The most serious complication of peritoneal dialysis is encapsulating peritoneal sclerosis (EPS). The prolonged inflammatory stimuli, fibrogenic cytokine overexpression, and angiogenesis that underlie EPS ultimately result in increased production of fibrous tissue, encapsulating the bowel loops.

In recent years, inhibitors of mammalian target of rapamycin (mTOR) as an alternative agent for calcineurin inhibitor toxicity have been widely used in organ transplantation. These agents have also been used since the 1990s in endovascular medicine for drug-eluting stents because of antiproliferative effects on vascular smooth muscle cells and potent anti-inflammatory properties by direct action on human immune cells.

Because of the shared characteristics of EPS and other fibrotic processes, we hypothesized that everolimus, an mTOR inhibitor, can reverse the process responsible for the eventual development of EPS.

We allocated 32 non-uremic albino Wistar rats to 4 groups: control group, 2 mL isotonic saline injected intraperitoneally (IP) daily for 3 weeks; CG group, 2 mL/200 g (0.1%) chlorhexidine gluconate (CG) injected IP daily and ethanol (15%) dissolved in saline, for 3 weeks; resting group, CG (weeks 0 – 3), plus peritoneal rest (weeks 3 – 6); and Evo-R, CG (weeks 0 – 3), plus 0.3 mg/L everolimus in drinking water (weeks 3 – 6).

At the end of the study, we performed a 1-hour peritoneal equilibration test with 25 mL 3.86% PD solution, and examined the dialysate-to-plasma ratio of urea (D/P urea), dialysate white blood cell count, ultrafiltration (UF) volume, and morphologic change in the parietal peritoneum.

Exposure to CG for 3 weeks resulted in alterations in peritoneal transport (increased D/P urea, decreased UF volume, \( p < 0.05 \)) and morphology (increased inflammation, neovascularization, fibrosis, and peritoneal thickness, \( p < 0.05 \)). Peritoneal rest has some beneficial effect only on UF failure and dialysate cell count (\( p < 0.05 \)). However, everolimus was more effective than peritoneal rest with regard to vascularity and peritoneal thickness (\( p < 0.05 \)).

Everolimus has beneficial effects on UF failure, inflammation, and fibrosis. Everolimus may have therapeutic value in the management of EPS.

Key words
Encapsulating peritoneal sclerosis, everolimus, fibrosis, rat model

Introduction
Encapsulating peritoneal sclerosis (EPS), which was first described by Gandhi et al. (1), is the most dangerous complication of peritoneal dialysis (PD). Despite its low rates of incidence and prevalence, EPS carries a high mortality risk. This inflammatory process affects the visceral peritoneal membrane diffusely and transforms it into a dense fibrous surrounding sheath. Duration of PD, peritonitis attacks, and use of bioincompatible PD fluids are the major determinants of EPS.

The biologically active peritoneal compartment is capable of almost all immunologic reactions under various stimuli, including antigen presentation, memory–effector T lymphocyte production, mononuclear cell burst, and ultimately, fibrotic process (2,3). Peritoneal mesothelial cells and immune cells of the peritoneal cavity orchestrate the entire process. An increased inflammatory response and proliferation of mesothelial cells with epithelial-to-mesenchymal
transition underlie the pathophysiology of EPS (4). Under various stimuli, mesenchymal transformation of epithelial cells in various tissues, through proliferation and inflammation responses, are responsible for tissue-specific fibrotic processes such as cardiac fibrosis and tubulointerstitial fibrosis of kidney (5,6).

Everolimus—and related compounds such as rapamycin—is a macrolide immunosuppressive agent that inhibits the activity of mammalian target of rapamycin (mTOR), a cellular enzyme that plays a key role in cell growth and proliferation. It has been used in organ transplantation since 1989 (7,8). Inhibitors of mTOR block the T-cell cycle at the late G1 phase by inhibiting the proliferative signals of T-cell growth factor—namely, interleukin-2 (9). Rapamycin derivatives inhibit growth factor–stimulated proliferation in vascular smooth muscle cells (10), in liver fibrosis (11), and in pulmonary fibrosis (12) by inhibiting mesenchymal cell proliferation. A recent randomized trial showed that everolimus-eluting stents in de novo coronary lesions (13) are more effective than are metallic stents at preventing in-stent restenosis.

