Methylglyoxal induces peritoneal thickening by mesenchymal-like mesothelial cells in rats

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Abstract

Background. The epithelial-to-mesenchymal transition (EMT) of mesothelial cells was observed in patients on peritoneal dialysis and may be involved in peritoneal thickening. Conventional peritoneal dialysis fluids (PDFs) that contain glucose degradation products (GDPs), such as methylglyoxal (MGO) and formaldehyde (FA), are bioincompatible. The aim of this study is to analyse the participation of EMT in peritoneal thickening induced by GDPs in rats.

Methods. Rat mesothelial cells were cultured with various GDPs, and the gene expression of Snail was analysed by polymerase chain reaction (PCR). Sprague-Dawley rats were administered intraperitoneally 20 mM MGO/PDFs, 20 mM FA/PDFs or 0.1% chlorhexidine gluconate (CHX)/15% ethanol/saline every day for 21 days. On Day 22, the expression of transforming growth factor-β (TGF-β), collagen 1, matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor (VEGF), Snail and receptor for advanced glycation end-products (RAGE) was analysed by PCR, enzyme-linked immunoassay or immunohistological staining.

Results. In cell-culture experiments, the expression of Snail was enhanced by MGO, but not FA. In rats treated with 20 mM MGO, peritoneal fibrous thickening with the proliferation of mesenchymal-like mesothelial cells was observed. The expression of TGF-β, collagen 1, MMP-2, VEGF, Snail and RAGE increased significantly (P < 0.01). In FA- or CHX-treated rats, the peritoneum was thickened with sparse collagen fibres, but mesenchymal-like mesothelial cells were not observed.

Conclusions. MGO induced peritoneal fibrous thickening with the proliferation of mesenchymal-like mesothelial cells in vivo. These cells may be transdifferentiated from mesothelial cells by EMT via Snail and play an important role in peritoneal fibrous thickening.

Keywords: epithelial-to-mesenchymal transition (EMT); methylglyoxal; matrix metalloproteinase-2 (MMP-2); peritoneal thickening; transforming growth factor-β (TGF-β)

Introduction

Peritoneal dialysis (PD) is a blood-purifying treatment for patients with end-stage renal disease. But long-term PD results in peritoneal thickening with functional decline, such as ultrafiltration failure and increased transport of small solutes across the peritoneum. Conventional PD fluids (PDFs) are considered unphysiological and bioincompatible because of a low pH and glucose degradation products (GDPs), such as methylglyoxal (MGO), glyoxal, formaldehyde (FA) and 3-deoxyglucosone. Glucose is widely used as an osmotic agent in PDFs, and GDPs are generated from the degradation of glucose during heat sterilization and storage [1,2]. GDPs show toxic effects on the proliferation and function of peritoneal cells [3]. MGO, an extremely toxic GDP with strong oxidative activity, enhances production of vascular endothelial growth factor (VEGF) that induces neovascularization and vascular permeability [4]. In addition, MGO induces the formation of advanced glycation end-products (AGEs) [2,5]. MGO may cause peritoneal injury with morphologic change and loss of function via the production of VEGF and accumulation of AGEs in the peritoneum [4–6].

Recent studies showed that the epithelial-to-mesenchymal transition (EMT) of mesothelial cells may induce peritoneal thickening in patients on PD [7]. Transforming growth factor-β (TGF-β) induces peritoneal fibrosis and the EMT of mesothelial cells [7–11]. In such events, matrix metalloproteinase (MMP)-2 degrades components of the extracellular matrix such as fibronectin and type IV collagen, which comprise the basement membrane, and plays important roles in angiogenesis, the EMT of mesothelial cells, inversion of transdifferentiated mesothelial cells and migration of cells that promote
fibroplasia [6,7,11–14]. The EMT of mesothelial cells enhances production of VEGF [7,8,15]. EMT may induce peritoneal thickening with ultrafiltration loss and increased solute transport, but this has not been fully elucidated. The aim of this study is to elucidate the mechanisms of peritoneal thickening. MGO induces peritoneal thickening through the excessive proliferation of α-smooth muscle actin (α-SMA)-positive mesenchymal-like cells [6]. In the present study, we investigated the effects of MGO on peritoneal thickening via the transdifferentiation of mesothelial cells to mesenchymal-like cells in rats.

