Mineralocorticoid receptor blockade ameliorates peritoneal fibrosis in new rat peritonitis model

Hayato Nishimura,1 Yasuhiko Ito,1 Masashi Mizuno,1 Akio Tanaka,2 Yoshiki Morita,1 Shoichi Maruyama,1 Yukio Yuzawa,1 and Seiichi Matsuo1

1Department of Nephrology and Renal Replacement Therapy, Nagoya University Graduate School of Medicine, and 2Department of Clinical Pharmacology, Chubu Rosai Hospital, Nagoya, Japan

Submitted 27 November 2007; accepted in final form 12 March 2008

LONG-TERM PERITONEAL DIALYSIS (PD) is accompanied by functional and histopathological alterations in the peritoneum (6, 10, 34). The characteristic feature of chronic peritoneal damage in PD is decreased ultrafiltration capacity associated with submesothelial fibrosis, accumulation of extracellular matrix, and neoangiogenesis (57). The decrease in ultrafiltration capacity after prolonged PD is one of the important reasons for its discontinuation (24). The pathogenesis of peritoneal fibrosis (PF) is attributed to a combination of bioincompatible factors in the dialysate, including high glucose (6), high osmolality, advanced glycation products (56), glucose degradation product in the dialysate, including high glucose (6), high osmolality, and neoangiogenesis (57). The decrease in ultrafiltration capacity associated with PD is decreased ultrafiltration capacity associated with PF. We developed a new model of PF in rats based on mechanical scraping of the peritoneum. This model is characterized by acute-phase inflammation (neutrophil and macrophage infiltration on days 0–3) and late-phase PF (α-smooth muscle actin-positive fibroblast infiltration, type III collagen accumulation, and neoangiogenesis on days 7–14). Peritoneal thickening peaked on day 14. MR was expressed in rat peritoneum and a rat fibroblast cell line. Expression of its effector kinase [serum- and glucocorticoid-induced kinase-1 (Sgk1)], transforming growth factor-β (TGF-β), plasminogen activator inhibitor-1 (PAI-1), and CD31-positive vessels increased during the course of PF. Rats were treated with spironolactone, angiotensin receptor blockade (ARB), or angiotensin-converting enzyme inhibitor (ACEI)-ARB-spiroloactone starting at 6 h after peritoneal scraping. All parameters, including peritoneal thickening, number of macrophages and CD31-positive vessels, and expression of monocyte chemotactic protein-1, TGF-β, PAI-1, and Sgk1, were significantly suppressed by spironolactone (10 mg·kg⁻¹·day⁻¹). The effects of spironolactone (10 and 20 mg·kg⁻¹·day⁻¹) were very similar to those of triple blockade. ARB, but not ACEI, significantly reduced peritoneal thickening. Furthermore, peritoneal function assessed by peritoneal equilibration test was significantly improved by spironolactone. Our results suggest that MR is a potential target to prevent inflammation-induced PF in patients on peritoneal dialysis.

serum- and glucocorticoid-induced kinase-1; renin-angiotensin-aldosterone system; ultrafiltration failure; neoangiogenesis

MATERIALS AND METHODS

Animals and Experimental Design

All animal studies were approved by the Animal Experimentation Committee of Nagoya University Graduate School of Medicine (approval number 19250, Nagoya, Japan). Sprague-Dawley rats (Chubu Kagaku Shizai, Nagoya, Japan; 7 wk old, 210–230 g initial body wt) were acclimatized for ~1 wk before any treatments. Animals were maintained under conventional laboratory conditions and given free access to water and food. At the beginning of the experiments, rats...
were incised at the abdominal midline under anesthesia with pentobarbital sodium, and the right parietal peritonea were mechanically scraped twice per second for 60 s with the top of 15-mL centrifuge tubes. Direction of scraping was changed vertically or horizontally every 5 s. After scraping, the abdominal incision was sutured. After surgery, rats had free access to 1% NaCl in tap water.

