The Therapeutic Potential of Human Umbilical Mesenchymal Stem Cells From Wharton’s Jelly in the Treatment of Rat Peritoneal Dialysis-Induced Fibrosis

Yu-Pei Fan, a, *, Ching-Hsi Hsia, b Kuang-Wen Tseng, c Chih-Kai Liao, d, * Tz-Win Fu, e, * Tsui-Ling Ko, f, * Mei-Miao Chi, g, h Yang-Hsin Shih, b, h, * Pei-Yu Huang, i, * Yi-Chia Chiang, j, *, Chih-Ching Yang, k, l Yu-Show Fu, m, n

Key Words. Umbilical cord • Mesenchymal stem cells • Transplantation • Peritoneal dialysis • Peritoneal fibrosis induced by dialysis • Submesothelial thickening

ABSTRACT

A major complication in continuous, ambulatory peritoneal dialysis in patients with end-stage renal disease who are undergoing long-term peritoneal dialysis (PD) is peritoneal fibrosis, which can result in peritoneal structural changes and functional ultrafiltration failure. Human umbilical mesenchymal stem cells (HUMSCs) in Wharton’s jelly possess stem cell properties and are easily obtained and processed. This study focuses on the effects of HUMSCs on peritoneal fibrosis in in vitro and in vivo experiments. After 24-hour treatment with mixture of Dulbecco’s modified Eagle’s medium and PD solution at a 1:3 ratio, primary human peritoneal mesothelial cells became susceptible to PD-induced cell death. Such cytotoxic effects were prevented by coculturing with primary HUMSCs. In a rat model, intraperitoneal injections of 20 mM methylglyoxal (MGO) in PD solution for 3 weeks (the PD/MGO 3W group) markedly induced abdominal cocoon formation, peritoneal thickening, and collagen accumulation. Immunohistochemical analyses indicated neoangiogenesis and significant increase in the numbers of ED-1- and α-SMA-positive cells in the thickened peritoneum in the PD/MGO 3W group, suggesting that PD/MGO induced an inflammatory response. Furthermore, PD/MGO treatment for 3 weeks caused functional impairments in the peritoneal membrane. However, in comparison with the PD/MGO group, intraperitoneal administration of HUMSCs into the rats significantly ameliorated the PD/MGO-induced abdominal cocoon formation, peritoneal fibrosis, inflammation, neoangiogenesis, and ultrafiltration failure. After 3 weeks of transplantation, surviving HUMSCs were found in the peritoneum in the HUMSC-grafted rats. Thus, xenografts of HUMSCs might provide a potential therapeutic strategy in the prevention of peritoneal fibrosis. 

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SIGNIFICANCE

This study demonstrated that direct intraperitoneal transplantation of human umbilical mesenchymal stem cells into the rat effectively prevented peritoneal dialysis/methylglyoxal-induced abdominal cocoon formation, ultrafiltration failure, and peritoneal membrane alterations such as peritoneal thickening, fibrosis, and inflammation. These findings provide a basis for a novel approach for therapeutic benefits in the treatment of encapsulating peritoneal sclerosis.

INTRODUCTION

Peritoneal dialysis (PD) is an attractive treatment for patients with end-stage renal failure. Several lines of evidence have shown that long-term peritoneal dialysis is often accompanied by functional and histopathological alterations in the peritoneum [1–4]. The characteristic features of chronic peritoneal damage in peritoneal dialysis include decreased ultrafiltration capacity associated with submesothelial fibrosis, accumulation of extracellular matrix, and angiogenesis [5, 6]. The decrease in ultrafiltration capacity following

*Contributed equally.

Correspondence: Yu-Show Fu, Ph.D., Department of Anatomy, School of Medicine, National Yang-Ming University, No. 155, Sec. 2, Li-Nung Street, Taipei 112, Taiwan, Republic of China. Telephone: 011-886-2-28267254; E-Mail: ysfu@ym.edu.tw; or Chih-Ching Yang, M.D., Ph.D., Department of Planning, Ministry of Health and Welfare, Executive Yuan, Taipei 11558, Taiwan, Republic of China. E-Mail: mdyangcc@mohw.gov.tw

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prolonged peritoneal dialysis is one of the important reasons for its discontinuation [7]. The pathogenesis of peritoneal fibrosis is attributed to a combination of bioincompatible factors in the dialysis solution, including high glucose levels [3], high osmolality, advanced glycation products [8], glucose degradation products [9], uremic inflammation [5], and acute peritonitis with inflammation [1, 10, 11]. At 2–6 years, ultrafiltration failure occurs in approximately 30% of patients undergoing PD [12]. The most important and difficult challenge for these patients is how to preserve peritoneal membrane integrity for a long term.

Several approaches in therapeutic interventions for the treatment of peritoneal fibrosis have been reported to be promising. These include the use of pharmacological strategies to inhibit the mesothelial-to-mesenchymal transition [13], elevated activities of submesothelial fibroblast-derived myofibroblasts [14], high levels of advanced glycation end products and their receptors [15], inflammation [16] and angiogenesis [17]. However, these pharmacologically based therapies might have limitations and undesirable side effects. By contrast, human embryos, umbilical cord blood, and bone marrow are common sources of stem cells with self-renewal capacity and differential ability, and, therefore, the offer potential benefits for many diseases [18]. Recent studies have demonstrated that transplantation of human and rat bone marrow-derived mesenchymal stem cells can attenuate peritoneal fibrosis by virtue of paracrine release of cytokines and chemokines [19, 20].

Human mesenchymal cells from Wharton’s jelly of the umbilical cord (HUMSCs) possess stem cell properties and are capable of differentiating into neurogenic, osteogenic, chondrogenic, adipogenic, and myogenic cells in vitro [21–23]. We also found that HUMSCs in the striatum and spinal cord were still viable 4 months after transplantation, without the need for immunological suppression, suggesting that HUMSCs might be a good stem cell source for transplantation [24–26]. Our previous study showed that sufficient amounts of HUMSCs in rat livers can secrete cytokines, reduce activation of hepatic stellate cells, enhance liver cell repair, and effectively cure liver fibrosis [27]. The potential of human umbilical mesenchymal stem cells to repair peritoneal sclerosis has not yet been evaluated to our knowledge. In this study, we investigated the effects of HUMSCs from Wharton’s jelly transplanted into rats in an established rat peritoneal sclerosis model.

