Blocking TGF-β1 Protects the Peritoneal Membrane from Dialysate-Induced Damage

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ABSTRACT

During peritoneal dialysis (PD), mesothelial cells undergo mesothelial-to-mesenchymal transition (MMT), a process associated with peritoneal-membrane dysfunction. Because TGF-β1 can induce MMT, we evaluated the efficacy of TGF-β1-blocking peptides in modulating MMT and ameliorating peritoneal damage in a mouse model of PD. Exposure of the peritoneum to PD fluid induced fibrosis, angiogenesis, functional impairment, and the accumulation of fibroblasts. In addition to expressing fibroblast-specific protein-1 (FSP-1), some fibroblasts co-expressed cytokeratin, indicating their mesothelial origin. These intermediate-phenotype (Cyto-/FSP-1+) fibroblasts had features of myofibroblasts with fibrogenic capacity. PD fluid treatment triggered the appearance of CD31+/FSP-1+ and CD45+/FSP-1+ cells, suggesting that fibroblasts also originate from endothelial cells and from cells recruited from bone marrow. Administration of blocking peptides significantly ameliorated fibrosis and angiogenesis, improved peritoneal function, and reduced the number of FSP-1+ cells, especially in the Cyto+/FSP-1+ subpopulation. Conversely, overexpression of TGF-β1 in the peritoneum by adenovirus-mediated gene transfer led to a marked accumulation of fibroblasts, most of which derived from the mesothelium. Taken together, these results demonstrate that TGF-β1 drives the peritoneal deterioration induced by dialysis fluid and highlights a role of TGF-β1-mediated MMT in the pathophysiology of peritoneal-membrane dysfunction.


Peritoneal dialysis (PD) is an alternative treatment of end-stage renal disease and is based on the use of the peritoneum as a semipermeable membrane across which ultrafiltration and diffusion take place.1,2 Exposure to nonphysiologic PD solutions and episodes of infection cause damage to the peritoneal membrane, which undergoes submesothelial fibrosis, angiogenesis, and hyalinizing vasculopathy.3–8 These morphologic alterations are associated with increased rates of small-solute transport and with ultrafiltration dysfunction of the peritoneal membrane.3,5–8 Inflammatory cells and pathologic fibroblasts, especially myofibroblasts, are considered to be mainly responsible for peritoneal membrane deterioration during long-term PD.3,9,10 Peritoneal myofibroblasts may have at least a...
dual origin: (1) from resident fibroblasts through an activation process and (2) from the mesothelium via mesothelial-to-mesenchymal transition (MMT). Mesothelial cells that have undergone a myofibroblast conversion acquire the capacity to synthesize extracellular matrix components as well as pro-inflammatory and pro-angiogenic factors, thereby contributing to the deterioration of the peritoneal membrane. The presence of mesothelial cells with mesenchymal phenotype, either in the PD effluent or the peritoneal tissue of PD patients, is correlated with high transport rates. In other fibrotic disorders, myofibroblasts may also originate from the local conversion of endothelial cells, through an endothelial-to-mesenchymal transition (EnMT), and from bone-marrow-derived circulating cells (fibrocytes) that are recruited to the injured tissues. The presence of these myofibroblast sub-populations in the damaged peritoneum and their contribution to the fibrotic process induced by PD has not been described so far.

TGF-β1 is a prototypical inducer of epithelial-to-mesenchymal transition in several tissues and organs. TGF-β1 is also a key factor for the myofibroblastic differentiation of resident fibroblasts and recruited fibrocytes and for the mesenchymal conversion of endothelial cells via EnMT. TGF-β1 binds to a specific type I serine-threonine kinase receptor and triggers different Smad-dependent and Smad-independent signaling pathways, which result in a profound molecular reprogramming including the downregulation of the intercellular adhesion molecule E-cadherin and the upregulation of mesenchymal-associated molecules such as Snail, fibronectin, collagen I, and α-smooth muscle actin (α-SMA).