**Experiment 1.** For determination of the general characteristics of this model, rats were killed after 6 h and on days 1, 3, 7, and 14 (n = 4 at each time point). Before they were killed, the animals were anesthetized with pentobarbital sodium, and samples were obtained from the parietal peritonea. Parietal peritoneum was harvested for light and electron microscopy and immunohistochemistry for cytoketan, ED-1, α-smooth muscle actin (α-SMA), type III collagen, ED-1, and CD31. Thickness of the peritoneum and immune-activator inhibitor-1 (PAI-1). Peritoneum of the left side, which was not scraped, was used as control.

**Experiment 2A.** Rats were randomly assigned to seven groups after scraping and orally treated daily starting at 6 h after scraping: vehicle (group I, n = 12); MR blockade with spironolactone at 5 mg kg\(^{-1}\) day\(^{-1}\) (group II, n = 5), 10 mg kg\(^{-1}\) day\(^{-1}\) (group III, n = 12), or 20 mg kg\(^{-1}\) day\(^{-1}\) (group IV, n = 6); triple blockade with angiotensin-converting enzyme inhibitor [ACEI (temocapril)] at 10 mg kg\(^{-1}\) day\(^{-1}\) + angiotensin receptor blocker (ARB) with olmesartan at 10 mg kg\(^{-1}\) day\(^{-1}\) + spironolactone at 10 mg kg\(^{-1}\) day\(^{-1}\) (group V, n = 12); ARB with olmesartan at 10 mg kg\(^{-1}\) day\(^{-1}\) + ACEI with temocapril at 10 mg kg\(^{-1}\) day\(^{-1}\) (group VI, n = 10); and ACEI with temocapril at 10 mg kg\(^{-1}\) day\(^{-1}\) (group VII, n = 8). Doses were based on previous reports (9, 23, 47). Blood pressure was measured by tail-cuff plethysmography (model BP-98A, Softron, Tokyo, Japan) once a week. Before they were killed, the animals were anesthetized with pentobarbital sodium, and blood samples were obtained on day 14. Thereafter, parietal peritoneal samples were procured. Thickness of the peritoneum and immunohistochemistry of TGF-β, PAI-1, type III collagen, ED-1, and CD31 were examined in harvested samples.

**Experiment 2B.** For examination of peritoneal thickness and mRNA expression, untreated rats (group VIII) and rats treated with spironolactone at 10 mg kg\(^{-1}\) day\(^{-1}\) (group IX) were killed on days 3, 7, and 14 (n = 6 at each time point).

**Experiment 3.** For determination of the impact of scraping on the peritoneum and the effects of spironolactone, the peritoneum of each rat was scraped bilaterally for 60 s. At 14 days after bilateral scraping, a peritoneal equilibration test was performed in each animal with (group X) or without (group XI) spironolactone at 20 mg kg\(^{-1}\) day\(^{-1}\) and in control (nonscraped) rats (group XII, n = 6 each).

**Histology and Immunohistochemistry**

Parietal tissue was processed for routine histology, immunohistochemistry, and electron microscopy, as described previously (20, 21). Part of the parietal peritoneal tissue was fixed in 10% buffered formalin and embedded in paraffin using conventional techniques. Sections (3 μm) were stained with hematoxylin and eosin in Masson’s trichrome for histological analysis. Another tissue sample was embedded in OCT compound (Sakura Finetechical, Tokyo, Japan) and frozen in liquid nitrogen for immunostaining. The remaining peritoneal tissues were fixed in 2% glutaraldehyde for 2 h and then postfixed in aqueous 1% osmium tetroxide. Tissues were dehydrated and embedded in Epon 812. Ultrathin sections were observed by electron microscopy (model H-7100, Hitachi, Tokyo, Japan).