**MATERIALS AND METHODS**

The use of human umbilical cord and laboratory animals in this study was approved by the Research Ethics Committee and the Animal Research Committee of the College of Medicine at National Yang-Ming University, Taipei, Taiwan.

**Preparation of Human Umbilical Mesenchymal Stem Cells**

With the written consent of the parents, fresh human umbilical cords were obtained postpartum and collected in Hanks’ balanced saline solution (HBSS; Gibco 14185-052; Thermo Fisher Scientific, Waltham, MA, https://www.thermofisher.com) maintained at 4°C. Following sterilization in 75% ethanol for 30 seconds, the umbilical cord vessels were cleared off while still in the HBSS. The mesenchymal tissue (in Wharton’s jelly) was then diced into cubes of approximately 0.5 cm³ and centrifuged at 250g for 5 minutes. The supernatant fraction was then removed, the precipitate (mesenchymal tissue) washed with serum-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco 12100-046; Thermo Fisher Scientific) and centrifuged at 250g for 5 minutes. Following aspiration of the supernatant fraction, the precipitates (mesenchymal tissue) were treated with collagenase at 37°C for 18 hours, washed, and further digested with 2.5% trypsin (Gibco 15090-046; Thermo Fisher Scientific) at 37°C for 30 minutes. Fetal bovine serum (FBS; HyClone SH30071.03; GE Healthcare Life Sciences, Pittsburgh, PA, http://www.gelifesciences.com) was then added to the mesenchymal tissue to neutralize the excess trypsin. The dissociated mesenchymal cells were further dispersed by treatment with 10% FBS-DMEM and counted under the microscope with the aid of a hemocytometer. The mesenchymal cells were then used directly for cultures.

**Peritoneal Mesothelial Cell Culture**

Human peritoneal mesothelial cells (HPMCs) harvested from omental tissues of consenting patients undergoing abdominal surgery were used for the culture. A selected intact mesothelial membrane was firmly clamped onto a base of cylindrical rings of various diameters (2–5 cm) to form isolation wells. The HPMCs were detached from the serosa by trypsin digestion (0.05%, weight per volume) and resuspended in DMEM supplemented with 10% FBS, antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) (Thermo Fisher Scientific), and 2 mmol/lL-glutamine. Several antibodies were used to check every batch of initially isolated mesothelial cells to ensure they were positive for the mesothelial markers cytokeratin and vimentin, and negative for the smooth-muscle marker desmin. The majority of the initial cultures exhibited the cobblestone appearance characteristic of pure mesothelial cells. HPMCs were used at the passages 3–4.

**Assay of HPMCs Damage in HPMC Culture Alone or HPMC and HUMSC Cocultures**

To explore the effect of HUMSCs on HPMC damage induced by PD, HPMCs were cultured alone or with HUMSCs in a special transwell system. The coculture system consisted of upper and lower chambers separated by a distance not physically traversable by the cells. The chambers, however, shared the same medium, which covered both cultures, thus allowing access to both cultures by humoral factors. Forming the bottom of the upper chamber was a porous membrane with multiple pores with a diameter of 8 μm that allowed medium across the membrane only but no actual mixing of the cells.

Primary HUMSCs were cultured in the upper chamber of the transwell coculture system, with HPMCs cultured in the lower chamber. These HUMSCs and HPMCs were treated with DMEM and with mixtures of DMEM and PD solution at ratios of 1:2, 1:3, and 1:4, respectively, for 24 hours. Then the upper transwell was removed, and the HPMCs in the bottom chamber were treated with propidium iodide (PI) to count the proportion of damaged cells.

**Assessment of Cell Damage**

PI is a fluorescent dye that binds to DNA but does not penetrate intact cell membranes. The permeability of the cell membrane is increased when the cell suffers damage and loses its membrane integrity. PI is then incorporated into the cell and binds to DNA. Positive staining of the nuclei thus indicates loss of membrane

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integrity and, therefore, is an index of cell injury. After performing the treatments, the cells were washed twice with 0.1 M phosphate buffer (PB) for 5 minutes each time, and then stained with 5 mg/ml PI for 30 minutes. The cells were then washed thoroughly with Tris buffer (50 mM Tris-HCl, pH 7.3). The staining fluorescence intensity, as measured by FACSort (BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com) was used to determine the percentage of damaged cells.

**Animal Model of Methylglyoxal in Peritoneal Dialysis-Induced Peritoneal Fibrosis**

The PD animal model was set up to use PD solution with methylglyoxal, as described previously by Hirahara et al. [28]. In brief, animals used in this study were male Sprague-Dawley rats, 8 weeks of age, weighing approximately 250 g. The animals were housed in an air-conditioned room maintained at a constant temperature of 23°C ± 2°C and a relative humidity of 50% ± 10%, were kept under a 12-hour light-dark cycle, and had free access to sufficient pellet food and water. The peritoneal dialysis fluid used in the study was a 4.25% glucose-based PD solution (4.25% glucose, 100 mmol/l NaCl, 35 mmol/l sodium lactate, 2 mmol/l CaCl₂, 0.7 mmol/l MgCl₂; Baxter Healthcare, Tokyo, Japan, http://www.baxter.com) and prepared by adding 20 mM methylglyoxal (MGO; Sigma M0252; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) to the PD solution to induce peritoneal fibrosis. The PD solution was prepared and adjusted to pH 5.2 just before the daily intraperitoneal injections (100 ml/kg body weight).