TGF-β1 is considered a master molecule in the develop-
Figure 2. Administration of TGF-β1-blocking peptides P17 or P144 decreases PD-induced peritoneal membrane thickness in a mouse model. Mice received a daily instillation of standard PD fluid for 5 weeks and intraperitoneally administered with control peptide (4 mg/kg per day: PDF, n = 10), P17 (4 mg/kg per day: PDF + P17, n = 11), or P144 (4 mg/kg per day: PDF + P144, n = 11). A control group of mice that were instilled with saline was also included (Control; n = 7). Peritoneal samples were prepared and analyzed as described in the Concise Methods. (A) Standard PD fluid exposure increases matrix deposition and the thickness of the peritoneal membrane, whereas TGF-β1-blocking peptide administration significantly reduces these effects when measured in Masson’s-trichrome-stained sections (representative slides). Magnification: ×200. (B) Box plots graphic represent 25th and 75th percentiles and median, minimum, and maximum values of thickness (μm). (C) Quantitative RT-PCR shows that PD fluid exposure upregulates the expression of the...
ment of peritoneal dysfunction because its overexpression has been correlated with worse PD outcomes.30–32 The relevance of TGF-β1 in peritoneal damage is further suggested in experimental animal models in which the TGF-β1 gene is transduced into the peritoneal cavity with adenovirus vectors, recapitulating the structural and functional alterations observed in PD patients.33–35 Overexpression of molecules counteracting TGF-β1-trigerred Smad signaling, including Smad-7 and bone morphogenic protein-7, prevents and reverses PD fluid-induced peritoneal worsening.36–40 Furthermore, inhibition of TGF-β1-induced Smad-independent pathways ameliorates the peritoneal membrane alterations in different experimental models.41 However, the potential protective effects for the peritoneal membrane of agents directly targeting TGF-β1 have not been explored.

In this study, we used two synthetic peptides (P17 and P144) designed to directly bind TGF-β1 and block its biologic function and that have been demonstrated to act systemically and to have robust antifibrotic effects in various organs such as the liver, heart, and skin.42–45 We show that intraperitoneal administration of these blocking peptides to mice exposed to PD fluid preserved the peritoneal morphology and function and decreased the accumulation of different subpopulations of “fibroblast-specific protein-1” (FSP-1) fibroblasts, especially of those co-expressing the mesothelial marker cytokeratin. These cells with intermediate phenotype (Cyto*/FSP-1+) displayed myofibroblast-like characteristics including fibrogenic capacity. These results point to TGF-β1-mediated MMT as a key process in the peritoneal damage induced by PD.

RESULTS

TGF-β1-Blocking Peptide P17 Counteracts TGF-β1- and Effluent-Induced MMT In Vitro

First, we analyzed the effect of TGF-β1-blocking peptide on the MMT of mesothelial cells in vitro. Treatment of omentum-derived mesothelial cells with TGF-β1 and control peptide induced the phosphorylation of Smad-2/3 and the expression of Snail at early time points (6 hours) and downregulated the expression of E-cadherin at 24 hours. These effects were prevented by co-treatment with TGF-β1-blocking peptide P17 (Figure 1A, A and B). In addition, peptide P17 completely blocked TGF-β1-mediated upregulation of the mesenchymal markers collagen I and fibronectin at 72 hours (Figure 1B). These data were further confirmed with mesothelial cells exposed to effluents from PD patients suffering peritonitis to induce MMT (Figure 1C).

TGF-β1-Blocking Peptides Ameliorate PD-Induced Peritoneal Membrane Structural and Functional Alterations

We analyzed whether the TGF-β1-blocking peptides P17 and P144 might prevent the deterioration of the peritoneal membrane in a mouse model of PD fluid exposure. Histologic analysis of parietal peritoneum biopsies from animals exposed to PD fluid (PDF group) showed a loss of the mesothelial cell monolayer and an increase in the peritoneal membrane thickness when compared with mice exposed to saline solution (control group) (Figure 2, A and B). Administration of TGF-β1-blocking peptides to PD fluid-treated mice (PDF + P17 and PDF + P144 groups) significantly reduced the peritoneal thickness and preserved the mesothelium (Figure 2, A and B). These data were confirmed by quantitative reverse-transcriptase (RT)-PCR, using RNA samples extracted from some peritoneal biopsies, to analyze the expression of mesenchymal markers. Exposure to PD fluid provoked the upregulation of collagen I, fibronectin, and α-SMA mRNAs; treatment with P17 or P144 interfered with the induction of these mesenchymal-associated molecules (Figure 2C). To determine the effect of PD fluid exposure, with or without blocking peptides, on the expression of TGF-β1, the levels of this cytokine were measured in the effluents of the different groups. Exposure to PD fluid sharply induced the production of TGF-β1, and administration of P17 or P144 significantly reduced its concentrations, suggesting the existence of a positive feedback in the peritoneal expression of TGF-β1 (Figure 2D). As shown in Figure 2E, there was a correlation between submesothelial thickness and the expression of TGF-β1, indicating that this cytokine played a central role in peritoneal fibrosis.

To test the effect of blocking peptides on PD fluid-induced angiogenesis, blood vessels of the parietal peritoneum were stained with an anti-CD31 antibody. There was a significant increase in the number of vessels in PD fluid-instilled mice when compared with the control saline-treated mice, and administration of peptides P17 or P144 to PD fluid-instilled mice significantly reduced this angiogenesis (Figure 3, A and B). To further explore the effects of TGF-β1 blockade on angiogenesis, the effluent levels of vascular endothelial growth factor (VEGF) were measured in the different experimental conditions. PD fluid exposure strongly increased the concentration of VEGF in the peritoneal cavity, and administration of peptides P17 or P144 significantly reduced the levels of this factor (Figure 3C). A correlation between
vessel formation and the production of VEGF was observed, reinforcing the notion of the relevance of this growth factor in peritoneal angiogenesis (Figure 3D).