Immunostaining for type III collagen, α-SMA, and monocyte/macrophages (ED-1) was performed on buffered formalin-fixed tissues. Sections were deparaffinized, rehydrated, incubated in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase, and washed in 10% normal goat serum (Dako, Glostrup, Denmark) in PBS to block nonspecific binding. Subsequently, sections were incubated for 1 h with rabbit anti-type III collagen antibody (Cosmo Bio, Tokyo, Japan), mouse anti-α-SMA antibody (1A4, Dako), or mouse anti-rat monocyte/macrophage antibody (ED-1, BMA Biomedicals, Augst, Swizerland). For cytoketan, neutrophil, CD31, TGF-β, and PAI-1 staining, 4-μm sections were cut with a cryostat, air-dried, and finally fixed in acetone at room temperature for 10 min. Endogenous peroxidase activity was inhibited using 0.1% NaOAc and 0.3% hydrogen peroxide in PBS, and nonspecific protein-binding sites were blocked with normal goat serum. Sections were then incubated with rabbit anti-TGF-β1, -2, and -3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-PAI-1 antibody (Santa Cruz Biotechnology), rabbit anti-CD31 antibody (BD Bioscience, San Jose, CA), mouse rat granulocyte antibody (BD Pharmingen, Franklin Lakes, NJ), or rabbit anti-cytokeratin antibody (Dako), followed by a conjugate of polyclonal goat anti-rabbit IgG antibody or anti-mouse IgG antibody, and horseradish peroxidase-labeled polymer (Histofine Simple Stain, Nichirei, Tokyo, Japan) as a secondary reagent. Enzyme activity was finally detected using a 3,3-diaminobenzidine tetrahydrochloride liquid system or 3-amino-9-ethylcarbazole (Dako).

**Morphological Analysis**

To assess the extent of peritoneal thickening, the submesothelial compact zone was identified as the membrane area extending from the surface mesothelium to the upper limit of the muscular tissues. We measured peritoneal thickness at six random points using a Zeiss Z1 microscope and Axiosvision Windows software version 4.4 (Carl Zeiss, Oberkochen, Germany), and mean thickness was calculated. The number of ED-1-positive cells and CD31-positive vessels was counted in 10 random 750 × 500 μm submesothelial areas. Tissue samples were observed under a microscope at ×200 magnification, and the area of the cytokeratin-positive mesothelial cell layer relative to total peritoneal surface area was calculated. The type III collagen-positive area of the submesothelial compact zone was measured using the MetaMorph 6.3 image analysis program (Universal Imaging, West Chester, PA). Peritoneal TGF-β and PAI-1 expression was analyzed and semiquantitatively classified as follows: 0, no staining; 1, mild staining; 2, moderate staining; 3, pronounced staining. For each peritoneal tissue, the “peritoneal expression score” was assessed, and the mean of individual scores was calculated for the untreated, ARB, spironolactone, and triple-blockade groups.

**Peritoneal Equilibration Test**

Rats in group IX were euthanized on day 14 for baseline assessment of ultrafiltration and membrane transport. At 4 h after intraperitoneal infusion of 30 ml of 2.5% Dianeel (glucose-based PD solution, Baxter Healthcare, Tokyo, Japan), animals were killed as described by Margetts et al. (33). An accurate ultrafiltration volume was measured, and blood samples were obtained. Creatinine, blood urea nitrogen, glucose, albumin, and total protein were measured in plasma and PD effluent. Albumin concentration was determined by ELISA using goat anti-rat albumin antibody (Cappel, Aurora, OH); glucose and creatinine levels were determined by enzymatic methods.

**Cell Culture Study**

A rat renal fibroblast cell line (NRK-49F) was purchased from the American Type Culture Collection (Manassas, VA) and maintained according to American Type Culture Collection guidelines. Briefly, NRK-49F cells were grown in complete medium containing Dulbecco’s modified Eagle’s medium (Sigma, Tokyo, Japan) supplemented with 5% fetal bovine serum (Hyclone) in humidified air with 5% CO\(_2\) at 37°C. Under subconfluent conditions, cells were washed twice with PBS, and culture medium was replaced with serum-free medium for 48 h to render the cells quiescent.

**RNA Preparation From Rat Parietal Peritonea and Rat Fibroblasts**

Rat parietal peritoneal tissues (30 mg) were immersed in RNA-later (Ambion, Austin, TX) for 1 day. The mixture was ground for
2 min with 5-mm tungsten carbide beads at a frequency of 27 Hz using a mixer-mill grinder according to the manufacturer’s instructions (Tissuelyser, Qiagen, Hilden, Germany). The ground solution was then centrifuged for 3 min at 10,000 g to compact the debris, and the supernatant was treated according to the manufacturer’s instructions. For rat tissues and fibroblasts, total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit or RNeasy Mini Kit (Qiagen). RNA concentrations were estimated using a spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Tokyo, Japan).