Materials and methods

Mesothelial cell culture

Rat mesothelial cells were isolated from the parietal peritoneum of male Sprague-Dawley rats. The methods of isolation and the cell cultures have been previously reported [13]. The 80–85% confluent cells at passages 4–7 were cultured with Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, MO, USA) in the presence of several concentrations of various GDPs, i.e. MGO (Nakarai, Kyoto, Japan), glyoxal (Wako Chemicals, Osaka, Japan), FA (Wako Chemicals) or 3-deoxyglucosone (Dojin, Kumamoto, Japan). TGF-β (R&D Systems, Minneapolis, MN, USA) was used as a positive control for the EMT of mesothelial cells. The experiments were repeated three times.

Animals

Animals used in this study were male Sprague-Dawley rats [Crj (CD) SD-IGS rats, 6 weeks of age, weighing about 215 g, Charles River Japan, Kanagawa, Japan]. The animals were housed in an air-conditioned room, at a constant temperature of 23 ± 2°C and a relative humidity of 50 ± 10% and kept under a 12-h light/dark cycle with free access to sufficient pellet food and water. We performed our experiments in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Table 1. PCR primer sequence

<table>
<thead>
<tr>
<th>Primer</th>
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<tr>
<td>E-cadherin forward primer</td>
<td>5′ GGCCCAGGAGCTGACAAAC 3′</td>
</tr>
<tr>
<td>E-cadherin reverse primer</td>
<td>5′ CCAGGGTGCGGTACTTCTT 3′</td>
</tr>
<tr>
<td>Cytokeratin forward primer</td>
<td>5′ GTGATGCAATGTTGTGC 3′</td>
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<td>Cytokeratin reverse primer</td>
<td>5′ GACCCTCAGTCACAAG 3′</td>
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<tr>
<td>TGF-β forward primer</td>
<td>5′ TCCCGGGGCTAGTGCAG 3′</td>
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<tr>
<td>TGF-β reverse primer</td>
<td>5′ AGGTAAACCGGCAAATGTTTGTCA 3′</td>
</tr>
<tr>
<td>Col1 forward primer</td>
<td>5′ GATGTGCCACTCTGACTGG 3′</td>
</tr>
<tr>
<td>Col1 reverse primer</td>
<td>5′ ACATCGATGATGGCAAGCCG 3′</td>
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<tr>
<td>MMP-2 forward primer</td>
<td>5′ ACAGCGTGCCATGGGCAGT 3′</td>
</tr>
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<td>MMP-2 reverse primer</td>
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</tr>
<tr>
<td>VEGF forward primer</td>
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<tr>
<td>VEGF reverse primer</td>
<td>5′ CTCTCATGCTGCTGGGCTTGG 3′</td>
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<tr>
<td>Snail forward primer</td>
<td>5′ TGCAACATCCGAAGCCACA 3′</td>
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<tr>
<td>Snail reverse primer</td>
<td>5′ TCTTCAATCGGAGTTGCTG 3′</td>
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<tr>
<td>RAGE forward primer</td>
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<td>RAGE reverse primer</td>
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<td>GAPDH forward primer</td>
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</tr>
<tr>
<td>GAPDH reverse primer</td>
<td>5′ ATGGTGGTGAAGGACCCAGTA 3′</td>
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PCR: polymerase chain reaction, TGF-β: transforming growth factor-β, VEGF: vascular endothelial growth factor, MMP-2: matrix metalloproteinase-2, Col1: collagen 1, RAGE: receptor for advanced glycation end-products, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
Intraperitoneal injection of PDFs

The PDFs were prepared by adding 6.6, 20 mM MGO or 20 mM FA to a PDF (2.5% glucose, 100 mM NaCl, 35 mM sodium lactate, 2 mM CaCl2 and 0.7 mM MgCl2) and then were sterilized by filtration. The PDFs were prepared and adjusted to pH 5.0 just before injection every day. Rats were divided into five groups (n = 6/group) and given the following solutions intraperitoneally for 21 days: group 1, 100 mL/kg of PDF without adding GDPs; group 2, 100 mL/kg of PDF containing 6.6 mM MGO; group 3, 100 mL/kg of PDF containing 20 mM MGO; group 4, 100 mL/kg of PDF containing 20 mM FA and group 5, 15 mL/kg of 0.1% chlorhexidine gluconate (CHX)/15% ethanol/saline. The concentrations of MGO, FA or CHX were decided based on previous reports [5,6,12,13]. The PDFs were administrated intraperitoneally by a simple needle puncture under anaesthesia with diethyl ether. If the solution remained in the peritoneal cavity, it was drained before the injection. On the 22nd day of the experiment, 50 mL/kg of PDF containing 2.5% glucose (Midperiq L250, Terumo Co., Tokyo, Japan) was intraperitoneally injected and the drained dialysate was collected 90 min later to analyse TGF-β, VEGF and MMP-2. The parietal peritoneum was also sampled for histological analysis or gene expression analysis.