To analyze the functional relevance of the observed morphologic changes of the peritoneum, a peritoneal ultrafiltration test was performed on the last day of treatments. Mice of the different groups were instilled with 2 ml of PD solution, and 30 minutes later the total peritoneal volumes were recovered. As shown in Figure 4A, the volumes recovered from PD fluid-exposed animals were lower than those from saline-treated mice, and a significant increase of the volumes recovered was obtained in mice exposed to PD fluid that were administered peptides P17 or P144. We observed that the loss of ultrafiltration correlated with peritoneal thickness (Figure 4B), angiogenesis (Figure 4C), and with the production of VEGF (Figure 4D). These results demonstrated that the blockade of TGF-β1 ameliorated the deleterious effects of PD fluid on the peritoneum, reducing fibrosis and angiogenesis and ultimately improving peritoneal membrane function.

TGF-β1-Blocking Peptides Reduce the Accumulation of FSP-1+ Fibroblasts in Response to PD

Another characteristic histologic change of the peritoneal membrane during PD is the accumulation of fibroblasts expressing FSP-1 in the submesothelial compact zone.39,46 In the peritoneum from control saline-treated mice, there was no expression of FSP-1. By contrast, FSP-1+ fibroblasts were present in the submesothelial compact region of mice treated with PD fluid (Figure 5, A and B). A remarkable finding was the clustering tendency of FSP-1+ fibroblasts, mainly restricted to the upper submesothelial area, but in some cases, especially those with prominent fibrosis, FSP-1+ fibroblasts also appeared in the deeper compact zone (Figure 5A). PD fluid-instilled mice treated with peptides P17 or P144 showed significant decreased submesothelial accumulation of FSP-1+ fibroblasts (Figure 5, A and B).

TGF-β1-Blocking Peptides Exert a Major Effect on PD Fluid-Induced MMT

To characterize the different subpopulations of pathologic fibroblasts in the injured peritoneum during PD fluid exposure, two-color immunofluorescence analysis was performed using the anti-FSP-1 antibody in conjunction with antibodies against cytokeratin (mesothelial marker), CD45 (pan-leukocyte marker expressed by fibrocytes), or CD31 (endothelial marker). As expected, in the peritoneum from control mice the expression of cytokeratin was exclusively restricted to the preserved mesothelium (Supplemental Figure S1) and the expression of CD31 was confined to deeper vessels located in the muscular tissue (Figure 3 and Supplemental Figure S1). Furthermore, in the control peritoneum there was no expression of FSP-1 (Figure 5 and Supplemental Figure S1) and CD45+ cells were barely detected (Supplemental Figure S1). In the peritoneal tissue of PD fluid-instilled mice there was accumulation of FSP-1+ fibroblasts, and different numbers of these fibroblasts co-expressed cytokeratin (Figure 6, A and B), CD45 (Figure 7, A and B), or CD31 (Figure 8, A and B). The most abundant subpopulations, in terms of number per field, were those co-expressing Cyto+ /FSP-1+ and CD45+/FSP-1+, whereas only a few fibroblasts were CD31+/FSP-1+ (Figure 9A). The average percentages of the different subpopulations of activated fibroblasts were 37% for Cyto+/FSP-1+, 34% for CD45+/FSP-1+, and 5% for CD31+/FSP-1+ (Figure 9B). The remaining 24% were single positive for FSP-1 (Figure 9B).

Administration of peptides P17 or P144 to PD fluid-treated mice significantly reduced the number per field of Cyto+/FSP-1+ (Figure 6, A and B), CD45+/FSP-1+ (Figure 7, A and B), and CD31+/FSP-1+ (Figure 8, A and B). Of note, the number of fibroblasts single positive for FSP-1 did not change upon blocking peptide administration (Figure 9A and Supplemental Figure S2). Proportionally, the most prominent reduction by the blocking peptides was observed in the Cyto+/FSP-1+ subpopulation (Figure 9, A and B), suggesting a major effect of TGF-β1 on the generation of pathologic fibroblasts through the mesenchymal conversion of mesothelial cells.

