The Selection of Peritoneal Mesothelial Cells Is Important for Cell Therapy to Prevent Peritoneal Fibrosis

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Long-term peritoneal dialysis (PD) causes chronic peritoneal damage. Peritoneal mesothelial cells (PMCs) play an important role in peritoneal function. We investigated the possibility of cell therapy using the PMCs to prevent peritoneal damage in PD patients. We harvested human PMCs from the PD effluent of PD patients. The PMCs were separated based on morphological characteristics into epithelial-like (Epi) cells and fibroblast-like (Fib) cells by the limiting dilution method. We transplanted these cells into nude mice whose parietal and visceral peritoneum were scratched by mechanical scraping. The transplanted cells were detected at the parietal and visceral peritoneum. Compared with the positive control, the Epi cell therapy group showed very few adhesions and exhibited no thickening of the parietal and visceral peritoneum. However, the group with Fib cell therapy could not inhibit peritoneal adhesion and thickening. In addition, hepatocyte growth factor was expressed by the grafted Epi cells but not Fib cells. Fib cells expressed vascular endothelial growth factor stronger than Epi cells. These two types of cells from the same patient showed different characteristics and effects for cell therapy. These findings suggest that the PMCs from the PD patient showed different characteristics, such as Epi cells and Fib cells, and the selection of PMCs is important for cell therapy on the point of not only the direct cellular interactions but also cytokine secretion from the grafted cells. Furthermore, the differences in the morphological cell characteristics may influence their role in peritoneal regeneration.

Introduction

IN PATIENTS UNDERGOING long-term peritoneal dialysis (PD), the peritoneum may be damaged by repeated stimulations with peritoneal dialysate. Peritoneal mesothelial cells (PMCs) have been reported to play an important role in peritoneal fibrosis (PF), which involves the epithelial-mesenchymal transition (EMT) of mesothelial cells and the neovascularization of the peritoneum.¹ It is thought that changes in the peritoneum are associated with multiple factors, including the stimulation from the long duration resulting from PD treatment, infection, the uremic state, the use of hypertonic dialysate, high concentrations of glucose and lactate, acidic pH, glucose degradation products, and the activation of inflammatory cytokines and various growth factors.² The pathogenesis of peritoneal damage is not well understood, and therapeutic targets for peritoneal damage have not yet been established.

PMCs are an important component in the structure and function of the peritoneum. Recent reports have suggested that PMCs may possess the ability to regenerate and differentiate.^{3,4} It is thought that PMC transplantation could restore chronic peritoneal damage in PD patients, and the first

mesothelial cell transplantation was reported in 1989.³ Several additional studies have followed up this work.^{5,6} Bertram *et al.* reported that the intraperitoneal transplantation of isologous mesothelial cells prevented adhesion in a rat peritoneal abrasion model.⁵ However, Hekking *et al.* reported that mesothelial cells transplanted into animal models of experimental peritonitis contributed to the activation and increased duration of the inflammatory state.⁶ The efficacy of mesothelial cell transplantation is still unclear. We investigated whether PMC therapy prevents PF and studied important factors associated with cell therapy in a mouse peritoneum-scraping model.

Materials and Methods

Epithelial- and fibroblast-like PMCs harvested from human PD effluent

Human mesothelial cells were harvested by centrifugation of 50 mL of dialysis fluid taken from stable patients undergoing continuous ambulatory PD. Cells were cultured in K-1 modified medium, which consisted of K1 medium (DMEM/F12 medium; Gibco) supplemented with 10% fetal calf serum, 5 µg/mL insulin, 2.75 µg/mL transferrin, 3.35 ng/mL