Adequate attention was paid to maintaining a hygienic environment and to preventing infectious peritonitis. Furthermore, a sterility test was performed using the dialysate drained on the 22nd day to check for the presence of aerobic bacteria, anaerobic bacteria and fungi. All rats were confirmed to be uninfected.

Histological analysis

The parietal peritoneum was sampled from the corresponding sites of each rat and fixed with a 10% FA/0.1 M...
phosphate buffer (pH 7.2). The peritoneal specimens were embedded in paraffin to prepare tissue sections with a thickness of 2–3 µm. To determine the thickness of the peritoneum, the sections were sliced perpendicularly to the peritoneal surface. Each section was stained with haematoxylin-eosin (HE) to analyse cell type and with Azan to identify collagen fibres.

**Immunohistochemistry**

The peritoneal tissue sections prepared from paraffin blocks were dewaxed with xylene. After being treated with 0.25% trypsin/1 mM EDTA for 2 h at room temperature, the sections were blocked with 10% goat serum for 30 min. These sections were treated for 2 h at room temperature with a monoclonal antibody against α-SMA conjugated with Cy3 (Sigma Chemical Co., St Louis, MO, USA) at a dilution of 1:400 to identify mesenchymal cells, and a monoclonal antibody against cytokeratin conjugated

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**Fig. 4.** Histopathological findings of parietal peritoneum. The parietal peritoneum was analysed histologically with HE stain (A, B, C, E, G) or Azan stain (D, F, H). Control rat: A, 6.6 mM MGO-treated rat: B, 20 mM MGO-treated rat: C, D. FA-treated rat: E, F. CHX-treated rat: G, H. ×400.
MGO induces peritoneal thickening by mesenchymal-like mesothelial cells with fluorescein isothiocyanate (Sigma Chemical Co.) at a dilution of 1:50 to identify mesothelial cells. Also, the sections were treated for 2 h at room temperature with a rabbit anti-human MMP-2 antibody (Sigma Chemical Co.) or a rabbit anti-human VEGF antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:1000 and 1:100, respectively, followed by staining with Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR, USA) for 1 h. The sections were mounted with the VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA, USA). Fluorescence was detected with the Axiocvert 200 M fluorescence microscope (Carl Zeiss GmbH, Gottingen, Germany). Negative staining was confirmed by incubation without the primary or secondary antibody for immunofluorescence staining.

Immunohistochemistry for AGE was analysed with frozen sections because AGE was formed as artefacts during the preparation of paraffin blocks. The parietal peritoneum fixed with FA was embedded in Tissue-Tek OTC compound (Miles, Inc., IN, USA) and immediately frozen. The frozen tissue samples were sectioned at a thickness of 2–3 µm with a cryostat. These sections were treated with 0.25% trypsin/1 mM EDTA for 2 h at room temperature. After being blocked with 10% goat serum for 30 min, these sections were incubated for 2 h at room temperature with a monoclonal antibody against AGE (Transgenic Ltd, Kumamoto, Japan) at a dilution of 1:1000, followed by staining with Alexa Fluor 568-conjugated goat anti-mouse IgG (Molecular Probes Inc.) for 1 h. These sections were then incubated for 2 h at room temperature with a monoclonal antibody against cytokeratin conjugated with fluorescein isothiocyanate at a dilution of 1:50 and mounted with the VECTASHIELD mounting medium with DAPI.

Analysis of TGF-β, VEGF and MMP-2 in the drained dialysate

The concentrations of TGF-β and VEGF in the drained dialysate were quantified with a Quantikine TGF-β1 immunoassay kit (R&D Systems) and a Quantikine VEGF immunoassay kit (R&D Systems), respectively.