Cells with Intermediate Phenotype (Cyto+/FSP-1+) Possess Features of Myofibroblasts and Fibrogenic Capacity

To analyze if myofibroblasts may derive from mesothelial cells in the injured peritoneum, a two-color immunofluorescence analysis was performed using the anti-α-SMA and anti-cytokeratin antibodies. Exposure to PD fluid induced the accumulation of Cyto+/α-SMA+ fibroblasts, and administration of peptides P17 or P144 significantly reduced the number of this myofibroblast subpopulation (Supplemental Figure S3). The number per field of Cyto+/α-SMA+ fibroblasts, in each experimental condition, was approximately one half of the Cyto+/FSP-1+ subpopulation (Supplemental Figure S3). By using three-color immunofluorescence analysis, we estimated that approximately 50% of Cyto+/FSP-1+ fibroblasts co-expressed α-SMA (data not shown).

To further demonstrate the pathologic nature of the fibroblasts with intermediate phenotype (Cyto+/FSP-1+), the capacity of these cells to produce extracellular matrix components was explored in cell culture experiments. Kinetic analysis of extracellular matrix component synthesis, during MMT in vitro, demonstrated that the upregulation of fibronectin and collagen I started at 48 hours and remained until the last day (Figure 10A and data not shown). Three-color immunofluorescence analysis of mesothelial cells treated with TGF-β1 for 72 hours showed that almost all fibronectin-expressing cells were Cyto+/FSP-1+ (Figure 10B). TGF-β1-blocking peptide P17 prevented the induction of FSP-1 and fibronectin (Figure 10B). Similarly, triple staining of effluent-derived mesothelial cells with no epitheloid phenotype showed that the most abundant fibronectin-producing cell population was Cyto+/FSP-1+ and only a small proportion of the cells were Cyto+/α-SMA+ (Figure 10C). These results indicated that the cell with intermediate phenotype (Cyto+/FSP-1+) had already acquired fibrogenic capacity.
Overexpression of TGF-β1 Induces the Peritoneal Accumulation of FSP-1+ Fibroblasts, Most of Which Co-Express Cytokeratin

In previous studies, it was shown that TGF-β1 overexpression reproduced the structural and functional alterations of the peritoneum observed in PD patients and that MMT appeared during the early stages (days 4 to 7) of peritoneal damage.33–35 To characterize the different subpopulations of activated fibroblasts in response to TGF-β1 overexpression, adenovirus-mediated gene transfer experiments were carried out. Mice were infected with control adenovirus or adenovirus encoding active TGF-β1 by intraperitoneal injection. Animals were killed on day 4 after infection. Parietal peritoneal sections were subjected to dual staining using anti-FSP-1 antibody in conjunction with antibodies against cytokeratin, CD45 or CD31.

As expected, mice infected with control adenovirus showed normal peritoneal histology with a mesothelial cell monolayer, a thin submesothelial compact zone, and few vessels located in the muscular tissue. Furthermore, the expression of FSP-1 in these control mice was absent and the expression of CD45 was barely detected (Supplemental Figure S4). In contrast, mice infected with adenovirus harboring TGF-β1 showed loss of the mesothelial cell monolayer, a fibroproliferative response, and a strong accumulation of FSP-1+ fibroblasts (Figure 11A). Importantly, the most abundant subpopulation of FSP-1+ fibroblasts was that co-expressing Cyto+/FSP-1+ (Figure 11, A and B), representing approximately 64% of the activated fibroblasts (Figure 11C). There was also a relevant accumulation of CD45+/FSP-1+ cells (Figure 11, A and B), with an average percentage of 29% (Figure 11C), whereas single-positive FSP-1+ and CD31+/FSP-1+ fibroblasts were only marginally represented (Figure 11C). These results further demonstrated that TGF-β1 exerted a major effect on mesothelial cells, which through the MMT process constituted the main source of pathologic fibroblasts in the injured peritoneum.