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sodium selenium (ITS-X; Gibco), 50 nM hydrocortisone (Sigma), 6.25 ng/mL hepatocyte growth factor (HGF; Sigma), 2.5 mM nicotinamide (Sigma), and 6.25 ng/mL fibroblast growth factor (FGF-b; Calbiochem). The cell suspensions were transferred into the wells of 96-well plates pre-coated with fibronectin (BD Bioscience). Cells were seeded at 1×10^2 – 1×10^3 cells/well. After 14–21 days, the cell colonies were separated into two morphologically different groups. The morphologic features of the first group of cell colonies included a cobblestone appearance which resembled that of epithelial cell colonies. The second group was composed of the fibroblast-like (Fib) cell colonies. These cells were maintained with K-1 modified medium, and the cells were replated in wells of six-well plates or 75-cm² flasks pre-coated with fibronectin (BD Bioscience) to expand the cell population. Samples were used for phase-contrast imaging and immunocytochemistry.

Immunocytochemistry

Cell morphology was analyzed under an FSX100 inverted phase-contrast microscope (Olympus Corporation). For immunofluorescence staining, cells were washed and fixed in 4% phosphate-buffered paraformaldehyde (15 min at room temperature [RT]) and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) (15 min at RT). After washing with PBS, the cells were treated with 1% bovine serum albumin in PBS for 15 min at RT before incubation with primary antibodies that were specific for pan-cytokeratin (Santa Cruz Biotechnology), FSP-1 (abcam), and collagen type I (Millipore) in 1% BSA in PBS for 1 h at 37°C. The cells were then washed with PBS before incubation with goat anti-mouse IgG-FITC-conjugated secondary antibody (Santa Cruz Biotechnology) and chicken anti-rabbit IgG(H+L)-Alexa Flour secondary antibody (Invitrogen) for 1 h at 37°C in the dark. The nucleus was counterstained with DAPI, and the cells were visualized under the FSX100 microscope (Olympus). The images were evaluated by Image J.

Electrical cell-substrate impedance sensing

The resistance of cells in culture was measured using electrical cell-substrate impedance sensing (ECIS). All tested cell lines were grown on fibronectin-coated dishes. Cell adhesion measurements were based on changes in the ratio of resistance/capacitance to the current flow applied to the electrode arrays at different frequencies (Applied Biophysics).⁷ A frequency scan was performed to determine the frequency at which the greatest difference in R_{ep} was obtained between the cell-covered and cell-free electrodes. The baseline was established using culture medium ($400 \mu\text{L} \cdot \text{well}^{-1}$) alone and compared with values obtained using electrodes covered with a monolayer of cells in $400 \mu\text{L}$ of medium. The optimal frequency for studying resistance appeared to be 400 Hz. For the resistance/capacitance measurements, cells were inoculated at 1.0×10^4 cells $\cdot \text{well}^{-1}$, unless otherwise indicated, in $400 \mu\text{L}$ in duplicate. Wounding was performed by electroporation using voltage pulses of 5 V and 40 kHz for 30 s.

In vitro cell labeling

To choose transplanted cells, epithelial-like (Epi) cells and Fib cells were labeled by Vybrant DiO cell-labeling solution

(Molecular Probes). DiO marked the cell cytoplasm with distinctive fluorescent colors. Cell labeling was performed following the manufacturer's protocol.