Fig. 5. Characterization of cells in the peritoneum. In the parietal peritoneum in the rats treated with MGO, cytokeratin-positive cells (green; A, D, G), α-smooth muscle actin-positive cells (red; B, E, H) and dual-positive cells (yellow/orange; C, F, I) were analysed by immunofluorescent staining with nuclear counterstain (blue, DAPI). In the control rat, mesothelial cells were positive for cytokeratin, but not α-smooth muscle actin (A, B, C). In the 6.6 mM MGO-treated rat, some mesothelial cells, forming a single layer, showed both cytokeratin and α-smooth muscle actin signals (D, E, F). In the 20 mM MGO-treated rat, cytokeratin-positive cells were present both at the surface and in the interstitium of the peritoneum, and some of these cells showed α-smooth muscle actin signals (G, H, I). The distribution of cytokeratin did not necessarily correspond with that of α-smooth muscle actin.
The MMP-2 in the drainage was analysed with gelatin zymography [6,13]. After electrophoresis under non-reducing conditions on 8% polyacrylamide gels containing 1 mg/mL of gelatin, the gels were treated with 2.5% Triton-100/0.1 M NaCl/50 mM Tris–HCl (pH 7.5) for 2 h, and incubated for 18 h at 37°C in 50 mM Tris–HCl (pH 7.5)/10 mM CaCl2. The gels were stained with 0.1% Coomassie Brilliant Blue. MMP-2 was detected as unstained 64-kDa proteolytic bands in the stained gels. The relative concentrations of MMP-2 in the dialysate were quantified by scanning proteolytic bands on the zymograms with a scanner (CanoScanFB 620S, Canon, Tokyo, Japan) and ImageJ quantitation program (National Institutes of Health, Bethesda, MD, USA).

Fig. 6. Deposition of AGE and production of VEGF and MMP-2 in the peritoneum. In the peritoneum, cytokeratin-positive cells (green; B, F, J), AGE-positive cells (red; C), VEGF-positive cells (red; G) and MMP-2-positive cells (red; K) were analysed by immunofluorescent staining with nuclear counterstain (blue, DAPI). Merged figures are shown in (A) and (D) (cytokeratin and AGE), (E) and (H) (cytokeratin and VEGF) or (I) and (L) (cytokeratin and MMP-2). In control rats, AGE (A), VEGF (E) and MMP-2 (I) were not detected in the cytokeratin-positive cells (green). In rats treated with 20 mM MGO, AGE (red; C) was deposited in the cytokeratin-positive (yellow/orange; D) or negative cells (red; D). VEGF (red; G) and MMP-2 (red; K) were also detected in cytokeratin-positive cells (yellow/orange; H or L) in addition to interstitial cells (red; H or L). Control rat: A, E, I. 20 mM MGO-treated rat: B, C, D, F, G, H, J, K, L.
MGO induces peritoneal thickening by mesenchymal-like mesothelial cells

Analysis of gene expression by real-time polymerase chain reaction

Gene expression was analysed by real-time polymerase chain reaction (PCR). Total RNA was extracted from the parietal peritoneum or cultured mesothelial cells using the RNeasy Kit with the DNase Set (QIAGEN GmbH, Hilden, Germany). The prepared total RNA concentration was determined spectrophotometrically using a GeneQuant DNA/RNA calculator (Amersham Pharmacia Biotech Ltd, Little Chalfont, UK). Total RNA from the peritoneum or the cultured cells (5 or 0.5 μg, respectively) was reverse-transcribed with 200 units of Superscript II reverse transcriptase (GIBCO BRL, Rockville, MD, USA). The resulting cDNA was amplified with SYBR Premix EX Taq DNA polymerase (Takara Shuzo Co., Ltd, Tokyo, Japan) using a thermal cycler (755 Fast Real-Time PCR System, Applied Biosystems, Foster, CA, USA). After an initial denaturation at 95°C for 10 s, real-time PCR was performed for 40 cycles under the following conditions: denaturation at 95°C for 5 s, annealing and extension at 60°C for 34 s. The PCR primer sequences are listed in Table 1. After real-time PCR, a dissociation analysis was performed to confirm the specificity of the amplification. Results were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a constitutive control to allow comparison of RNA levels among different samples. Data are expressed as relative quantities compared to the control.

Statistical analysis

Data are presented as the mean ± SD. Statistical comparisons were carried out with an analysis of variance (ANOVA), followed by Dunnett’s multiple comparison test. Statistical analyses were performed using the SAS system for Windows, version 8.2 (SAS Institute Inc., Cary, NC, USA). A *P*-value of <0.05 was accepted as significant.