DISCUSSION

During the last few years it has been speculated that the MMT of mesothelial cells might be a potential target for therapeutic intervention to preserve peritoneal morphology and function.3,9,10 In the complex microenvironment that occurs during PD fluid-induced tissue injury a wide range of cytokines and factors are upregulated, making it difficult to assign priorities or hierarchy for their effects on MMT and on the onset and progression of peritoneal damage.3,10 Nonetheless, TGF-β1 has been proposed to be a key mole-
In this study, we demonstrate that administration of two TGF-β1-blocking peptides to mice exposed to PD fluid significantly decreases peritoneal fibrosis and angiogenesis, partially preserves membrane function, and significantly blocks the accumulation of FSP-1+ and α-SMA+ fibroblasts. We have previously shown that in response to PD, the pathologic fibroblasts may originate from resident fibroblasts and from the mesothelium via MMT.11,12,39 Herein, we provide evidence for the first time that peritoneal FSP-1+ fibroblasts may also derive from bone-marrow-derived circulating cells (CD45+) and from the local conversion of endothelial cells (CD31+) via EnMT. The percentage of mesothelial-cell-derived FSP-1+ fibroblasts, 37%, in the injured peritoneum is in agreement with that found in renal fibrosis, in which approximately 36% come from local conversion of tubular epithelial cells via epithelial-to-mesenchymal transition.22 In contrast, the percentages of FSP-1+ fibroblasts deriving from circulating CD45+ cells and from endothelial cells in the PD-fluid-exposed peritoneum differ sharply from those observed in the fibrotic kidney. Whereas in renal fibrogenesis approximately 14% to 15% of pathologic fibroblasts derive from bone-marrow CD45+ cells22,23 and 30% to 50% from CD31+ endothelial cells,19 in the injured peritoneum we have estimated percentages of 34% and 5% for the CD45+/FSP-1+ and CD31+/FSP-1+ subpopulations, respectively. These divergent observations may suggest that tissue- and organ-specific microenvironments dictate the different proportions of pathologic fibroblast subpopulations. Furthermore, it might be speculated that the contribution of the different fibroblast subpopulations to the peritoneal structural and functional deterioration is not necessarily dependent on their relative abundance. In this sense, we have observed that the most abundant Cyto+/FSP-1+ subpopulation correlates with peritoneal fibrosis better than the other subpopulations (Supplemental Figure S5), whereas the less representative CD31+/FSP-1+ subpopulation shows the strongest correlation with the loss of ultrafiltration (Supplemental Figure S6). The fibroblasts single positive for FSP-1, which represented up to 24%, correlate neither with peritoneal fibrosis nor with the loss of ultrafiltration (Supplemental Figures S5 and S6). The origin of these FSP-1 single-positive cells is not fully clear: They might derive from resident fibroblasts or from other fibroblast subpopulations (e.g., Cyto+/FSP-1+ after losing the expression of cytokeratins). It is worthwhile to note that myofibroblasts are commonly observed in peritoneal biopsies from PD patients.13,14 Nevertheless, the heterogeneous origin of these myofibroblasts and their roles in the setting and progression of different features related with peritoneal deterioration have not been explored in depth. Only a few studies have been carried out with PD patients, which have shown significant correlations among high peritoneal transport rates and the presence of MMT-derived fibroblasts in the effluents or in peritoneal tissue specimens.15–18

TGF-β1 is a strong inducer of MMT and EnMT3,11,13,16,20,21 and appears to be a key factor for the myofibroblastic differentiation of resident fibroblasts and recruited fibrocytes.23,24,29 In this study, we show that administration of two different TGF-β1-blocking peptides to PD fluid-treated mice significantly reduced the number of fibroblasts of all subpopulations except of those being FSP-1 single positive. One important finding is that the most prominent reduction by the blocking peptides is exerted on the Cyto+/FSP-1+ subpopulation, suggesting that the MMT process is tightly regulated by TGF-β1 levels in the

**Figure 4.** - TGF-β1-blocking peptide P17 or P144 treatments improve peritoneal ultrafiltration. A 30-minute ultrafiltration test was performed on the last day of treatments. (A) The volumes recovered from animals exposed to PD fluid (PDF; n = 10) are lower than those from mice instilled with saline solution (Control; n = 7), and an increase of net ultrafiltration is obtained in mice exposed to PD fluid that were administered P17 (PDF + P17; n = 11) or P144 (PDF + P144; n = 11). Box plots represent 25th and 75th percentiles and median, minimum, and maximum values. Numbers above boxes depict mean ± SEM. Symbols represent the statistical differences between groups. (B to D) Correlations between ultrafiltration and peritoneal thickness (μm), angiogenesis (CD31+ staining), and VEGF levels (pg/ml) in the whole group of mice (Spearman regression, n = 39).
context of PD fluid exposure. This hypothesis is reinforced by the fact that TGF-β1 overexpression results in a stronger accumulation of Cyto-1+ fibroblasts than any other subpopulation of fibroblasts; however, it cannot be ruled out that this effect could be due to a better accessibility of mesothelial cells to TGF-β1 than any other peritoneal cell. Furthermore, in vitro cell culture experiments demonstrate that treatment of mesothelial cells with blocking peptide prevents the MMT induced by TGF-β1 and by PD effluents.

We are aware that agents directly blocking TGF-β1 cannot be easily used in the clinical practice of PD, at least for long-

Figure 5. TGF-β1-blocking peptides reduce the number of FSP-1+ fibroblasts in the compact zone of the parietal peritoneum. (A) Immunofluorescence microscopy analysis of parietal peritoneal tissue sections stained for FSP-1 (red) with 4,6-diamidino-2-phenylindole (DAPI) counterstaining shows accumulation of FSP-1+ fibroblasts in the submesothelial space in the PDF group (n = 10), and the administration of P17 or P144 peptides reduces the number of FSP-1+ cells per field (PDF+P17, n = 11; PDF+P144, n = 11). The expression of FSP-1 is absent in animals instilled with saline (Control, n = 7). Representative pictures are presented. Magnification: ×200. (B) The reductions in the number of FSP-1+ fibroblasts by P17 or P144 peptides are significant. Box plots represent 25th and 75th percentiles and median, minimum, and maximum values. Numbers above boxes depict mean ± SEM. Symbols show statistical differences between groups.