Animal PF model and transplantation of human mesothelial cells

The experimental protocol was approved by the Animal Ethics Review Committee of the Okayama University Graduate School of Medicine and Dentistry and Pharmaceutical Sciences. We developed a mouse model of PF based on the mechanical scraping of the peritoneum. Male BALB/c nu/nu mice, aged 7–8 weeks, were purchased from CREA (CREA), maintained under conventional laboratory conditions, and given free access to water and food. At the beginning of the experiments, mice were incised at the abdominal midline under anesthesia with pentobarbital sodium, and the right and left parietal peritonea and visceral peritonea were mechanically scratched evenly 33 times each with gauze. This scratched model was referenced by Dr. Nishimura report,⁸ and we modified the methods to demonstrate peritoneal adhesion and thickness more clearly. After scratching, the abdomen was closed. After surgery, we randomly assigned each mouse to one of four groups: sham operation (negative control: $n=6$), peritoneum scratched mice with an intraperitoneal injection of 1 mL of PBS (positive control: $n=6$), peritoneum-scratched mice transplanted with Dio-labeled human Epi cells on postoperative day 1 and 2 ($n=5$), and peritoneum-scratched mice transplanted with Dio-labeled human Fib cells on postoperative day 1 and 2 ($n=4$). Transplanted Epi and Fib cells were diluted to 1×10^7 cells/mL PBS. One milliliter of cell suspension was intraperitoneally injected each day. After postoperative day 14, mice were sacrificed with ether, and samples of parietal peritoneum and visceral peritoneum with the intestinal tract were collected. The sections were used for histological analyses, measurements of adhesion numbers. We measured the adhesion number in the abdominal cavity on visual observation. We distinguished the adhesion as the attachment between parietal peritonea and visceral peritonea. Next, we evaluated the peritoneal thickness using five fields in a slide of each group on microscopic analysis. We observed the section with $\times 400$ fields.

Immunofluorescence analysis in vitro and in vivo

The peritoneum was fixed with 4% paraformaldehyde and embedded in paraffin. The thickness of the peritoneal membrane was measured in tissue sections stained with hematoxylin-eosin under light microscopy. For immunofluorescence microscopy, 4- μm -thick frozen tissue sections were incubated with primary antibodies against type 1 collagen (Millipore), fibronectin (Santa Cruz), and proliferating cell nuclear antigen (PCNA) (PharMingen) for 1 h at 37°C. Sections were then incubated for 1 h with a fluorescein-conjugated secondary antibody (FITC-goat anti-rabbit IgG; Invitrogen). HGF and vascular endothelial growth factor (VEGF) staining was also performed. Frozen sections were incubated with primary antibodies against HGF (Institute of Immunology, Co., Ltd.) and VEGF (Santa Cruz) for 1 h at 37°C. Sections were then incubated for 1 h at 37°C with a fluorescein-conjugated secondary antibody (FITC goat anti-rabbit IgG; Invitrogen). These sections were viewed under an

FIG. 1. Selection of peritoneal mesothelial cells (PMCs) from human peritoneal dialysis (PD) effluents. Phase-contrast imaging revealed two types of cells established from the effluent of PD patients isolated by the limited dilution method. **(A)** Cobblestone appearance of PMCs (Epi cells). **(B)** Spindle-shaped PMCs (fibroblast-like [Fib] cells). Scale bar: 100 μ m. **(C-F)** Immunocytochemistry analysis. **(C)** Epi cells: type I collagen, green; pan-cytokeratin, red; DAPI, blue. **(D)** Fib cells: type I collagen, green; pan-cytokeratin, red; DAPI, blue. **(E)** Epi cells: FSP-1, green; pan-cytokeratin, red; DAPI, blue. **(F)** Fib cells: FSP-1, green; pan-cytokeratin, red; DAPI, blue. Scale bar: 10 μ m. **(G)** Evaluation of type I collagen production between Epi cells and Fib cells. Fib cells expressed type I collagen strongly than Epi cells (* $p < 0.05$). **(H)** Evaluation of FSP-1 positive cells between Epi cells and Fib cells. Fib cells expressed FSP-1 stronger than Epi cells (** $p < 0.01$). **(I, J)** Evaluation of cell proliferation and cell attachment using the electrical cell-substrate impedance sensing (ECIS) method. **(I)** Capacitance: the capacitance of Fib cells decreased more than that of Epi cells between 2.5 and 30 h. After 30 h, capacitance levels did not differ between these cells. **(J)** Resistance: The resistance of Epi cells peaked at 70 h and then stayed fixed at this level; cell-cell contacts were considered to be formed at this time point. In Fib cells, resistance increased over time; cell-cell contacts were not formed. At 3 h, the difference between the resistance in Epi cells and Fib cells was ~ 100 ohms. ECIS analysis: positive control (PC) (black, $n = 2$), Epi cells (red, $n = 3$), Fib cells (blue, $n = 3$).