**Animal Grouping**

Rats were divided into 4 groups and given the following solutions: group 1 rats received only normal saline for 3 weeks (referred to as the saline 3W group); group 2 rats were treated with PD solution every day for 3 weeks (referred to as the PD 3W group); group 3 rats were treated with PD solution containing 20 mM MGO for 1, 2, or 3 weeks (referred to as the PD/MGO 1W, 2W, or 3W group, respectively); and group 4 rats were treated with PD solution containing 20 mM MGO for 3 weeks. At the first injection of PD+MGO, the rats also received a single injection of 10⁷ HUMSCs into the peritoneal cavity simultaneously. The volume of a pellet of HUMSCs is approximately 20 μl. The cell pellet was mixed in the PD/MGO solution. The rats treated with PD/MGO for 3 weeks were referred to as the PD/MGO 3W+HUMSCs group. Adequate attention was paid to maintaining a hygienic environment and to preventing infectious peritonitis. Furthermore, a sterility test was performed on the drained dialysate to check for the presence of aerobic bacteria, anaerobic bacteria, and fungi to confirm the uninfected status of the rats. After the experiments, samples were taken from corresponding sites in the visceral and parietal peritoneum for histological analyses.

**Protein Extraction and Western Blotting**

Peritoneal tissues were rinsed twice with PBS (4°C) and lysed with a lysis buffer containing 150 mmol/l NaCl, 1.5 mmol/l MgCl₂, 5 mmol/l EDTA, 1% Triton X-100, 1% NP40, 10 mmol/l NaF, 1 mmol/l Na₂VO₄, and a protease inhibitor cocktail (Roche, Indianapolis, IN, http://www.roche.com). The protein concentrations of the tissue homogenates were determined using the bicinechonic acid protein assay kit (Thermo Fisher Scientific). SDS-PAGE (10% acryl amide gel) and Western blot analyses were performed to quantify the amounts of type I collagen and α-tubulin.

**Quantitative Analysis of Peritoneal Tissues From Fresh Peritoneum**

Peritoneal collagen was determined using the Sircol Soluble Collagen Assay kit (Biodyne Science S1000; Biocolor, Carrickfergus, U.K., http://www.biocolor.co.uk). The rat peritoneal tissues were excised and frozen in liquid nitrogen. Peritoneal tissues of 50 mg each were homogenized and then centrifuged in 2.5 mg of pepsin in 0.5 M acetic acid solution. The supernatant was reacted with the Sicol dye reagent, and then centrifuged at 25,000g for 30 minutes. The resulting pellet was mixed with an alkali reagent. The red eluant was read immediately in a spectrophotometer at 540 nm (i.e., the wavelengths corresponding to the maximal absorbance of Sirius red).

**Histology and Immunohistochemistry**

At the end of the experiments, the rats were perfused with fixative (4% paraformaldehyde and 7.5% picric acid in 0.1 M PB). The peritoneum was excised and immersed in the same fixative at 4°C for 24 hours, and then switched to PB containing 30% sucrose before cryosectioning. Successive sections of the tissues were sliced at a thickness of 10 μm using a cryomicrotome and adhered to gelatin-pretreated slides.

Sections were stained with hematoxylin and eosin or 0.1% Sirius red for histological analysis. A parallel tissue sample was embedded in optimum cutting temperature compound (Sakura Finetechanical, Tokyo, Japan, http://www.sakura-finetek.com/) and frozen for immunostaining. To trace the survival of HUMSCs, immunostaining using anti-human-specific nuclear antigen was performed. The peritoneum sections were first reacted with a primary antibody (mouse anti-human-specific nuclear antigen, 1:25; Chemicon MAB1281, 1:100 [EMD Millipore, Billerica, MA, https://www.emdchem.com]) at 4°C for 18 hours, washed with 0.1 M PBS, reacted with secondary antibodies (biotin-conjugated goat anti-mouse-IgG, 1:300 diluted [Sigma-Aldrich]) at room temperature for 1 hour. The sections were then washed with 0.1 M PBS for 5 minutes 3 times. After the chromogenic reaction, the sections were placed under a cover slip and observed under a microscope.

To evaluate the changes of inflammatory cells and blood vessels, immunohistochemistry was performed by using primary antibodies against ED-1 (alternative name, CD68) to label macrophage (1:500; Chemicon; EMD Millipore) and mouse anti-α-smooth-muscle actin antibody (1:400; Sigma-Aldrich), followed by secondary antibodies (biotin-conjugated goat anti-mouse-IgG, 1:300 diluted; Sigma-Aldrich) and avidin-biotin-peroxidase complex (ABC kit, PK-4000; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). The 3,3′-diaminobenzidine (5 mg in 3.5 μl of 30% H₂O₂ and 10 ml of 50 mM Tris buffer) acted as a chromogenic reagent. Tissue sections were dehydrated, set in Permolt (SP15-500; Fisher Scientific, Pittsburgh, PA, https://www.fishersci.com), and placed under a cover slip.

**Analysis of Blood Vessel Profiles**

The sections were incubated in 25 μg/ml Griffonia simplicifolia 1 (GS1)-lectin (Vector Laboratories) for 60 minutes to label the capillaries and microcirculatory vessels [29]. Vessel density, defined as the proportion of the area occupied by the labeled blood vessels to that of the entire peritoneum, was measured and computed using ImagePro (MediaCybernetics, Rockville, MD, http://www mediacy.com).
Quantification of Transforming Growth Factor-β
Protein samples from rat peritoneum tissue lysed in a protein lysis buffer containing phosphatase and protease inhibitors were collected for enzyme-linked immunosorbent assay (ELISA). To activate latent transforming growth factor (TGF)-β to the immunoreactive form, 1 N HCl and 1.2 N NaOH/0.5 M HEPES was prepared for acid activation and neutralization. TGF-β was determined using Quantikine ELISA kit (R&D Systems, Minneapolis, MN, https://www.rndsystems.com), according to the manufacturer’s protocol.