Figure 6. TGF-β1-blocking peptides reduce the number of fibroblasts derived from mesothelial cells. (A) Immunofluorescence microscopy analysis of parietal peritoneal sections stained for cytokeratin (green) and FSP-1 (red) with DAPI counterstaining shows accumulation of transdifferentiated mesothelial cells in the submesothelial space (cytokeratin positive cells) in the PDF group (n = 10), some of which co-express FSP-1 (yellow cells in the Merge panel). The administration of P17 or P144 peptides reduces the number of cytokeratin/FSP-1 double-positive cells per field (PDF+P17, n = 11; PDF+P144, n = 11). Representative slides are presented. Magnification: ×200. Arrows show examples of double-positive cells. (B) The reductions in the number of cytokeratin/FSP-1-positive fibroblasts by P17 or P144 peptides are significant. Box plots represent 25th and 75th percentiles and median, minimum, and maximum values. Numbers above boxes depict mean ± SEM. Symbols show statistical differences between groups.
term treatments, because TGF-β1 has important modulating functions of the immune and inflammatory responses. It can be expected that further molecular studies of the TGF-β1 signaling pathways involved in the mesenchymal conversion of mesothelial cells will provide more specific strategies for the preservation of the peritoneal membrane with minimum side effects.

**CONCISE METHODS**

**Peptides and Adenoviral Vectors**

P17 is a small 15-mer peptide (KRIWFIPRSSWYERA) that was identified to bind TGF-β1 using a phage-displayed peptide library and demonstrated to block the activity of TGF-β1 in vitro and in vivo.42 P144 is a synthetic 14-mer peptide designed from the TGF-β1 type III receptor (TSLDASIIWAMMQN) that has been shown to bind TGF-β1 and to inhibit its activity in vitro and in vivo.43,44 As a control,
we used the irrelevant 14-mer peptide SVSRARPRLLLLGL designed from the NS5B protein of hepatitis C virus genotype 2a (amino acids 3005 to 3018). P17, P144, and control peptides were synthesized by the F-moc solid-phase method as described previously. The purity of peptides was at least 95% as judged by HPLC. The adenoviral vector expressing active TGF-$\beta_1$ was kindly provided by David Dichek, University of Washington, Seattle) and has been previously described.

Culture of Mesothelial Cells and Treatments

Mesothelial cells were obtained from omentum samples and from PD effluents using the methods described previously. Cells were cultured in Earle’s M199 medium supplemented with 20% fetal calf serum, 50 U/ml penicillin, 50 $\mu$g/ml streptomycin, and 2% Biogro-2 (Biologic Industries, Israel). The purity of effluent and omentum-derived mesothelial cell cultures was determined by the expression of the standard mesothelial markers intercellular adhesion molecule-1, cytokeratins, and calretinin. The study presented here is adjusted to the Declaration of Helsinki and was approved by the Ethics Committee of the Hospital Universitario de la Princesa (Madrid, Spain). Written consent was obtained from the PD patients included in this study to use effluent samples. Verbal informed consent was obtained from omentum donors that submitted to elective surgeries.

To induce MMT in vitro, omentum-derived mesothelial cells were seeded on wells coated with 50 $\mu$g/ml of collagen I (Roche Boehringer GmbH, Mannheim, Germany) and treated for different time periods (6 hours to 6 days) with human-recombinant TGF-$\beta_1$ (1 ng/ml; R&D Systems, Inc., Minneapolis, MN) or with effluents from PD patients suffering episodes of peritonitis diluted one half with culture medium. These treatments are proven to be good in vitro models of MMT. When indicated, TGF-$\beta_1$-blocking peptide P17 was used at a final concentration of 150 $\mu$g/ml. This dose of P17 was similar to that used in previous studies.

For quantitative RT-PCR analysis, mesothelial cells were lysed in TRI reagent (Ambion, Inc., Austin, TX), and RNA was extracted as per fabricant instructions. Complementary DNA was synthesized from 2 $\mu$g of total RNA by reverse transcription (RNA PCR Core Kit, Applied Biosystems, Inc.). Real time-PCR was carried out in a Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) using a SYBR Green Kit (Roche Diagnostics GmbH) and specific primers sets for Snail, E-cadherin, collagen I, and fibronectin (Supplemental Table S1). Primers specific for histone H3 were used as a control.

