and either treated them or not with Celecoxib for 15 d. The analysis of peritoneal influx of inflammatory cells showed a significant reduction of total cell counts in Celecoxib-treated mice. The decrease of cell infiltration was more evident in macrophages (CD11b<sup>+</sup>), because the reduction of T cells (CD3<sup>+</sup>) did not reach statistical differences (Figure 6F). These results

Figure 3. Effects of standard and low-GDP solutions on EMT of MCs and on COX-2 expression in vitro. Omentum-derived MCs were incubated for 24 or 48 h with control medium (C), standard PD fluid containing high GDPs (HGDP), or solution containing low GDPs (LGDP) diluted one half with culture medium. Cells were also treated with TGF-β plus IL-1β (TGF). (A) Expression of E-cadherin was determined by Western blot. (B) The synthesis of PGE<sub>2</sub> was measured in culture medium supernatant by ELISA, and results are depicted as picograms per milligram of total cellular proteins. (C) The expression of COX-2 was analyzed by Western blot. Bars in A and C represent the fold induction of E-cadherin and COX-2 expression over the control after normalization with tubulin expression. The experiment was repeated at least three times, and a representative experiment is shown.

Figure 4. COX-2 expression in PD effluent MCs and peritoneal transport rate. (A) Linear correlation between COX-2 mRNA expression and Cr-MTC in the PD patient group. (B) Differences in COX-2 mRNA expression in MCs obtained from patients with low and low-average versus high and high-average (Cr-MTC ≤11 versus >11 ml/min) peritoneal transport rates. Bars represent means ± SE.
demonstrated that COX-2 inhibition ameliorated the deleterious effects of PD fluid exposure of PM by reducing inflammation and fibrosis, which in turn resulted in improved ultrafiltration. The data also indicated that inflammation was an upstream event in peritoneal fibrosis and probably in vessel permeability, and that inflammation-independent mediators operated in new vessel formation.

Exposure to Low-GDP Solution Results in Decreased PM Inflammation and Structural Alteration

Because the incubation of MCs with low-GDP solutions had little impact on EMT and on the expression of COX-2, we analyzed whether the exposure of mouse PM to biocompatible fluids resulted in ameliorated PM worsening. Mice were daily instilled with standard PD fluid or with a solution containing low GDP concentration. The inflammatory response was analyzed at days 7 and 35, and PM thickness was determined at day 35. The analysis of peritoneal influx of inflammatory cells during the time course showed that total cell counts increased in the standard fluid–treated group but not in the group instilled with low-GDP fluid. The number of infiltrating cells in the group treated with low-GDP fluid was significantly smaller than in the standard fluid–treated group at day 35 (Figure 7A). The initial percentage of recruited T lymphocytes (CD3+ cells) at day 7 was significantly reduced in the low-GDP fluid–treated group, whereas at day 35, there was no difference among groups (Figure 7B). The recruitment of macrophages (CD11b+ cells) was reduced in the group treated with low-GDP fluid at both time points, but the differences among groups reached statistical significance only at day 35 (Figure 7C). Finally, at day 35, the low-GDP fluid–treated group showed a significant decrease of submesothelial fibrosis compared with the standard fluid–treated group (Figure 7D). These findings demonstrated that mice exposed to PD fluid with low content of GDPs showed less PM worsening.

DISCUSSION

The possible involvement of COX-2 in PM deterioration during PD has remained elusive. Prostaglandins are produced in the peritoneal cavity of PD patients, mostly during peritonitis, and are implicated in increased peritoneal permeability to macromolecules.16,17 Interestingly, whereas the inhibition of prostaglandin synthesis, by intraperitoneal administration of indomethacin, results in a decrease of hyperpermeability to macromolecules during peritonitis, it has no effect on permeability in clinically stable and uncomplicated PD patients.20,21 These findings suggest that only the augmented prostaglandins, probably those produced by the inducible COX-2, are involved in peritoneal transport dysfunction.

Herein, we demonstrate that effluent MCs with nonepithelioid phenotype show increased expression of COX-2 and that peritoneal transport rate correlates with COX-2 expression levels, suggesting that MCs that have undergone an EMT are an important source of prostaglandins and thus contribute to peritoneal inflammatory response in PD patients. We also show that effluent MC phenotypes and the expression of Snail...
and COX-2 are associated with the PD fluids used in patients. Treatments with standard PD fluids have a greater impact on EMT of MCs and on the expression of Snail and COX-2 than treatments with low-GDP solutions or with a combination of standard and icodextrin-based fluids.