The present study demonstrates beneficial effects of everolimus, an mTOR inhibitor, in an “EPS regression” rat model.

Materials and methods

We used 24 nonuremic albino female Wistar rats at 8 weeks of age for the present study, according to the guidelines of the National Research Council. Rats were housed in polycarbonate cages maintained at 24°C with 12-hour light–dark cycles and were fed a standard laboratory diet and given free access to water. The Animal Ethics Committee of Ege University Hospital approved the study design.

To produce an EPS model, we intraperitoneally (IP) infused rats with 0.1% chlorhexidine gluconate (CG) and 15% ethanol dissolved in injection saline as previously described (14). It has been reported that CG injection IP for 3 weeks is sufficient to induce marked epithelial cell proliferation, fibroblastic activity, and neovascularization, and accumulation of CD34+ cells in peritoneum (15). Based on this finding, we decided on a 3-week period of treatment of IP CG injection.

We allocated the 24 rats to 4 groups: control group \((n = 8)\), daily IP injection with 2 mL isotonic saline for 3 weeks; CG group \((n = 8)\), daily IP injection with 2 mL of 200 g chlorhexidine gluconate (CG) for 3 weeks; resting group \((n = 8)\), daily IP injection with CG (weeks 0 – 3), plus peritoneal rest (weeks 4 – 6); Evo-R (regression) group \((n = 8)\), daily IP injection with CG (weeks 0 – 3), plus 0.3 mg/L everolimus [Certican: Novartis, Basel, Switzerland] in drinking water (weeks 4 – 6). The daily dose of everolimus was 0.3 mg/kg, similar to the dose per kilogram used in kidney transplant recipients.

At the end of the study, we performed a 1-hour peritoneal equilibration test with 25 mL 3.86% PD solution (Dianeal: Eczacibasi–Baxter Healthcare, Istanbul, Turkey). After 1 hour, rats underwent ketamine HCl anesthesia (60 mL/kg body weight), and blood samples were immediately collected by direct cardiac puncture. Dialysate samples were obtained by midline abdominal incision, with insertion of a short dialysis catheter to prevent dialysate leakage.

Blood and dialysate urea were measured using an enzymatic kinetic method (Randox Laboratories, San Francisco, CA, U.S.A.). Glucose concentration was determined using a glucose oxidase method. We calculated dialysate-to-plasma ratio \((D/P)\) of urea and end-dialysate-to-initial-dialysate concentration \((D_1/D_0)\) of glucose. Net ultrafiltration \((UF)\) was calculated as the difference between the instilled and the drained dialysate volumes. Dialysate cell count is expressed as white blood cell (WBC) count per cubic milliliter of dialysate.

In the histology examination, samples of the anterior abdominal wall were fixed in 4% buffered formalin solution at room temperature, processed routinely, and then embedded in paraffin. Sections of 4 μm were stained with hematoxylin and eosin and Masson trichrome for light microscopy examination.

All slides were blindly examined by the same pathologist. The microscopic images were recorded at 200× magnification. Number and reactivity of mesothelial cells, the presence of inflammation and mast cells, fibroblastic activity and fibrosis, vascularization, and peritoneal thickness were evaluated and semi-quantitatively scored. The semi-quantitative scoring for inflammation, fibroblastic activity, and neovascularization was based on counts of mononuclear cells, fibroblasts, and capillaries at high-power magnification (score: 0–3). Fibrosis was also scored based on evaluation of edema and collagen density. The number of mesothelial cells was expressed as decreased, normal, and increased. Mesothelial cells were classified normal (flat cells) or reactive (cubic transformation of flat cells). Fibrosis was evaluated as early (edema and a few lacy collagens),
middle (lacy and mature collagen), and late (mature collagen fibrils).

Parietal peritoneal surfaces were evaluated by morphometry. “Peritoneal thickness” was defined as the thickness of the submesothelial zone measured from the inner surface of abdominal muscle to the mesothelium (peritoneal cavity) as previously described (16). The “submesothelial area”—an area from the abdominal muscular surface to the peritoneal cavity—was also analyzed. We used the Axiovision LE 4.5 Imaging System (demo version: Carl Zeiss, Jena, Germany) to analyze the images, evaluating at least a 450 μm length of parietal peritoneum and submesothelial zone. All sectioned blood vessels within the compact zone were counted.