Table 1. Infiltration of inflammatory cells in peritoneal scraping model

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3 h</th>
<th>24 h</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil, mm⁻²</td>
<td>12.5 ± 10.7</td>
<td>389.7 ± 33.2†</td>
<td>446.9 ± 48.3†</td>
<td>318.6 ± 76.7†</td>
<td>108.0 ± 19.5</td>
<td>43.5 ± 17.8</td>
</tr>
<tr>
<td>Macrophage, mm⁻²</td>
<td>10.6 ± 3.1</td>
<td>70.9 ± 18.9</td>
<td>620.9 ± 94.5†</td>
<td>631.1 ± 53.3†</td>
<td>492.1 ± 36.3†</td>
<td>267.4 ± 24.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.005; †P < 0.0001 vs. control.

Fig. 1. Pathological findings of rat peritoneal scraping model. EM, electron microscopy; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; PAI-1, plasminogen activator inhibitor-1. Scale bars: 100 μm (Masson’s trichrome and immunohistochemistry) and 20 μm (electron microscopy).
First-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. A total of 1 µg of parietal peritoneal RNA or 2 µg of rat fibroblast RNA was then reverse transcribed. MR mRNA expression was detected by PCR, as described previously (36). PCR products for MR (190 bp) were electrophoresed on 2% agarose gels in Tris-borate-EDTA buffer and then stained with ethidium bromide. Synthetic oligonucleotides [5'-TGC ATG ATC TCG TGA C-3' (sense) and 5'-AAG TTC TTC CTG GCC GGT AT-3' (antisense)] were used as specific primers for MR (36). To validate gene expression changes, real-time PCR analysis was performed with an Applied Biosystems Prism 7500HT sequence detection system using TaqMan gene expression assays according to the manufacturer’s specifications (Applied Biosystems, Foster City, CA). TaqMan probes and primers for MR (assay identification nos. Rn00565562_m1 and Rn01512388_m1), TGF-β1 (Rn00572010_m1), type III collagen (Rn01437683_m1), monocyte chemoattractant protein-1 (MCP-1; Rn00580555_m1), PAI-1 (Rn00561717_m1), and Sgk1 (Rn00570285_m1) and 18S ribosomal RNA (4326317E) were Assay-on-Demand gene expression products (Applied Biosystems). 18S ribosomal RNA was used as an endogenous control. The thermal cycler conditions were as follows: 10 min at 95°C followed by two-step PCR for 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate. Amplification data were analyzed with Applied Biosystems Sequence Detection software version 1.3.1. To normalize the relative expression of the genes of interest against the 18S ribosomal RNA control, standard curves were prepared for each gene, as well as 18S ribosomal RNA, in each experiment. In quantitative and nonquantitative PCR, negative control experiments were performed without polymerase, primers, and cDNA.

**SDS-PAGE and Western Blotting Analysis**

To detect MR protein, we modified a previously reported technique (36). Briefly, lysates were mixed 1:1 with sample buffer for SDS-PAGE and separated under nonreducing conditions on 10% gels. Separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, London, UK), and membranes were blocked with 5% (wt/vol) nonfat milk in PBS (PBS-M). Membranes were then probed with a polyclonal anti-MR antibody (Santa Cruz Biotechnology) diluted in PBS-M, washed in PBS containing 0.1% Tween 20, and then probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA) in PBS-M (1:2,000 in PBS-M). After they were washed again in PBS containing 0.1% Tween 20, bands were developed using enhanced chemiluminescence (Perbio Science, Helsingborg, Sweden) and captured on autoradiographic film (Kodak, Tokyo, Japan).

**Statistical Analysis**

Values are means ± SE. Comparisons among groups of animals were performed by one-way ANOVA followed by Dunnett’s multiple comparison test. For statistical analysis on mRNA expression between spironolactone treatment and nontreatment, two-tailed Student’s t-test was performed. Comparisons of TGF-β and PAI-1 peritoneal expression between animals were evaluated by Mann-Whitney test. Differences were considered to be statistically significant if P < 0.05. All analyses were performed using SPSS (Chicago, IL).
RESULTS