Fig. 7. Concentrations of TGF-β, VEGF and MMP-2 in the drained dialysate. The concentrations of TGF-β (A) and VEGF (B) in the drained dialysate were quantified with an enzyme-linked immunoassay. MMP-2 was analysed with gelatin zymography and results are shown as relative quantities compared to the value of the control (C). #1 *P* = 0.057, #2 *P* = 0.055, **P < 0.01.

Fig. 8. Gelatinases in the drained dialysate. The drained dialysate was analysed with gelatin zymography. Gelatinases were detected as unstained proteolytic bands in the gel stained with Coomassie Brilliant Blue. Latent MMP-2 was enhanced in the 20 mM MGO-, FA- and CHX-treated group. Lane 1: MMP marker (CVMICON International Inc., Temecula, CA, USA). Lane 2: drained dialysate from the control rat. Lane 3: drained dialysate from the 20 mM MGO-treated rat. Lane 4: drained dialysate from the 20 mM FA-treated rat. Lane 5: drained dialysate from the CHX-treated rat.
Results

Mesothelial cell culture

The gene expression of Snail was assessed in mesothelial cells cultured in the presence of MGO, glyoxal, FA, 3-deoxyglucosone or TGF-β. MGO, glyoxal and 3-deoxyglucosone stimulated the gene expression of Snail at a concentration of 0.33 mM or more, 3.3 mM or more and 10 mM, respectively, but FA did not (Figures 1, 2A and 3A). MGO suppressed the gene expression of E-cadherin, but not cytokeratin (Figures 2C, D and 3B). TGF-β enhanced the gene expression of Snail and TGF-β but suppressed that of cytokeratin and E-cadherin (Figures 1–3). MGO and TGF-β induced the expression of Snail but no morphologic change to a mesenchymal-like phenotype was observed in the cultured rat mesothelial cells (data not shown).

Histological findings of peritoneum in vivo

In control rats, a monolayer of mesothelial cells with negative signals for α-SMA and weak positive signals for cytokeratin were observed on the surface of the very thin peritoneum (Figures 4A and 5A–C).

In rats receiving PDFs containing 6.6 mM MGO, increased cubical mesothelial cells formed a monolayer at the surface of the peritoneum (Figure 4B). Some mesothelial cells showed not only cytokeratin but also α-SMA signals (Figure 5D–F).

In rats receiving PDFs containing 20 mM MGO, peritoneal fibrous thickening with increased numbers of microvessels and cellular infiltration was observed (Figure 4C and D). The peritoneal tissue consisted of dense collagen fibres, but at the surface of the peritoneum, collagen was scarce (Figure 4D). At the surface of the peritoneum, a group of mesenchymal-like cells with cytokeratin and/or α-SMA excessively proliferated (Figures 4C and 5G–I). In some of these cells, cytokeratin and α-SMA were not colocalized. AGE accumulated in these mesenchymal-like cells with or without cytokeratin at the surface of the peritoneum (Figure 6B–D). In addition to endothelial cells and interstitial infiltrated cells, these mesenchymal-like cells at the surface of the peritoneum produced VEGF and MMP-2 (Figure 6F–H and J–L). However, some cytokeratin-positive mesenchymal-like cells were negative for MMP-2.

In the CHX- or FA-treated rats, peritoneal thickening with sparse collagen fibres, oedema, increased numbers of microvessels and cellular infiltration was observed.

Fig. 9. Gene expression of TGF-β, collagen 1, VEGF and MMP-2. The gene expression of TGF-β (A), collagen 1 (B), VEGF (C) and MMP-2 (D) in the parietal peritoneum was analysed by real-time PCR. Results were normalized with respect to GAPDH and are shown as relative quantities compared to the value of the control. **P < 0.01.
MGO induces peritoneal thickening by mesenchymal-like mesothelial cells

Expression of TGF-β, collagen 1, VEGF, MMP-2, Snail and RAGE

The concentrations of TGF-β, VEGF and MMP-2 in the drained dialysate increased in rats treated with 20 mM MGO (Figures 7 and 8). The gene expression of TGF-β, collagen 1, VEGF and MMP-2 was also enhanced in the 20 mM MGO-treated rats (Figure 9). The MMP-2 concentration in the dialysate increased in rats treated with 20 mM FA or CHX (Figures 7C and 8). Most of the MMP-2 in the drained dialysate existed as a latent form, and the active form of MMP-2 was scarcely detected. MMP-9, another major gelatinase, was hardly detected (Figure 8).