Results are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using analysis of variance, unpaired t-tests, and Mann–Whitney tests. A p value of less than 0.05 was considered significant.

Results

Table I summarizes the results.

Functional parameters

Our experimental model of EPS induced by IP CG injection severely disturbed peritoneal functions. In the CG group, UF capacity, D/P urea, and D1/D0 glucose were much decreased as compared with those parameters in the control group (–6.4 ± 1.5 mL vs. 8.4 ± 0.7 mL, 0.92 ± 0.01 vs. 0.57 ± 0.06, and 0.16 ± 0.03 vs. 0.45 ± 0.04 respectively; all p < 0.05). Although peritoneal rest had some beneficial effects on functional parameters as compared with those parameters in the CG group, only the improvement in D/P urea reached statistical significance (0.80 ± 0.04 vs. 0.92 ± 0.01, p < 0.05). Although UF capacity was restored with the use of everolimus (0.43 ± 1.1 mL vs. –6.1 ± 0.8 mL, p < 0.05), D/P urea and D1/D0 glucose did not improve with everolimus as compared with peritoneal rest (0.86 ± 0.03 vs. 0.80 ± 0.04 and 0.24 ± 0.03 vs. 0.28 ± 0.05 respectively, both p > 0.05).

Morphologic parameters

As seen in Table I, injection with CG increased peritoneal thickness (134 ± 10 μm vs. 26 ± 5 μm, p < 0.05), inflammation (1.5 ± 0.1 vs. 1.0 ± 0.0, p < 0.05), vascularity (7.7 ± 1.2 vessels vs. 4.5 ± 2.0 vessels, p < 0.05), and fibroblastic activity (1.6 ± 0.1 vs. 0.5 ± 0.5, p < 0.05) as compared with values in the control group. Rest not only had no effect on morphologic parameters, it even resulted in an increase in thickness (225 ± 21 μm vs. 134 ± 10 μm, p < 0.05), inflammation (1.9 ± 0.2 vs. 1.5 ± 0.1, p < 0.05), vascularity (20.4 ± 4.2 vessels vs. 7.7 ± 1.2 vessels, p < 0.05), and fibroblastic activity (2.7 ± 0.2 vs. 1.6 ± 0.1, p < 0.05) as compared with values in the CG group. As compared with peritoneal rest, use of everolimus significantly improved peritoneal thickness (129 ± 11 μm vs. 225 ± 21 μm, p < 0.05) and vascularity (6.14 ± 1.6 vessels vs. 20.4 ± 4.2 vessels, p < 0.05). Reduced inflammation in the peritoneum was insignificant with everolimus (1.28 ± 0.18 vs. 1.9 ± 0.2, p > 0.05), and fibroblastic activity did not change (2.7 ± 0.18 vs. 2.7 ± 0.2, p > 0.05).

Table I

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>CG (n=8)</th>
<th>Resting (n=8)</th>
<th>Evo-R (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrafiltration (mL)</td>
<td>8.4±0.7</td>
<td>–6.4±1.5b</td>
<td>–6.1±0.8b</td>
<td>0.43±1.1b</td>
</tr>
<tr>
<td>D/P urea</td>
<td>0.57±0.06</td>
<td>0.92±0.01b</td>
<td>0.80±0.04b</td>
<td>0.86±0.03b</td>
</tr>
<tr>
<td>D1/D0 glucose</td>
<td>0.45±0.04</td>
<td>0.16±0.03b</td>
<td>0.28±0.05</td>
<td>0.24±0.03b</td>
</tr>
<tr>
<td>Peritoneal thickness (μm)</td>
<td>2.6±5</td>
<td>134±10b</td>
<td>225±21b</td>
<td>129±11b</td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.0±0.0</td>
<td>1.5±0.1</td>
<td>1.9±0.2</td>
<td>1.28±0.18</td>
</tr>
<tr>
<td>Vessels (n)</td>
<td>4.5±2.0</td>
<td>7.7±1.2</td>
<td>20.4±4.2b</td>
<td>6.14±1.6d</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>0.5±0.5</td>
<td>1.6±0.1</td>
<td>2.7±0.2b</td>
<td>2.7±0.18b</td>
</tr>
</tbody>
</table>

a All results given as mean ± standard error of the mean.
b p < 0.05 vs. Control.
c p < 0.05 vs. CG.
d p < 0.05 vs. Resting.