Characteristics of PF Model Induced by Peritoneal Scraping

Morphological and immunohistochemical analysis. In normal rats, histological examination confirmed a monolayer of cytokeratin-positive mesothelial cells covering the surface of the peritoneum and exiguous connective tissues. On electron microscopy, a continuous layer of mesothelial cells exhibited thin cytoplasm with microvilli formation (Fig. 1). From 6 to 24 h after scraping, light microscopy revealed numerous neutrophils with fibrin exudation on the surface of the peritoneal membrane. Immunohistochemistry showed a peak in infiltration of neutrophils at 6–24 h after scraping (Table 1). The profile of leukocyte accumulation revealed a switch from largely neutrophil influx at 6–24 h to predominantly mononuclear population on day 3 (Table 1, Figs. 1 and 2). Subsequently, the number of infiltrating cells gradually decreased. There was some damage in the muscles by day 3 (Fig. 1). The absence of cytokeratin-positive cells in the peritoneum at 6 and 24 h indicates complete removal of mesothelial cells by mechanical scraping. Cytokeratin-positive mesothelial cells were observed on 33.7 ± 10.8% and 68.7 ± 6.7% of the peritoneal surface on days 3 and 14, respectively (Figs. 1 and 2). The peritoneum thickened with time, particularly from days 7 to 14, exhibiting α-SMA-positive fibroblasts and type III collagen. On day 14, the submesothelial compact zone was occupied by large amounts of matrix, instead of cytoplasm (Fig. 1). The parietal peritoneum thickened markedly compared with normal.

Table 2. Profiles of systolic blood pressure and plasma aldosterone levels in control rats and rats treated with ARB, ACEI, spironolactone, or triple blockade

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Plasma Aldosterone, ng/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>18</td>
<td>121.1 ± 4.4</td>
<td>129.7 ± 3.9</td>
<td>131.1 ± 3.0</td>
<td>5.94 ± 0.33</td>
</tr>
<tr>
<td>ACEI</td>
<td>9</td>
<td>125.1 ± 5.6</td>
<td>132.3 ± 5.4</td>
<td>120.2 ± 2.9</td>
<td>5.14 ± 0.65</td>
</tr>
<tr>
<td>ARB</td>
<td>10</td>
<td>125.6 ± 4.1</td>
<td>132.7 ± 3.6</td>
<td>124.4 ± 4.2</td>
<td>5.53 ± 0.47</td>
</tr>
<tr>
<td>Spironolactone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>5</td>
<td>120.6 ± 4.8</td>
<td>ND</td>
<td>ND</td>
<td>7.54 ± 0.72</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>18</td>
<td>124.5 ± 4.2</td>
<td>131.2 ± 4.4</td>
<td>126.8 ± 3.1</td>
<td>6.84 ± 0.49</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>6</td>
<td>125.1 ± 4.0</td>
<td>137.6 ± 2.3</td>
<td>125.2 ± 4.6</td>
<td>6.21 ± 0.56</td>
</tr>
<tr>
<td>Triple treatment</td>
<td>11</td>
<td>123.8 ± 4.3</td>
<td>127.8 ± 2.7</td>
<td>129.5 ± 4.4</td>
<td>6.91 ± 0.40</td>
</tr>
</tbody>
</table>

Values are means ± SE. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blockade; ND, not determined.

Fig. 3. Expression of mineralocorticoid receptor (MR) mRNA and protein in rat peritoneum and fibroblasts. Amplification of MR mRNA by RT-PCR in NRK-49F cells (A) and rat peritoneum (B) at day 14. PCR products for MR (190 bp) were electrophoresed on 2% agarose gels in Tris-borate-EDTA buffer and then stained with ethidium bromide. C: MR protein expression in NRK-49F cells and rat peritoneum at day 14 as measured by Western blotting analysis. MR protein expression was detected at ~110 kDa in lysates of rat fibroblasts and peritoneum. ×1/2 indicates that protein was electrophoresed at 50% dilution.