The gene expression of Snail and receptor for AGE (RAGE) was enhanced in the 20 mM MGO-treated rats, but not in the FA- or CHX-treated rats (Figures 10 and 11).

Fig. 10. Gene expression of Snail. The gene expression of Snail in the parietal peritoneum was analysed by real-time PCR. Results were normalized with respect to GAPDH and are shown as relative quantities compared to the value of the control. **P < 0.01.

Fig. 11. Gene expression of the receptor for AGE (RAGE). The gene expression of the RAGE in the parietal peritoneum was analysed by real-time PCR. Results were normalized with respect to GAPDH and are shown as relative quantities compared to the value of the control. **P < 0.01.

Discussion

Long-term PD results in peritoneal thickening with functional decline, such as ultrafiltration failure and increased transport of small solutes across the peritoneum. GDPs in PDFs may be a cause of peritoneal thickening [6]. Also, it was suggested that conventional PDFs containing high concentrations of GDPs might induce the EMT of mesothelial cells in PD patients [16]. TGF-β induces EMT via Snail, a transcription factor, and then EMT enhances fibroplasias [7–11]. In our animal model treated with 20 mM MGO, the expression of TGF-β and Snail was enhanced. At the surface of the peritoneum, the mesenchymal-like mesothelial cells with cytokeratin and/or α-SMA excessively proliferated. In rats treated with 6.6 mM MGO, some mesothelial cells, forming a monolayer at the surface of the peritoneum, showed not only cytokeratin but also α-SMA signals. These results suggest that MGO might induce the conversion of mesothelial cells to mesenchymal-like mesothelial cells by EMT via TGF-β. As another explanation, some reports indicate the possibility that EMT is induced by hypoxia or reactive oxygen species independent of TGF-β [17,18]. MGO with strong oxidative activity might induce EMT via a signal pathway different from that of TGF-β. Alternatively, mesenchymal-like mesothelial cells might transdifferentiate from myofibroblasts via a process mediated by mesenchymal-to-epithelial transition [19]. The expression of TGF-β, collagen and MMP-2 in the mesenchymal-like mesothelial cells induced by MGO might result in peritoneal thickening with a loss of peritoneal function via reconstitution of the basement membrane or peritoneal tissue.

MGO is a potent promoter of the production of AGEs [2,5]. In a previous study using rats, the administration of 20 mM MGO resulted in the accumulation of AGEs, such as imidazolone and carboxyethyl lysine, in the peritoneum [5]. AGEs and MGO induced the expression of RAGE, and then the AGE–RAGE interaction induced the EMT of mesothelial cells and upregulated the expression of TGF-β and VEGF [20–22]. In our 20 mM MGO-treated rats, AGE was detected in the mesenchymal-like mesothelial cells at the surface of the peritoneum and the gene expression of RAGE, TGF-β and VEGF was enhanced. So, the mesenchymal-like mesothelial cells might transdifferentiate from the mesothelial cells in response to signals from RAGE binding with AGE.

MMP-2 is produced from mesenchymal cells, macrophages, endothelial cells and human mesothelial cells, and relates to cell migration, neovasculization and the EMT of mesothelial cells [6,7,11–13]. But cultured rat mesothelial cells hardly produce MMP-2 [13], and in our control rats, MMP-2 was not detected in mesothelial cells. In rats treated with 20 mM MGO, MMP-2 was produced by many mesenchymal-like cells at the surface of the peritoneum. But the distribution of MMP-2 did not necessarily correspond to that of cytokeratin, a marker for mesothelial cells. From these results, transdifferentiation from mesothelial cells to mesenchymal cells may result in the production of MMP-2. The level of MMP-2 reflects peritoneal thickening [23]. In this study, the production of MMP-2 was increased.
in the 20 mM MGO-, FA- and CHX-treated rats with peritoneal thickening. Interestingly, a group of mesenchymal-like mesothelial cells were observed in the 20 mM MGO-treated rats but not in the FA- or CHX-treated rats. MMP-2 may play an important role in peritoneal thickening with or without EMT.