CG = chlorhexidine gluconate; Evo-R = everolimus for regression of encapsulating peritoneal sclerosis; D/P = dialysate-to-plasma ratio; D1/D0 = end-dialysate-to-initial-dialysate concentration.
Figures 1 and 2 show macroscopic and microscopic morphologic changes respectively. In Figure 1, diffuse thickening over the peritoneal layer that encloses the abdominal organs are clearly visible. Figure 2 shows decreased thickness, cellularity, and new vessel formation in the peritoneum of animals receiving everolimus therapy.

Discussion

Mesothelial cells play a pivotal role in the biologically active peritoneal compartment. They are specialized cells capable of producing almost all inflammatory cytokines, growth factors, and chemokines under physiologic or pathologic conditions (2,3,17,18). Various stimuli from either bioincompatible PD fluids or peritonitis cause inflammation and result in peritoneal fibrosis (19). Pathologic changes in the peritoneal membrane with long-term PD are characterized by a decrease in, or loss of, mesothelial cells, by epithelial-to-mesenchymal transition, by enlargement of the submesothelial compact zone because of inflammation, and by interstitial fibrosis accompanied by accumulation of collagen and neovascularization.

In our study, 3 weeks of intraperitoneal CG injection resulted in loss of UF capacity, increased peritoneal thickness and inflammation, damage to mesothelial cell integrity, and ultimately, EPS. Peritoneal rest had advantages only in levels of D/P urea and D1/D0 glucose, and no effect on any functional and morphologic parameters of the peritoneum. We also showed that, after exposure to CG, peritoneal UF capacity, thickness, inflammation, and vascularity were better restored with everolimus than with peritoneal rest.

As shown with other therapeutic approaches to the management of EPS with somatostatin, colchicine, and tamoxifen, peritoneal rest may have no benefits in EPS. If we consider that stimulation of the peritoneum into fibrosis is an irreversible process (20), then taking preventive measures in the early EPS period would be meaningful. It is also important to note that, in our study, inflammation, thickness, and vascularity continued to increase during peritoneal rest, indicating that the peritoneum was still active in this period.

More recently, Fieren et al. reported that the incidence of EPS was increased in two centers in Holland after 2004 and that most cases were seen after kidney transplantation (21). They suggested that transplantation-related factors may have played an important role in EPS development. They noted that several immunosuppressive agents, such as the calcineurin inhibitors cyclosporine and especially tacrolimus, have profibrotic properties (22). On the other hand, corticosteroids may have beneficial effect on EPS that occurs post kidney transplantation (23). A trend to decreasing the corticosteroid dose and using calcineurin inhibitors may play a role in the development of EPS. Our results suggest that using everolimus
in the immunosuppressive protocol of kidney transplant patients who come from PD may prevent development of EPS.

Reduced inflammatory activity and neo-angiogenesis (as shown in Table I and Figure 2) may ultimately lead to less thickness and increased UF capacity. However fibroblastic activity of peritoneum did not change during the everolimus treatment period. This finding reflect the severity of our experimental peritonitis model, which is also a possible explanation for the lack of beneficial effects of the short period of peritoneal rest in this model.
Regulation by mTOR protein of lipopolysaccharide- and interferon γ–induced inflammatory gene transcription (24) may underlie the lessened inflammatory activity of peritoneum seen in the everolimus group. The ultimate result of less neoangiogenesis was seen as less vascularity in the everolimus group of our experimental model. Less peritoneal thickness in the everolimus group may be a result of the effects of this agent in preventing differentiation of fibroblasts to myofibroblasts (the way that fibroblasts control fibrogenesis) (12,25).

Conclusions
Everolimus, an mTOR inhibitor, can restore functional and morphologic derangements of peritoneum better than resting can. Studies in humans should be undertaken to find out if everolimus use after transplantation may prevent the dangerous complication of EPS in PD patients.

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