Fig. 4. Effects of MR blockade on scraping model. Thickness of the peritoneum was reduced with angiotensin-converting enzyme inhibitor (ACEI), angiotensin receptor blockade (ARB), spironolactone (Spiro) at 5, 10, and 20 mg/kg, and triple blockade (triple). *P < 0.05; #P < 0.001 vs. untreated control (Cont).
rat peritoneum (285.2 ± 25.4 vs. 5.3 ± 0.4 μm) and then decreased to 61.2 ± 13.0 μm on day 28 (n = 4). There was an increase in blood vessel density, which peaked at day 14, as assessed by anti-CD31 staining (Figs. 1 and 2). Immunohistochemistry showed a rapid increase in TGF-β and PAI-1 expression from day 3 and a peak at day 14 (Figs. 1 and 2). This model was thus characterized by acute inflammation in the early phase and by strong expression of α-SMA, associated with prominent collagen deposition and microvessel proliferation, on day 14. There were no histological changes in the nonscraped side.

Real-time PCR analysis during development of PF. We examined mRNA expression in the peritoneum by real-time PCR to confirm expression patterns in this peritoneal scraping model. Peritoneal type III collagen, TGF-β, and PAI-1 mRNA expression increased by 14.3-, 12.9-, and 69.0-fold, respectively, and peaked at day 7. In contrast, MCP-1 increased rapidly by 39.3-fold over baseline from day 3 (see Fig. 6).

Expression of MR in Scraped Peritoneum Model and Rat Fibroblasts

Next, we analyzed MR expression in rat fibroblasts and scraped peritoneum. RT-PCR analysis revealed expression of MR (190 bp) and GAPDH (307 bp) products in rat fibroblasts and scraped peritoneum at day 14 (Fig. 3, A and B). No PCR

![Graphs](image-url)
products were yielded in the absence of cDNA from fibroblasts or scraped peritoneum and in the absence of primers (data not shown). Western blotting analysis with an MR-specific antibody also yielded a prominent band at $\sim 110$ kDa in NRK-49F lysates and scraped peritoneal lysates at day 14 (Fig. 3C). The relative ratio of MR mRNA to 18S ribosomal RNA in the peritoneum, as detected by real-time PCR using two types of Taqman probe (Rn00565562_m1 and Rn01512388_m1), did not change after disease induction.

**Effects of MR Blockade on Systemic Blood Pressure and PF**

We investigated the effects of spironolactone and renin-angiotensin system blockade on this model. Systolic blood pressure was not affected compared with untreated rats (Table 2). There was no significant difference in plasma aldosterone concentration among experimental groups (Table 2). MR inhibition with spironolactone resulted in a significant reduction in peritoneal thickness compared with no treatment (Fig. 4): 285.2 $\pm$ 25.4, 201.9 $\pm$ 60.8, 88.8 $\pm$ 39.2, 75.4 $\pm$ 16.8, 78 $\pm$ 9.3, 179.4 $\pm$ 32.9, and 217.3 $\pm$ 27.8 $\mu$m in groups I, II, III, IV, V, VI, and VII, respectively. ARB with olmesartan also effectively reduced peritoneal thickness. However, ACEI did not effectively reduce the peritoneal thickening. The effects of spironolactone (10 and 20 mg⋅kg$^{-1}$⋅day$^{-1}$) were very similar to those of triple blockade with ACEI, ARB, and spironolactone. On semiquantitative assessment by immunohistochemistry, TGF-$\beta$ expression was significantly suppressed by ARB, spironolactone, and triple blockade (Fig. 5); the PAI-1 score also decreased with spironolactone and triple blockade (Fig. 5). Type III collagen deposition and the number of ED-1-positive macrophages and CD31-positive vessels were significantly suppressed by spironolactone at 10 mg⋅kg$^{-1}$⋅day$^{-1}$ (Fig. 5). For verification of the results of immunohistochemical studies, quantitative analysis by real-time PCR for MCP-1, type III collagen, TGF-$\beta$, and PAI-1 mRNA in the peritoneal membrane was performed at days 3, 7, and 14. Upregulation of type III collagen and TGF-$\beta$ was significantly inhibited by spironolactone at each time point. PAI-1 and MCP-1 mRNA expression was suppressed at days 3 and 7 and at days 3 and 14, respectively (Fig. 6). Notably, expression of Sgk1, an effector kinase of MR, was upregulated and peaked at day 7 in the scraped peritoneum and was significantly inhibited in spironolactone-treated rats (Fig. 6).