Peritoneal Equilibration Test
To analyze peritoneal functions, peritoneal permeability of glucose was estimated by a peritoneal equilibration test modified from that of Twardowski et al. [30] and applied to the rats. First, 30 ml of 4.25% glucose PD was injected into the peritoneal cavity. Four hours later, a tail blood sample and a dialysate sample drained from the abdominal cavity were obtained. Drained dialysate was collected immediately (0 minute [D₀]) and 4 hours after administration (D₄). Glucose levels were assessed in the Department of Medical Technology, Taipei Veterans General Hospital, using Binesion (Dimension Rxt. Max, http://www.binesion.net/). The ratio of the glucose levels in D₀ and D₄ in drained dialysate was defined as the D₄/D₀ glucose level. Net ultrafiltration volume was obtained from the volume of dialysate that was drained 4 hours after administration. The dialysate to plasma creatinine ratio at 4 hours after administration (D₄/P₄ creatinine) was used as the solute transport parameter.

Positron Emission Tomography Imaging
Static abdominal and pelvic positron emission tomography (PET) imaging was performed 45 minutes after an intravenous injection of 10 millicurie of sterile ¹⁸F-fluorodeoxyglucose ([¹⁸F]-FDG). A GE/Scanditronix PET camera (PC4096-15WB; GE Healthcare, Uppsala, Sweden, http://www.gelifesciences.com) was used to make positron images. A VAX computer (Digital Equipment Corporation, Maynard, MA) was used as the network server. PET images were visualized using a VAX workstation in which DECnet with Pathwork (Digital Equipment Corporation) was used to connect personal computers to the VAX so that PET images could be visualized with software running on Windows 3.1 (Microsoft, Redmond, WA, https://www.microsoft.com). PET images were obtained preoperatively and visually analyzed to identify areas of localized [¹⁸F]-FDG uptake compared with the surrounding tissues. These areas were considered abnormal and suspicious of recurrent lesions. All PET interpretations were performed blinded to the conventional imaging and surgical findings.

Human Cytokine Array
A human protein cytokine kit (R&D Human Cytokine Array; R&D Systems) was used to detect the expression and levels of 85 cytokines, chemokines, or proteins. HUMSCs (5 × 10⁵) were seeded in 100-mm culture dishes and cultured in 2% bovine serum albumin serum-free DMEM. When the HUMSCs reached confluence, the medium was collected, centrifuged at 300g for 5 minutes, and, finally, filtered using a 0.22-μm syringe filter. The HUMSC-conditioned medium (1 ml; 500 μg of protein) was incubated with the membranes containing an array of human cytokine antibodies (n = 3). The levels of cytokine expression were determined by the intensity of immunoreactivity, following the manufacturer’s instructions.

Statistical Analysis
All data are presented as mean ± SE. One-way analysis of variance was used to compare all means and Fisher’s least significant difference for the posteriori test. In all statistical analyses, p < .05 was considered significant.

RESULTS
Effects of PD Solution on the Morphology and Viability of Primary HPMCs and HUMSCs
To investigate whether peritoneal dialysis solution causes cell damage, in vitro cultures of the HPMCs and HUMSCs were incubated for 24 hours in mixtures of serum-free DMEM and 4.25% dextrose PD solution at ratios of 1:2, 1:3 or 1:4, respectively, then subjected to cell damage assessments by inverted phase-contrast microscopy and flow cytometry. No significant changes were observed in morphology (Fig. 1A–1D) in the HUMSCs following incubation in any combinations of the incubation media. There was no statistical change in damage rate (1.33%, 1.33%, 0.88%, and 0.95% in DMEM, 1:2, 1:3, and 1:4 mixtures of DMEM:PD solution, respectively) (Fig. 1A1–D1, 1E). Confluent primary HPMCs exhibited a cobblestone appearance (Fig. 1F). HPMCs treated with a 1:2 mixture had no morphological change (data not shown). However, exposure of HPMCs to a 1:3 mixture showed observable morphological disturbances and apoptosis-like cell debris (Fig. 1G) that were preventable by coculturing with HUMSCs (Fig. 1I, 1H). Analyses of cell viability in the HPMCs by flow cytometry following PI staining indicated overlapping of fluorescence histograms. Figure 1J shows three distinguishable peaks, from left to right, indicating, respectively, the background, and low and high levels of PI fluorescence. Treatment of HPMCs with a 1:3 mixture for 24 hours caused a shift to the high PI fluorescent peak, indicative of cell damage (DMEM:PD solution: 1:3) (Fig. 1J), an effect again preventable by coculturing with HUMSCs (DMEM:PD solution: 1:3 with HUMSCs) (Fig. 1J, 1I). The PI fluorescence assay showed that the percentage of cell death in PD-treated HPMCs was significantly increased by 62.13% ± 8.62% (DMEM:PD solution: 1:3) (Fig. 1J) as compared with monoculturing of HPMCs (p < .05) (Fig. 1K, 1N). How- ever, in transwell coculturing of fibroblasts and HPMCs, there was no significant change in the percentage of damage of HPMCs after treatment with DMEM:PD solution at the 1:3 ratio, as compared with monoculturing of HPMCs when exposed to the 1:3-mixture culture medium (p > .05). Using the transwell culturing system, coculturing HPMCs with HUMSCs reduced the percentage of HPMC death to 26.95% ± 7.79% (DEME: PD solution: 1:3 with HUMSCs; p < .05) (Fig. 1M, 1N) as compared with monoculturing of HPMCs when exposed to the 1:3 mixture culture medium, indicating a protective role of HUMSCs in peritoneal dialysis solution-induced HPMC death.

Suppression of PD/MGO-Induced Abdominal Cocoon Formation and Peritoneal Thickening After Transplantation of HUMSCs
The peritoneum in the control rats (the saline 3W group) (Fig. 2A), the PD 3W group (Fig. 2B), and the PD/MGO 1W group (Fig. 2C) appeared transparent and glossy. Treatment of rats with PD/MGO
for 2 weeks resulted in the partial encasement of the intestines by a thickened and fibrotic peritoneum (the PD/MGO 2W group) (Fig. 2D). The fibrotic peritoneum became whitish, thickened, and was extended to wrap around other proximal organs such as the stomach, liver and intestines, forming an abdominal cocoon in the PD/MGO 3W group (Fig. 2E). In the PD/MGO 3W+HUMSCs group, there was no observable formation of abdominal cocoon, but loops of small intestine were partially encased by a fibrotic peritoneum (Fig. 2F).