That COX-2 upregulation in MCs paralleled that of the EMT inducer Snail prompted us to analyze whether it could play a role in the transdifferentiation process of these cells; however, in vitro experiments demonstrated that inhibition of COX-2 did not prevent TGF-β plus IL-1–induced EMT of MCs and did not affect the expression of EMT-associated molecules. In contrast, it was recently shown that in MCs stimulated with high glucose, the inhibition of COX-2 prevented TGF-β and ECM upregulation.22 An explanation to these apparent discrepancies could be that the inhibition of COX-2 in vitro prevents the indirect effect of high glucose on ECM production, via inhibition of TGF-β synthesis,22 but not the direct effect of inflammatory cytokines on the EMT of MCs.

We demonstrate that Celecoxib treatment preserves the PM of PD fluid–instilled mice. Because inflammation is an early response of peritoneum to PD fluid–mediated insult, the beneficial effect of Celecoxib could be a consequence of its anti-inflammatory properties.9 It is known that tissue injuries in adult mammals caused by traumas or surgeries induce inflammation and scar formation as a consequence of an imperfect healing process.23 In contrast, during the fetal period or soon after birth, injury-induced inflammatory response is weak and the healing takes place without scar formation.23 In addition, it has been shown that mice deficient in macrophages, neutrophils, and mast cells (PU.1 null mice) are impaired to mount a standard inflammatory response and show scar-free healing.24 Thus, the participation of inflammation in tissue repair does not seem to be essential.25 It is tempting to speculate that the inhibition of inflammation, by Celecoxib treatment, contributes to the reduction of peritoneal fibrosis in mice exposed to PD fluid. Our data indicate that PD fluid–induced angiogenesis is only slightly reduced in mice treated with Celecoxib. This may be explained by the fact that not only inflammatory cells but also injured MCs are able to produce different proangiogenic factors26,27; therefore, whereas Celecoxib treatment may block the production of angiogenic factors by inflammatory cells, it has no effect on the synthesis of these factors by MCs. In addition, under stress conditions, resident peritoneal fibroblasts may account for the production of angiogenic factors.28,29 The partial recovery of PM function in animals exposed to PD fluid and treated with Celecoxib might be explained by the inhibition of PGE2 synthesis, which results in decreased vascular permeability.6 Our findings are further supported by the fact that mice exposed to low-GDP fluid, which does not induce EMT
of MCs and COX-2 expression in vitro, show fewer infiltrating inflammatory cells and decreased fibrosis than mice treated with standard fluid, demonstrating the benefit of using biocompatible PD solutions.

The results presented in this work indicate that inflammation is an early response to PD fluid exposure, which in turn leads to peritoneal fibrosis, whereas angiogenesis does not seem to be entirely dependent on inflammatory reaction. The inhibition of COX-2 or the prevention of its expression by anti-inflammatory cells (macrophages) were also determined by flow cytometry at days 7 and 35. Peritoneal thickness was measured at day 35, and results are depicted as percentage of fibrosis induction, 100% being the mean value of the high-GDP group. Box plots represent 25th and 75th percentiles and median, minimum, and maximum values.

CONCISE METHODS

Patients

We included 23 clinically stable PD patients: 12 men and 11 women. The causes of renal failure were nephrosclerosis (n = 6), glomerulonephritis (n = 6), diabetes (n = 4), chronic pyelonephritis (n = 2), polycystic kidney disease (n = 1), unknown cause (n = 2), and other causes (n = 2). The baseline characteristics of the patients and the differences between the subgroups according to the phenotype of effluent MCs are shown in Table 1. The age of patients ranged from 21 to 83 yr (mean 67.88 ± 14.60). The mean period PD was 9.47 ± 7.44 mo (range 3 to 25). Most patients (21 of 23) received recombinant human erythropoietin during this study. The duration of active peritoneal inflammation was defined as the time (days) from elevation of cell count in PD effluent until normalization of cell count. Three patients showed peritonitis, and two experienced hemoperitoneum. All of the patients who experienced peritonitis or hemoperitoneum drained nonpetheliod MCs in the effluents. For these patients, at least 3 mo passed after the resolution of the pathologic conditions before sampling the effluent-derived MC. Peritoneal glucose load was calculated by the sum of glucose contained in each PD-fluid bag during the whole time on PD. urea-MTC and Cr-MTC were measured using standard methods. Ultrafiltration capacity was defined by a peritoneal exchange of 4 h using 3.86% glucose.