**MR Blockade Improved Peritoneal Function in Bilateral Scraping Model**

We examined peritoneal function by peritoneal equilibration test to evaluate the effects of MR blockade. At day 14, we found significantly impaired ultrafiltration with associated increases in glucose transport out of the peritoneum and high albumin permeability compared with the control group. In contrast, spironolactone significantly improved peritoneal dysfunction, including ultrafiltration, glucose transport, and albumin leakage. Creatinine and urea transport tended to be increased in the nontreatment group compared with the control group; however, the change was not statistically significant (Fig. 7).

**DISCUSSION**

According to a multicenter survey conducted in Japan, 34% of PD discontinuations are due to overhydration resulting from ultrafiltration failure or poor compliance with salt and fluid restrictions (24, 39). Retrospective and prospective studies have found that peritonitis impairs the ultrafiltration capacity of the peritoneum (41) and is an important risk factor for ultrafiltration failure (41, 50). Peritonitis was also reported to be the only independent factor affecting peritoneal function during the 1st yr on PD (8). Furthermore, the degree of associated peritoneal inflammation determines the changes in peritoneal function (7, 50). Histologically, acute peritoneal inflammation can cause morphological damage to the peritoneum (16, 42), and episodes of peritonitis are correlated with chronic peritoneal fibrotic changes (49). These reports indicate that peritonitis and inflammation can lead to structural changes and dysfunction of the peritoneal membrane. Therefore, we hypothesized that antifibrotic agents, together with antibiotics, may be useful in PD patients who develop bacterial peritonitis. In this study, we examined the utility of MR blockade in a novel PF model with acute inflammation.

We successfully generated a reproducible model of PF with acute inflammation induced by mechanical scraping. The pathological stages of this model were identified as 1) mesothelial exfoliation and exudation of fibrin on the surface of the membranes; 2) leukocyte accumulation after initial influx of neutrophils and monocytes; 3) resorption of fibrin and remesothelialization; and 4) progressive fibrotic changes and increased vessel density associated with membrane dysfunction.
This model was similar to bacterial peritonitis in terms of the leukocyte accumulation profile and progressive fibrotic changes with interaction between proinflammatory cytokines (MCP-1) and prosclerotic growth factors (TGF-β/H9252 and PAI-1) (2, 18, 27, 53). In disease progression, vessel density was correlated with the extent of PF as reported in bacterial peritonitis (40, 42). The induction of PF by mechanical scraping differs from that of bacterial peritonitis. However, the pathological changes in solute transport induced by scraping may mimic those observed in bacterial peritonitis, as described by Ni et al. (40). The pathological findings at day 14 were comparable with general morphological features in the peritoneal membrane of patients undergoing long-term PD with ultrafiltration failure and include thickened peritoneum with increased blood vessel density (34, 57). In this respect, the present model is unique and might be useful in exploring strategies for treatment and prevention of PF and membrane failure.

Angiotensin II concentration in PD effluent is significantly higher at the onset of infectious peritonitis than in normal continuous ambulatory peritoneal dialysis patients (59). Miyazaki and Yuzawa (35) showed that angiotensin II blockade with benazapril and candesartan reduced the thickness of the submesothelial compact zone and the number of CD31-positive vessels. In human studies, the ARB valsartan not only preserved residual renal function, but also increased peritoneal creatinine clearance 1 yr after initiation (52). These findings indicate that the rennin-aldosterone system plays a role in the progression of PF and deterioration of ultrafiltration function in PD patients. However, whether activation of MR is involved in the development of PF remains unclear. We hypothesized that MR blockade may ameliorate PF in the scraping model. We confirmed that MR mRNA and protein were consistently present in the rat peritoneal membrane and fibroblast cell line. Furthermore, expression of Sgk1, originally cloned as a glucocorticoid-responsive gene and recently recognized as an MR-signaling molecule, was upregulated in the scraping model. Sgk1 is thought to play a role in mineralocorticoid-induced tissue fibrosis (37). Sgk1 transcripts were shown to be elevated in several fibrotic diseases, such as diabetic nephropathy (28), glomerulonephritis (46), and aldosterone-induced renal injury (51). Interestingly, deoxycorticosterone-acetate-high salt treatment led to significant cardiac fibrosis and proteinuria in sgk1−/− mice, but not sgk1+/− mice, despite identical increases in blood pressure (54, 55). In our experiments, increase of Sgk1 in the scraping model was significantly suppressed by spironolactone. These results suggest that activation of MR is involved in development of PF in this model and that spironolactone has a beneficial effect on peritoneal thickness and function.