After perfusion fixation, the liver surfaces were smooth and glossy in the saline 3W (Fig. 2A1) and the PD 3W group rats (Fig. 2B1). In the PD/MGO 1W group, part of the liver surface...
was covered by a whitish, fibrotic peritoneum (Fig. 2C1). In rats treated with PD/MGO for 1 week (Fig. 2C2, 2C3), 2 weeks (Fig. 2D2, 2D3), or 3 weeks (Fig. 2E2, 2E3), the liver surfaces appeared encased by a thickened peritoneum, which appeared coarser, and the gross appearance of the liver edge became rounded. In the PD/MGO 3W+HUMSCs group, the liver surfaces remained smooth and lustrous (Fig. 2F2, 2F3).

To investigate changes in the thicknesses of the parietal and visceral peritoneums of rats treated with PD/MGO alone and those also transplanted with HUMSCs, histochemical staining was performed with H&E to distinguish changes in the mesothelial layer and the submesothelial zone in the peritoneal membrane. In the saline 3W group, an intact mesothelial monolayer of the parietal peritoneum and the visceral peritoneum on the liver covering a thin layer of submesothelial connective tissue was observed (Fig. 3A, 3A1, 3G, 3H). There were no morphological alterations in the PD 3W group (Fig. 3B, 3B1). Treatment with PD/MGO for 1 week caused a slight increase in the submesothelial zone (Fig. 3C, 3C1, 3G, 3H). A small thickening of the submesothelial zone was observed in rats treated with PD/MGO for 2 weeks (Fig. 3D, 3D1, 3G, 3H). After 3 weeks of PD/MGO treatment, a thick layer of submesothelial connective tissue appeared in the peritoneum (Fig. 3E, 3E1, 3G, 3H). In the PD/MGO 3W+HUMSCs group, the submesothelial became slightly thicker but nonetheless thinner than that in the PD/MGO 2W and PD/MGO 3W groups (Fig. 3F, 3F1, 3G, 3H).

**Figure 2.** Transplantation of HUMSCs prevents the PD/MGO-induced formation of abdominal cocoon and thickening of the parietal and visceral peritoneum. Photographs showing the opened abdominal cavity (A-F), the liver surface (A1–F1), the H&E-stained parietal peritoneum (A2–F2), and the H&E-stained visceral peritoneum (A3–F3). Control rats were treated with saline (A–A3). Experimental rats were treated with PD solution for 3 weeks (B3), PD/MGO for 1 week (C–C3), 2 weeks (D–D3), or 3 weeks (E–E3). The HUMSC-transplanted rats were treated with PD/MGO for 3 weeks. (G, H): Data for the parietal (G) and visceral peritoneal thicknesses (H) were respectively collected from six independent experiments, in each of which samples from six random fields per section and six sections per biopsy specimen were analyzed statistically using the Fisher’s least significant difference post hoc test. ∗, p < .05, compared with the saline 3W group; #, p < .05, compared with the PD/MGO 3W group or PD/MGO 3W + HUMSCs group.

Transplantation of HUMSCs Prevents PD/MGO-Induced Peritoneal Fibrosis

To examine possible effects of PD/MGO treatment and HUMSC transplantation on the peritoneal fibrosis, Sirius red staining was used to identify collagen fibers in the parietal peritoneums and the visceral peritoneum on the livers, shown as reddish patterns in the submesothelial connective tissue. Histological examinations indicated that Sirius red stained-collagen fibers were scarce in the thin submesothelial zone in the saline 3W group (Fig. 3A, 3A1, 3G, 3H), the PD 3W group (Fig. 3B, 3B1), and the PD/MGO 1W group (Fig. 3C, 3C1, 3G, 3H). By contrast, large numbers of the
Sirius red-stained collagen fibers and thickening of the submesothelial zone were observed in the PD/MGO 2W group (Fig. 3D, 3D1, 3G, 3H). After 3 weeks of PD/MGO treatment, the Sirius red-stained collagen fibers were even more abundant in the previously thickened submesothelium (Fig. 3E, 3E1, 3G, 3H). In the PD/MGO 3W+HUMSCs group, the Sirius red-stained collagen fibers and the submesothelial thickness were markedly decreased to nearly those in the saline 3W group (Fig. 3F, 3F1, 3G, 3H).

The amount of collagen in the fresh mesentery was also quantified using the Sircol assay. As shown in Figure 3I, collagen content was increased significantly ($p < .05$) in the PD/MGO 3W group as compared with the saline 3W group; #, $p < .05$, compared with the PD/MGO 3W+HUMSCs group using Fisher’s least significant difference post hoc test. Abbreviations: HUMSC, human umbilical cord mesenchymal stem cell; PD/MGO, peritoneal dialysis solution/methylglyoxal; PD + MGO 1W (2W; 3W), treated with peritoneal dialysis solution plus methylglyoxal for 1 week (2 weeks; 3 weeks); PD + MGO 3W + HUMSCs, treated with peritoneal dialysis solution plus methylglyoxal plus human umbilical cord mesenchymal stem cells for 3 weeks; Saline 3W, 3-week treatment with saline (control).