At the moment of effluent-derived MC sampling, seven patients were on continuous ambulatory peritoneal dialysis (CAPD) and 16 were on APD. Fourteen patients (five on CAPD and nine on APD) were treated with standard solution based on glucose and lactate, containing high GDP concentration (Dianeal; Baxter Healthcare Corp., Deerfield, IL). Seven of these patients (five on CAPD and two on APD) received one long dwell per day (generally overnight) with icodextrin-containing solution (Extraneal; Baxter). Nine patients (two on CAPD and seven on APD) were treated with low-GDP solutions buffered with lactate in five cases (Balance; Fresenius Medical Care, Bad Homburg, Germany) or bicarbonate in four cases (BicaVer; Fresenius). The distribution of patients according to PD techniques and PD fluids is shown in Supplemental Table S1.

This study abides by the Declaration of Helsinki and was approved by the ethics committee of Hospital Universitario de la Princesa (Madrid, Spain). Written consent was obtained from the PD patients included in this study to use effluent samples. Oral informed consent was obtained from omentum donors submitted to elective surgeries.

Culture of MCs and Reagents

MCs were obtained from PD effluents and from omentum samples using the methods described previously. To standardize effluent MC harvesting in the high-GDP group (n = 14), we obtained the cells
from a long dwell (generally overnight) with a PD fluid containing 2.27% glucose (Dianeal; Baxter). Effluent MCs from the low-GDP group were isolated from a long dwell with fluids containing 2.3% glucose and buffered with lactate (n = 5; Balance; Fresenius) or with bicarbonate (n = 4; BicaVera; Fresenius). From each patient, at least three independent effluent MC samples were obtained in a period of 2 mo, which showed phenotype stability, and one representative MC culture per patient was used in the study. All cells were cultured in mo, which showed phenotype stability, and one representative MC culture per patient was used in the study. All cells were cultured in Earle’s M199 medium, supplemented with 20% FCS, 50 U/ml peni-

fat-free milk and then incubated with specific antibodies against

To induce EMT in vitro, we treated omentum-derived MC for 24 or 48 h with a combination of human recombinant TGF-β (0.5 ng/ml) and IL-1β (2 ng/ml; R&D Systems, Minneapolis, MN), which has been proved to be a good model of EMT in vitro. When indicated, the selective COX-2 inhibitor NS398 (Alexis Biochemicals, San Diego, CA) was used at a final concentration of 20 μM. Wound-healing studies were carried out as described previously by performing a mechanical injury with a 1.5-mm cell scraper. To analyze the effect of GDPSs on EMT and COX-2 expression, omentum-derived MC were incubated for 24 or 48 h with standard PD fluid composed of 4.25% glucose and buffered with lactate (Stay Safe; Fresenius) or low-GDP solutions composed of 4.25% glucose and buffered with lactate or bicarbonate (Balance or BicaVera; Fresenius) diluted one half with culture medium. These MC cultures were negative for von Willebrand factor excluding endothelial cell contamination.

To analyze the effect of Celecoxib in early inflammatory response to PD fluid exposure, we used 20 mice. Mice (10 per group) were daily instilled with 1.5 ml of standard PD solution (Stay Safe; Fresenius) and then incubated with specific antibodies against COX-2, fibronectin, collagen I, E-cadherin, and α-tubulin (Becton Dickinson, Franklin Lakes, NJ). Membranes were incubated with goat anti-mouse IgG antibody conjugated with peroxidase (Pharmingen, San Diego, CA) and developed with enhanced chemiluminescence detection kit (Amersham Biosciences, Freiburg, Germany). Blot images were acquired with an LAS-1000 charged coupled device camera (Fujifilm, Cedex, France).

For the detection of VEGF and PGE2 in culture supernatants, the media of MCs cultured under the various conditions were replaced, and 18 h later supernatants were collected and stored at −80°C until their analysis. The concentrations of VEGF and PGE2 in supernatants were assessed by a standard ELISA kit (R&D Systems). The production of fibronectin under various conditions was measured in cell lysates by ELISA as recommended by the manufacturer (Biomedical Technologies, Stoughton, MA).