VEGF, which induces neovascularization and vascular permeability, is produced in the peritoneal cavity of patients undergoing long-term PD and may induce peritoneal functional decline, such as the increased transport of small solutes [4]. GDPs or EMT stimulates the production of VEGF [6–8,15,18]. In our 20 mM MGO-treated rats, VEGF was produced by many mesenchymal-like mesothelial cells in addition to endothelial cells and interstitial infiltrated cells. Microvessels in the peritoneum were also increased in number. In our previous report, MGO increased peritoneal permeability [6]. These results suggest that MGO induced the proliferation of mesenchymal-like mesothelial cells and then VEGF produced by these cells contributed to an increase in peritoneal permeability via neovascularization and an increase in vascular permeability. EMT may play an important role in the peritoneal functional decline, such as the increased transport of small solutes, by enhancing the production of VEGF.

CHX causes peritoneal injury with thickening, neovascularization, proliferation of myofibroblasts and loss of mesothelial cells in animal models [12,13,23]. But it is thought that CHX is not the main cause of peritoneal injury with thickening in PD patients because it is seldom if ever used in PD today. In this study, a CHX model was used as a positive control with peritoneal thickening because CHX-treated rats are usually used as a model of peritoneal injury [12,13,23]. In our CHX-treated rats, peritoneal thickening without mesenchymal-like mesothelial cells was observed. The extent of the peritoneal fibrosis and collagen expression induced by CHX was less than that evoked by 20 mM MGO. These results suggest that mesenchymal-like mesothelial cells play an important role in peritoneal fibrosis.

Snail, a transcription factor, is a master regulator for EMT and is induced to express by TGF-β [8–11]. It has been often reported that TGF-β decreased the expression of both E-cadherin and cytokeratin via the upregulation of Snail in the EMT of mesothelial cells. In contrast, there were some reports that TGF-β increased the expression of E-cadherin [11,24]. In our in vitro study, both MGO and TGF-β induced the expression of Snail, and MGO downregulated that of E-cadherin but not cytokeratin, while TGF-β suppressed the expression of both E-cadherin and cytokeratin. Although the reason for the differences is unclear, the expression of E-cadherin and cytokeratin may be regulated by other factors in addition to Snail. In our in vitro study, no morphologic change to the mesenchymal-like phenotype was observed in cultured rat mesothelial cells. It has been reported that MMP-2 is necessary for the EMT of epithelial cells [14], but cultured rat mesothelial cells hardly produce MMP-2 [13]. Indeed, in the present study, the gene expression level of MMP-2 in cultured rat mesothelial cells was <1/1000 of that in rats treated with 20 mM MGO (data not shown). Snail is a key regulator of EMT, but additional factors, such as MMP-2, may be necessary for the downregulation of cytokeratin expression and morphologic change to the mesenchymal-like phenotype in the EMT of rat mesothelial cells. More studies are required to clarify the mechanisms of EMT induced by MGO.

The proliferation of mesenchymal-like mesothelial cells was induced by 20 mM MGO in our animal model. Permitted daily exposure (PDE), which was recommended in guidelines established by the international conference on harmonization (Guideline for Residual Solvents Q3C. 1997), was performed to confirm that the concentration of MGO used in this animal experiment is appropriate as a clinical concentration [6]. From the result of the PDE analysis using our in vivo data, a risk of the proliferation of mesenchymal-like mesothelial cells in clinical PD exists at a concentration of >8 μM of MGO. Because conventional PDFs contain 2–33 μM MGO [1–3,22], the MGO in commercial PDFs has the potential to induce mesenchymal-like mesothelial cells to form. Moreover, conventional PDFs contain various GDPs, such as glyoxal and 3-deoxyglucosone, that induce the expression of Snail, besides MGO. Thus, it is possible that GDPs in commercial PDFs may induce mesenchymal-like mesothelial cells to proliferate in PD patients.

In conclusion, MGO induced peritoneal fibrous thickening with excessive proliferation of mesenchymal-like mesothelial cells in rats. These cells might be derived from mesothelial cells by EMT. MGO enhanced the production of TGF-β, collagen, MMP-2, VEGF and RAGE directly or via EMT. These factors may result in peritoneal functional decline, such as ultrafiltration failure and increased transport of small solutes, with reconstitution of the basement membrane, neovascularization or an increase in vascular permeability.

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References

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