Reduction of PD/MGO-Induced Inflammation, \(\alpha\)-Smooth-Muscle Actin Expression, Neoangiogenesis, and TGF-\(\beta\) Activity After Transplantation of HUMSCs

Macrophage infiltration in the parietal peritoneum was assessed immunohistochemically using ED-1 antibody for the identification and enumeration of macrophages. Only a few ED-1-positive macrophages were observed in the saline 3W group (Fig. 4A). After 3 weeks of PD/MGO treatment, the number of ED-1-positive macrophages was markedly increased in the upper part of the thickened peritoneum (Fig. 4B). Transplantation of HUMSCs into the rats prevented the PD/MGO-induced macrophage infiltration in the parietal peritoneum (Fig. 4C).
Immunohistochemical staining for α-smooth-muscle actin (α-SMA) as a myofibroblast marker in the parietal peritoneum was next performed. As shown in Figure 4A1, few α-SMA-positive cells were observed in the parietal peritoneum of the saline 3W group (Fig. 4A1). Treatment of rats with PD/MGO for 3 weeks caused an increase in the numbers of α-SMA-positive cells (Fig. 4B1), an effect prevented by HUMSC transplantation (Fig. 4C1).

Immunohistochemical labeling of GS1-lectin was performed to quantify microvessels in the parietal peritoneal tissue. In the saline 3W group, peritoneal GS1-lectin staining was sparse (Fig. 4A2), and the areas, lengths, and number of microvessels were low (Fig. 4D–F). After 3 weeks of treatment with PD/MGO, peritoneal GS1-lectin staining was intense (Fig. 4B2), with the areas, lengths, and number of microvessels significantly increased as compared with the saline 3W group (p < .05) (Fig. 4D–F). All parameters in the PD/MGO 3W+HUMSCs group, including GS1-lectin staining areas, lengths, and number of microvessels returned to levels not significantly different from the saline 3W group (p > .05) but significantly lower than those in the PD/MGO 3W group (p < .05) (Fig. 4D–F).

A significant increase in TGF-β concentration in the PD/MGO 3W group was observed compared with that of the saline 3W group. The PD/MGO 3W+HUMSCs group had lower level of TGF-β compared with the PD/MGO 3W group. There was no statistically significant difference between saline 3W and PD/MGO 3W+HUMSCs groups in terms of TGF-β level (Fig. 4G).

Inflammation was qualitatively evaluated by monitoring changes in metabolic activity using [18F]-FDG microPET imaging and quantified by estimation of the ratio of brain to intestinal glucose uptake. Low metabolic activity was determined at baseline before PD/MGO treatment (0 week treatment) (Fig. 5A, 5G). After PD/MGO treatment for 3 weeks, a significant increase in the metabolic activity was seen compared with 0 week treatment (p < .05) (Fig. 5B, 5G). After PD/MGO treatment for 8 weeks, the metabolic activity was significantly higher than at PD/MGO pretreatment (p < .05) and was no different from...
toward that found in the saline 3W group (\( p < .05 \)) compared with the PD/MGO 3W group (\( p < .05 \)), but significantly higher than the PD/MGO 3W group (\( p < .05 \)) (Fig. 6B).

The effectiveness of the peritoneal dialysis was estimated by the 4-hour dialysate to plasma ratio (\( D_4/P_4 \)) of creatinine removal. The \( D_4/P_4 \) ratio in the saline 3W rats was low (Fig. 6C). Treatment of rats with PD/MGO for 3 weeks caused a significant increase in the \( D_4/P_4 \) ratio as compared with the saline 3W group (\( p < .05 \)). In the PD/MGO 3W+HUMSCs group, the \( D_4/P_4 \) ratio decreased, and was significantly lower than that found in the PD/MGO 3W group (\( p < .05 \)) but was not significantly different from that in the saline 3W group (\( p > .05 \)) (Fig. 6C).

Immunohistochemical staining using anti-human-specific nuclear antigen was performed to detect the survival of HUMSCs in the parietal peritoneal tissues after transplantation. In the PD/MGO 3W+HUMSCs group, transplanted HUMSCs survived but were scattered in the visceral peritoneum of the intestines (Fig. 6D, 6E).

**Cytokines Released by HUMSCs**

To examine protein levels of cytokines released by HUMSCs, we performed human antibody-based protein array analysis of HUMSC-conditioned medium, which reacted to 85 cytokines. High levels of chemokine (C-X-C) motif ligand 1 (CXCL-1), macrophage migration inhibitory factor (MIF), interleukin 8 (IL-8), plasminogen activator inhibitor-1 (PAI-1), TIMP metalloproteinase inhibitor 1 (TIMP-1), pentraxin-related protein PTX3 (PTX3), and insulin-like growth factor-binding protein 3 (IGFBP3) were found in the conditioned medium of HUMSCs. Furthermore, there were small quantities of matrix metalloproteinase 9 (MMP-9) and thrombospondin 1 in the HUMSC-conditioned medium (Fig. 6F). These findings suggest that HUMSCs can release growth-associated human cytokines to execute the protective and anti-inflammatory effects on peritoneal fibrosis.

**DISCUSSION**

Previous studies have shown that HPMCs are susceptible to 3.86%–4.25% dextrose PD fluid (PDF)-induced cell death [31, 32]. In this context, our present study provided another approach to evaluate cell death in primary HPMCs and HUMSCs by prolonged treatment with a mixture of serum-free DMEM and 4.25% dextrose PDF. Large numbers of HPMCs were dead and morphologically changed, but cell viability in HUMSCs remained unaffected until treated with a 1:3 mixture for 24 hours. However, coculturing with HUMSCs effectively prevented the dextrose PDF-induced cell death and morphological alterations in the primary HPMCs (Fig. 1).

It is known that glucose degradation products in PDF are responsible for causing damage and fibrosis to the peritoneal membrane [33]. Previous experiments showed that heat-sterilized PDF contains several intermediate products of glycation, including MGO, which are cytotoxic to HPMCs [34–37]. In an in vivo study, thickness of the parietal and visceral peritoneal membranes increased approximately 2- and 5-fold, respectively, when treated with peritoneal dialysis solution containing 3.86% glucose for 12 weeks [38]. To obtain rapid peritoneal fibrosis, rats were treated with daily administration of 2.5% glucose PDF containing 20 mM MGO for 3 weeks [28]. Taking into consideration the results reported by Hirahara et al. [28], we successfully established the peritoneal fibrosis model as seen by thickening of the parietal...
peritoneum and formation of abdominal cocoon in rats receiving 4.25% PDF containing 20 mM MGO for 3 weeks (Fig. 2). Moreover, in our peritoneal fibrosis model, thickness of the visceral and parietal peritoneums increased significantly more than those previous reported, plausibly because of differences in the dialysis solution compositions (Fig. 2).