Peritoneal Exposure Model in Mice

A total of 112 female C57BL/6 mice with age between 12 and 16 wk were used in this study (Harlan Interfauna Iberica, Barcelona, Spain). The experimental protocol was in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and was approved by the Animal Ethics Committee of the Unidad de Cirugía Experimental of Hospital Universitario La Paz.

A customized vascular access port (Access Technologies, Skokie, IL) was implanted subcutaneously, and the catheter end was introduced into the peritoneal cavity by a chirurgical procedure. During the week of recovery, animals received daily 0.2 ml of saline solution containing 1 U/ml heparin. Thereafter, mice were subdivided into different groups. For histologic and functional analysis, 70 mice were used: 20 were daily instilled with 1.5 ml of standard PD fluid composed of 4.25% glucose and buffered with lactate (Stay Safe; Fresenius) and orally treated with vehicle (PDF group), 20 were instilled with PD fluid and orally treated with Celecoxib (2 μg/g body wt; PDF + Cel group), 15 were daily instilled with 1.5 ml of physiologic saline and orally treated with vehicle (Sal group), and 15 were instilled with physiologic saline and orally treated with Celecoxib (Sal + Cel group). Mice were treated with different conditions for 5 wk. The whole study was performed in four separate experiments. In one experiment, a peritoneal equilibrium test was performed during the last day of treatment with the various conditions. Mice were instilled with 1.5 ml of PD solution; 90 min later, animals were killed and the total peritoneal volumes were collected. The peritoneal net volume was determined by the difference between instilled and recovered volumes. For histologic analyses, specimens of the parietal peritoneum were collected. Sample were fixed in neutral-buffered formalin, embedded in paraffin, cut into 5-μm sections, and stained either with hematoxylin and eosin or Masson’s trichrome. The thickness of submesothelial tissue was determined by blinded microscopic analysis using a metric ocular. The presence of blood vessels was determined by immunostaining with an anti-CD31 mAb (Becton Dickinson). Blood vessels of five fields per mouse sample were counted, and the median was used for statistical analysis.

To analyze the effect of Celecoxib in early inflammatory response to PD fluid exposure, we used 20 mice. Mice (10 per group) were daily instilled with 1.5 ml of standard PD solution (Stay Safe; Fresenius)
and orally treated with Celecoxib or vehicle alone during 15 d. Then, the peritoneal influx of inflammatory cells was analyzed by flow cytometry using anti-CD11b and anti-CD3 mAb (Becton Dickinson).

During these experiments, the percentages of dropouts were similar in all of the groups (approximately 40%), the main cause being cather damage produced by the mouse itself and not as a consequence of treatments. There were no significant differences in weight gain among the various groups.

To compare the effects of PD fluids with different contents of GDPs on PM inflammation and fibrosis, we used 22 mice. Mice (11 per group) were daily instilled with 1.5 ml of standard PD fluid composed of 4.25% glucose and lactate (Stay Safe; Fresenius) or with low-GDP solution composed of 4.25% glucose and bicarbonate (Bicavera; Fresenius). The peritoneal influx of inflammatory cells was analyzed by flow cytometry at days 7 (five mice per group) and 35 (six mice per group). Peritoneal thickness was also determined at day 35. In this experiment, no dropout was observed as a result of technical improvement of surgical implantation of catheters.

Statistical Analysis
Results are given as means ± SD (Table 1). Figures 1, B through F, 2A, and 4B represent means ± SE. Comparisons between data groups were performed using the nonparametric Mann-Whitney rank sum U test. Linear correlation was determined by Spearman regression analysis (Figures 1D, 2B, and 4A). χ² and two-tailed Fisher exact tests were used to compare qualitative variables. P < 0.05 was considered statistically significant. We used SPSS 14.5 (Chicago, IL) and GraphPad Prism 4.0 (La Jolla, CA).

ACKNOWLEDGMENTS
This work was supported by grants SAF2007-61201 and PET2006-0256 from Ministerio de Educación y Ciencia to M.L.-C. and FIS PI 06/0098 and RETICS 06/0016 from Fondo Investigaciones Sanitarias to R.S. This work was also partially supported by Fresenius Medical Care and Gambro Europe.

We thank the nurses from the peritoneal dialysis units for help in recompilation of peritoneal effluents and omental samples. We also thank Javier Benito de la Víbora, DVM, and Carlota Largo Aramburu, DVM, PhD, for the assistance with mouse care.

DISCLOSURES
None.

REFERENCES


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