Mesothelial cells were stained for immunofluorescence analysis with antibodies against phosphorylated-Smad 2/3 (Santa Cruz Biotechnology, Santa Cruz, CA), pan-cytokeratin (Sigma-Aldrich, St Louis, MO), FSP-1 (Dako, Glostrup, Denmark), and fibronectin (Novus Biologicals, Littleton, CO). The cells were fixed in 4% formaldehyde in PBS and blocked with 10% horse serum in PBS with 0.3% Triton X-100. Cells and antibodies were first incubated in PBS with 0.1% Triton X-100 overnight at 4°C. After three washing steps (5 minutes in PBS with 0.1% Triton X-100), secondary Alexa-labeled antibodies were incubated with cells for 90 minutes at room temperature. Negative controls for immunofluorescence staining were con-

Figure 9. Relative effects of TGF-$\beta_1$-blocking peptides on the different subpopulations of fibroblasts. (A) Box plot graphics represent 25th and 75th percentiles and median, minimum, and maximum of the numbers of pathologic fibroblasts from different origin in the PDF, PDF + P17, and PDF + P144 groups. (B) Circles depict the average percentages of the different subpopulations of fibroblasts in the PDF, PDF + P17, and PDF + P144 groups.
ducted using 10% rabbit serum instead of primary antibody. Images were analyzed by computerized digital image analysis (AnalySIS, Soft Imaging System).

**PD Fluid Exposure Model in Mice**

A total of 44 female C57BL/6 mice between 12 and 16 weeks of age were used in this study (Harlan Interfauna Iberica, Barcelona, Spain). The experimental protocol used 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 the Hospital Universitario la Paz. PD fluid or saline solution were instilled via a peritoneal catheter connected to an implanted subcutaneous mini access port (Access Technologies, Skokie, IL) as described previously.\textsuperscript{16,46,50}

During the first week after surgery, the animals implanted with a peritoneal access port ($n = 44$) received 0.2 ml of saline with 1 IU/ml of heparin to facilitate wound healing. Thereafter, during a 5-week period, 8 mice were daily instilled with 1.5 ml of saline solution (control; $n = 8$), 12 mice were daily instilled with 1.5 ml of standard PD fluid composed of 4.25% glucose and buffered with lactate (Stay Safe, Fresenius, Bad Homburg, Germany) and treated with control peptide (4 mg/kg per day; PDF group), 12 mice were daily instilled with 1.5 ml of standard PD fluid and treated with P17 (4 mg/kg per day; PDF+P17 group), and 12 mice were daily instilled with 1.5 ml of standard PD fluid and treated with P144 (4 mg/kg per day; PDF+P144 group). The doses of P17 and P144 used in this study were similar to those used previously.\textsuperscript{42–44,51} Two animals of the PDF group and one in each of the others groups were not used in the final analysis, with the main causes of dropouts being catheter port infection or traumatic catheter removal (control group, $n = 7$; PDF group, $n = 10$; PDF+P17 group, $n = 11$; PDF+P144 group, $n = 11$). A peritoneal equilibrium test was performed during the last day of treatments. All of the mice were instilled with 2 ml of PD solution, and animals were anesthetized with isoflurane (MTC Pharmaceuticals, Cambridge, Ontario, Canada) 30 minutes later and sacrificed to recover the total peritoneal volumes. Parietal peritoneum samples were obtained from the contralateral side of the implanted catheter. Some of these samples (control group, $n = 5$; PDF group, $n = 6$; PDF+P17, $n = 7$; PDF+P144, $n = 7$) were used for peritoneal RNA extraction. Food and water were provided \textit{ad libitum} to the animals during the experiment.

**Overexpression of TGF-β1 in the Peritoneum by Adenovirus-Mediated Gene Transfer**

Eight female C57BL/6 mice (12 and 16 weeks old) were used in this experiment (Harlan Interfauna Iberica). The experimental protocol used 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 the Hospital Universitario la Paz. PD fluid or saline solution were instilled via a peritoneal catheter connected to an implanted subcutaneous mini access port (Access Technologies, Skokie, IL) as described previously.\textsuperscript{16,46,50}

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\caption{Cells with intermediate phenotype (Cyto\textsuperscript{+}/FSP-1\textsuperscript{−}) have fibrogenic capacity. (A) Quantitative RT-PCR shows that fibronectin is upregulated in mesothelial cells stimulated with TGF-β1 after 48 hours of treatment. (B) Immunofluorescence microscopy shows that almost all mesothelial cells treated with TGF-β1 for 72 hours are positive for cytokeratin (green), FSP-1 (red), and fibronectin (blue). Control cells and cells co-treated with TGF-β1 and peptide P17 are only positive for cytokeratin. (C) Triple immunofluorescence analysis of effluent-derived mesothelial cells with no epithelioid phenotype shows that the most abundant fibronectin-producing cells are positive for cytokeratin and FSP-1. However, a small proportion of fibronectin-producing FSP-1\textsuperscript{−} cells are negative for cytokeratin (arrow in Merge panel). Magnification: ×400.}
\end{figure}
Morphologic Analysis of Peritoneal Samples, Quantitative PCR, and Effluent Growth Factor Measurements