Multiple lines of evidence have suggested that TGF-\(\beta\) is the key mediator in the development of peritoneal fibrosis [39, 40]. TGF-\(\beta\) is involved in the pathways of extracellular matrix deposition, angiogenesis, immune modulation, cell cycle regulation, and transdifferentiation to the mesenchymal phenotype [39, 41, 42]. Margetts et al. [43, 44] directly showed the importance of TGF-\(\beta\) in peritoneal fibrosis (PF) using the models of adenovirus-mediated TGF-\(\beta\) gene transfer to the peritoneum, which caused PF and neoangiogenesis. Consistent with these findings, we found the PD+MGO rats had the highest TGF-\(\beta\) activity (Fig. 4G). At the same time, as revealed by Sirius red staining, Western blot analyses, and Sircol assays, PD/MGO treatment for 3 weeks caused a significant increase in protein levels and contents of collagen, which might sequentially lead to parietal and visceral fibrosis (Fig. 3). We also showed that PD/MGO treatment for 3 weeks increased \(\alpha\)-SMA-positive cells in the submesothelial thickening, implying PD/MGO-induced mesothelial to myofibroblast transition (Fig. 4A–4E).

In previous studies, i.p. or systemic administration of various drugs has been used to alleviate TGF-\(\beta\)-mediated fibrosis [45–53]. Recently, TGF-\(\beta\)1 small interfering RNAs (siRNAs) encapsulated in nanoparticles dissolved in PD fluid were injected into the peritoneum of mice with PF 3 times a week for 2 weeks. TGF-\(\beta\)1 siRNA nanoparticles knocked down TGF-\(\beta\)1 expression significantly in the peritoneum and inhibited peritoneal thickening with fibrous changes. TGF-\(\beta\)1 siRNA nanoparticles also inhibited the increase of expression of \(\alpha\)-SMA-positive myofibroblasts [54]. Those results suggest that suppression of TGF-\(\beta\)1, one of the causes of peritoneal fibrosis, may provide therapeutic benefit of peritoneal fibrosis in clinical treatment.

Glucose degradation products such as MGO in heat-sterilized PDF promote formation of advanced glycation end-products (AGEs) [55]. Mediated by their receptors, AGEs activate the transcriptional factor NF-\(\kappa\)B and upregulate TGF-\(\beta\)-1, inducing, in turn, inflammatory responses and peritoneal fibrosis [8, 56–58]. In rats receiving 4.25% glucose dialysate with the addition of lipopolysaccharide, the increased number of OX-1-positive leukocytes and ED-1 macrophages infiltrated the thickening of visceral peritoneum [59]. In the present study, microPET imaging and

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**Figure 6.** Transplanted HUMSCs are localized in the peritoneum and prevent the PD/MGO-induced functional losses of the peritoneal membrane. Comparison of PD effectiveness in rats treated with saline, PD/MGO alone, or PD/MGO+HUMSCs for 3 weeks. (A–C): Following 4 hours of PD treatment, peritoneal functions were assessed by monitoring the ascitic drain volume (A), ratios of peritoneal glucose uptake at \(D_0/D_4\) (B), and Cr removal as represented by \(D_4/P_4\) (C). Treatment with PD/MGO caused ultrafiltration failure in PD/MGO-treated rats and this effect was prevented by transplantation of HUMSCs. *, \(p < .05\), as compared with the saline 3W group; #, \(p < .05\), compared with the PD/MGO 3W groups by Fisher’s least significant difference post hoc test (n = 7). (D, E): Representative photomicrographs show the histology of the visceral peritoneum covering small intestines from rats transplanted with HUMSCs for 3 weeks immunostained for human-specific nuclei antigen. Arrows indicate the cell bodies that stained positively. (F): Several human cytokines are detectable in HUMSC-conditioned medium. Frames indicate CXCL-1, MIF, IL-8, PAl, TIMP-1, PTX3, and IGFBP3. Red lines indicate MMP-9 and thrombospondin-1. Arrowheads indicate the positive control. Abbreviations: Cr, creatinine; \(D_0/D_4\) peritoneal glucose uptake at time 0 and at 4 hours; \(D_4/P_4\), peritoneal dialysate and plasma following 4 hours of perfusion; HUMSC, human umbilical cord mesenchymal stem cell; PD, peritoneal dialysis solution; PD/MGO, peritoneal dialysis solution/methylglyoxal; PD + MGO 3W, treated with peritoneal dialysis solution plus methylglyoxal for 3 weeks; PD + MGO 3W + HUMSCs, treated with peritoneal dialysis solution plus methylglyoxal plus human umbilical cord mesenchymal stem cells for 3 weeks; Saline 3W, 3-week treatment with saline (control).
immunohistochemical analyses demonstrated elevated abdominal metabolic activities and infiltration of ED-1-positive macrophages in the PD/MGO 3W group, suggesting that PD/MGO treatment effectively induced inflammation in the rat abdominal cavity (Figs. 4, 5).

In primary rat mesothelial and human endothelial cells, MGO-containing PDF upregulated the expression of vascular endothelial growth factor (VEGF) and consequently highlighted angiogenesis [60]. In patients undergoing peritoneal dialysis, morphological changes such as submesothelial thickening, peritoneal vasculopathy, and ultrafiltration failure were reported [5, 61]. Lo et al. showed that injection of chlorhexidine gluconate in rats resulted in a significant increase in the vessel areas, diameters, and lengths [17]. In agreement with those findings, in our present study, immunohistochemical staining of GS1-lectin revealed that the areas, lengths, and numbers of capillaries and microcirculatory vessels were markedly increased in the PD/MGO 3W rats (Fig. 4).