Spironolactone is a steroid analog with structural similarity to aldosterone and, thereby, functions as a competitive antagonist. Aldosterone stimulates types I, III, and IV collagen synthesis via ERK1/2-dependent pathways in cultured fibroblasts (36). On analysis of molecules that might be involved in peritoneal damage in this model, spironolactone was found to suppress inflammatory (macrophage infiltration) and fibrotic processes. Spironolactone reduced peritoneal MCP-1, which was upregulated on day 3, and was considered to accelerate monocyte...
recruitment. MR was reported to be present in macrophages, and MR blockade with eplerenone suppressed aldosterone-induced NADPH oxidase of macrophages (25). This report suggests that spironolactone may directly suppress the macrophage oxidative stress in this model. Thickening of the peritoneum was markedly reduced by spironolactone through inhibition of MCP-1, PAI-1, and TGF-β. neoangio genesis may be suppressed by inhibition of TGF-β-induced VEGF expression. In this model, fibroblasts play the main role in the progression of fibrosis, inasmuch as the mesothelial cells were completely removed at 6 and 24 h after scraping. However, mesothelial cells were found to repopulate the fibrotic tissues from day 3. These cells, possibly relocated visceral mesothelial cells, might play a role in modulating fibrosis. The ARB olmesartan was also effective in reducing peritoneal thickness. Thus very high doses of olmesartan or temocapril may be more effective, as previously observed, in hypertensive renal injuries (9).

Multiple lines of evidence have suggested that TGF-β is the key mediator in the development of fibrosis (3, 4). TGF-β enhances the synthesis of extracellular matrix, partly through PAI-1 (17). Other functions of TGF-β include angiogenesis, immune modulation, cell cycle regulation, and transdifferentiation to the mesenchymal phenotype (3, 30). The observed changes in peritoneal membrane thickening and collagen deposition over time are attributable to increases in α-SMA-positive cells, suggesting myofibroblast differentiation induced by fibrogenic cytokines, such as TGF-β (3, 30). Margetts et al. (31, 32) directly showed the importance of TGF-β1 in PF with models of adenovirus-mediated TGF-β1 gene transfer to the peritoneum, which caused PF, transdifferentiation, neoangiogenesis, and increased peritoneal membrane solute transport. We demonstrated that overexpression of TGF-β1 and PAI-1, which peaked at day 7 and was associated with histological changes consistent with PF and neoangiogenesis, was suppressed by spironolactone. In particular, the elevation of PAI-1 in our model was marked. Aldosterone blockade was shown to prevent renal injury by suppressing inflammation and antifibrotic effects, such as TGF-β1, PAI-1, and connective tissue growth factor (11, 14, 15). Inhibition of MR is reported to be strongly linked to PAI-1 inhibition in animal models and PAI-1−/− mice (1, 11, 29). In normotensive rats, aldosterone infusion reportedly increases urinary TGF-β through the MR (22, 45). Taken together, these observations suggest that the complex interaction between mineralocorticoid and MR, as well as TGF-β and PAI-1, plays a role in the progression of PF in the scraping model and that spironolactone effectively suppresses this cytokine network, leading to prevention of PF and dysfunction.

In summary, this model allows further investigation into the relative contributions of inflammation and profibrotic cytokines in PF and may lead to new interventions for PF and membrane failure. The MR antagonist spironolactone is able to ameliorate progression of PF and preserve peritoneal membrane function in the peritoneal scraping rat model. Aldosterone blockade may also prevent inflammation-induced structural and functional damage to the peritoneum in PD patients. Clinical studies of MR blockade, together with antibiotic treatment, are warranted to more definitively address the efficacy and safety of aldosterone antagonism in PD patients with bacterial peritonitis.

ACKNOWLEDGMENTS
The technical assistance of Norihiko Suzuki, Naoko Asano, and Yuriko Sawa (Department of Nephrology, Nagoya University) is greatly appreciated.

GRANTS
This work was supported by the Aichi Kidney Foundation and Baxter Japan PD Grant 2007.

REFERENCES