For histologic analyses, specimens of the parietal peritoneum were obtained from the contralateral side to the tip of the implanted catheter. Cryostat sections (5 μm) were cut and stained with Masson’s trichrome (Merck, Darmstadt, Germany) to quantify fibrosis. The thickness of submesothelial tissue was determined by blinded microscope analysis using a metric ocular and was expressed as the mean of ten independent measurements for each animal. For quantitative RT-PCR analysis of peritoneal mesenchymal markers, frozen peritoneal tissues were homogenized for total RNA extraction in TRI reagent using a Polytron homogenizer. Complementary DNA was synthesized from 2 μg of total RNA by reverse transcription. Real-time PCR was carried out in a Light Cycler 480 using a SYBR Green Kit (Roche Diagnostics GmbH) and specific primers sets for α-SMA, collagen I, and fibronectin (Supplemental Table S1). Primers specific for 18S RNA were used as a control.

Frozen sections were stained for immunofluorescence analysis with antibodies to visualize vasculature (CD31; Serotec, Oxford, United Kingdom), mesothelial cells (pan-cytokeratin; Sigma-Aldrich), bone-marrow-derived cells (CD45; BD Biosciences Franklin Lakes, NJ), and pathologic fibroblasts (FSP-1; Dako or α-SMA; Abcam, Inc., Cambridge, United Kingdom). The frozen sections were fixed for 15 minutes in 4% formaldehyde in PBS, and blocked with 10% horse serum for 60 minutes in PBS with 0.3% Triton X-100. After three washing steps, secondary Alexa-labeled antibodies and sections were incubated for 90 minutes at room temperature. After another washing process, the preparations were mounted with a 4,6-diamidino-2-phenylindole (DAPI) nuclear stain (Vectashield; Vector Laboratories). Negative controls for immunofluorescence staining were conducted using 10% rabbit serum instead of primary antibody. Images were analyzed by computerized digital image analysis (AnalySIS, Soft Imaging System). The number of cells with single- or double-positive staining was counted and was expressed as the mean of ten independent measurements for each animal. The amounts of VEGF and TGF-β1 in the peritoneal effluents were determined by ELISA-based assays according to the manufacturers instructions (PeproTech, Rocky Hill, NJ; Invitrogen Corporation; Carlsbad, CA).

Figure 11. Overexpression of TGF-β1 in the peritoneum induces the accumulation of FSP-1+ fibroblasts, most of which derive from mesothelial cells. Mice were infected with adenovirus encoding active TGF-β1 by intraperitoneal injection, and animals were killed on day 4 after infection. (A) Parietal peritoneal sections were subjected to dual immunofluorescence analysis using anti-FSP-1 antibody (red) in conjunction with antibodies against cytokeratin, CD45, or CD31 (green). A strong accumulation of cytokeratin/FSP-1 double-positive fibroblasts is observed (yellow cells in the Merge panel). Representative slides are presented. Magnification: ×200. (B) Box plot graphics represent 25th and 75th percentiles and median, minimum, and maximum of the numbers of fibroblasts from different subpopulations. (C) Circle depicts the average percentages of the different subpopulations of fibroblasts.

for Care and Use of Laboratory Animals and was approved by the Animal Ethics Committee of the Unidad de Cirugía Experimental of the Hospital Universitario la Paz. Mice were injected intraperitoneally with 100 μl of suspensions containing 1 × 10⁶ plaque-forming units of an adenovirus expressing active TGF-β1 (n = 5) or 1 × 10⁷ plaque-forming units of the empty adenoviral vector that served as a control (n = 3). Food and water were provided ad libitum. Four days after the injection, animals were anesthetized with isoflurane (MTC Pharmaceuticals) and sacrificed. The parietal peritoneum of all animals was collected for histologic and immunofluorescence analysis.
Statistical Analysis
Results are presented as 25th and 75th percentiles; median, minimum, and maximum values in box plot graphics; and as mean ± SEM in bar graphics. In Figures 9 and 11 the percentages were calculated using the median of each fibroblast subpopulation. The data groups were compared with the nonparametric Mann–Whitney rank sum U-test and linear correlations were determined by Spearman regression using the SPSS statistic package version 14.5 (Chicago, IL) and GraphPad Prism version 4.0 (La Jolla, CA). P < 0.05 was considered statistically significant.

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DISCLOSURES
Javier Dotor is an employee of Digna Biotech. Francisco Borras-Cuesta is an adviser of Digna Biotech.

REFERENCES

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