For decades, transplantation of pluripotent stem cells has been considered a potential therapeutic candidate in regenerative medicine to cure fibrotic diseases. Previous studies have demonstrated that transplantation of bone marrow-derived mesenchymal stem cells (BM-MSCs) reduces inflammatory responses and accumulation of extracellular matrix, considered responsible for the functional impairments in renal and liver fibroses [62, 63].

It is interesting to find that the peritoneal fibrosis caused by different methods results in the different effects of BM-MSC transplantation. Intravenous injection of 5.0 × 10⁶ rat BM-MSCs into Sprague-Dawley rats via the tail vein apparently attenuated scraping-induced peritoneal inflammation, adhesions, and fibrosis. However, such a treatment showed only partial improvement in peritoneal fibrosis characteristics, with the reduction being less than 50% [19]. Similarly, in the chlorhexidine gluconate-induced peritoneal fibrosis model, intraperitoneal injection of 1.0 × 10⁷ human BM-MSCs reduced inflammation, thickening, and fibrosis of the parietal peritoneum, as well as functional impairments of the peritoneal membrane. Unfortunately, however, improvements attainable by such a treatment appeared to be only minor [20]. However, rat BM-MSC transplantation intraperitoneally in peritoneal fibrosis induced by dialysis may protect the peritoneal membrane from the deleterious effect of PD [64]. Similarly, HUMSC transplantation significantly ameliorated the PD/MGO 3W-induced peritoneal fibrosis to nearly normal levels following 3 weeks of intraperitoneal transplantation in this study. Furthermore, HUMSC transplantation suppressed TGF-β activity significantly.

Recent studies suggest that different interventions result in differing efficacies. In a study by Wang et al. [65], rat bone marrow-derived MSCs were injected either intraperitoneally or intravenously into Sprague-Dawley rats after peritoneal scraping. Intravenously injected MSCs were found to accumulate in the lung and to attenuate peritoneal adhesion by secreting TSG-6, but intraperitoneally injected MSCs were phagocytized by macrophages in the spleen and failed to attenuate peritoneal adhesion [65]. However, approximately one-thousandth of transplanted HUMSCs were found in the rat peritoneum in this study. These surviving HUMSCs were only scattered over the peritoneum of the surface of liver, spleen, and intestines. It was hard to find the HUMSCs in the parenchyma of peritoneal organs (Fig. 6). We suggest that the different kinds of mesenchymal stem cells have distinct properties of homing, migration, and chemotaxis.

The timing of HUMSC injection may represent different meanings and goals. At the first injection of PD+MGO in this study, the rats received a single injection of 10⁷ HUMSCs into the peritoneum. HUMSC transplantation demonstrated the prevention and protection of peritoneal fibrosis induced by dialysis. In another study, after 3 weeks of injection of PD+MGO, the rats received multiple injections of HUMSCs into the peritoneum (manuscript submitted for publication). The results revealed that HUMSC transplantation displayed reversal of and therapeutic effects on peritoneal fibrosis induced by dialysis (manuscript submitted for publication).

Several lines of evidence have demonstrated that the engrafted stem cells produce and release paracrine factors such as cytokines, chemokines, and growth factors that may contribute to therapeutic interventions. Using cytokine array analysis, TNF-α-stimulating gene-6 was singularly identified as contributory to the reduction of the peritoneal injury [19]. Moreover, human BM-MSCs suppress epithelial-to-mesenchymal transition (EMT) of primary HPMCs and peritoneal fibrosis by inhibiting expressions of TGF-β1 and fibronectin [20]. It has been also reported that TGF-β1 activation induces expression of type I collagen and α-smooth muscle actin, leading to EMT of mesothelial cells and peritoneal fibrosis [43]. Treatments of HMPCs with antifibrotic cytokines such as morphogenic protein 7 result in blockage of EMT and peritoneal fibrosis induced by high glucose levels [66]. Likewise, our previous study using cytokine array analysis demonstrated that large amounts of human neutrophil-activating protein-2, neurotrophin-3, and vascular endothelial growth factor receptor 3 were produced by HUMSCs in the transected spinal cords of rats, contributing to spinal-cord repair [25]. In addition, expressions of the cutaneous T-cell-attracting chemokine, leukemia inhibitory factor, and prolactin were increased significantly in HUMSC-grafted livers, which may help relieve CCL2-induced liver fibrosis [27]. From our cytokine assay results, CXCL-1, MIF, IL-8, PAI-1, TIMP-1, PTX3, IGFBP3, MMP-9, and thrombospondin-1 were found in the HUMSC-conditioned medium. The previous studies showed that both BM and amnion MSCs expressed CXCL-1, IL-8, and PAI-1 [67]. The expression of IL-8 and CXCL-1 might be involved in the mechanisms of angiogenesis and self-renewal [68]. Results of a wound healing study showed that burn wound-activated adipose tissue-derived MSCs increased secretion of CXCL-1, indicating the activation may promote wound healing [69]. It has been reported that MIF might be a potential therapeutic factor and capable of activating stem cells or progenitor cells for the treatment of degenerative brain disorders [70]. We speculate that some other cytokines and growth factors beyond the 85 we assessed may also stimulate the regeneration of the fibrotic peritoneum.

**CONCLUSION**

We have demonstrated that direct xenograft of HUMSCs into the rat intraperitoneum effectively prevented PD/MGO 3W-induced abdominal cocoon formation, ultrafiltration failure, and peritoneal membrane alterations such as peritoneal thickening, fibrosis, and inflammation. These findings provide a basis for a novel approach with therapeutic benefits in the treatment of encapsulating peritoneal sclerosis.

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**AUTHOR CONTRIBUTIONS**

Y-P.F.: collection and/or assembly of data, manuscript writing; C.C.H.: financial support; K.-W.T.: provision of study material; C-K.L., T-W.F., T-L.K., M-M.C., Y-H.S., P-Y.H. and Y-C.C.: collection and/or assembly of data; C-C.Y.: financial support, data analysis and interpretation; Y-S.F.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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