FSX100 microscope (Olympus). The images were analyzed by Image J.

Immunoblotting

SDS-PAGE and immunoblotting were performed as previously described.⁹ We used 20 µg protein for each lane. Primary rabbit antibodies against HGF (Santa Cruz), VEGF (Santa Cruz), bone morphogenic protein 7 (BMP-7; AVIVA Systems Biology), transforming growth factor-β1 (TGF-β1; BioVision), and actin (Sigma) were used. Secondary goat anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase was purchased from Sigma. The images were exposed by ECL plus western blotting detection

system (GE Healthcare). The bands were detected by ImageQuant LAS 4000 mini (GE Healthcare). The bands were evaluated by Image J.

Statistical analysis

The number of parietal adhesions was compared between groups using Mann–Whitney test, because the *p*-value of the number of parietal adhesion was 0.00 in Levene test and the number of visceral adhesions and the peritoneal thickness were compared between groups using ANOVA tests. The number of western blot analysis and cell immunocytochemistry staining were compared between groups using Student *t*-tests. Significance was defined as *p* < 0.05.

FIG. 2. Treatment of the injured peritoneum with cell therapy using PMCs. We implanted the two types of mesothelial cells, Epi cells and Fib cells, into the peritoneal cavities of peritoneal fibrosis (PF) model mice. Implanted Epi cells labeled with DiO (green) attached to the surface of the parietal (A) light microscopy image, (B) FITC, (C) merge and visceral (D) light microscopy image, (E) FITC, (F) merge peritoneum. Some implanted cells migrated into the peritoneum. Fib cells exhibited similar migration and attachment patterns (K). Scale bar: 100 µm. (G–N) Evaluation of cell proliferation in adhered PMCs. The adhesion ability was examined by staining with proliferating cell nuclear antigen (PCNA: red). With regard to the Epi cells, several implanted DiO-positive cells were observed in the injured parietal peritoneum (G). Many PCNA-positive cells were also observed in the injured peritoneum (H) DAPI was observed (I) PCNA and DiO double-positive cells were observed in the parietal peritoneum (J) Fib cells behaved similar pattern (K–N). Implanted DiO-positive cells (K) PCNA-positive cells (L) DAPI was observed (M) PCNA and DiO double-positive cells (N) Scale bar: 100 µm.

Results

Selection of PMCs from PD effluents

We harvested human PMCs from the dialysis effluents of PD patients using a limiting dilution method. The PD effluent contained many types of cells, including mesothelial cells, lymphocytes, eosinophils, macrophages, and fibroblasts. We separated these cells from the PD effluent and isolated each cell type using a limiting dilution method. We selected two types of cells, namely Epi cells and Fib cells, based on their respective morphologies. We characterized the two cell types on the basis of features such as their cytoskeletal protein expression and extracellular matrix (ECM) production. Epi cells exhibited a cobblestone-like appearance

(Fig. 1A). In contrast, Fib cells exhibited a spindle-shaped appearance (Fig. 1B). The Fib cells produced more type I collagen than the Epi cells significantly (Fig. 1C vs. 1D, 1G: $*p < 0.05$). Mesothelial cells are thought to be derived from mesenchymal cells and to possess the ability to undergo an EMT.¹⁰ Mesothelial cells derived from mesenchyme are known to express vimentin. Our two subpopulations of cells expressed vimentin (data not shown). These results suggest that the two types of cells, Epi cells and Fib cells, exhibited mesenchymal cell characteristics. However, these two types of cells possessed different characteristics, particularly in their ability to produce ECM. In addition, Fib cells expressed FSP-1 stronger than Epi cells significantly (Fig. 1E vs. F, 1H: $**p < 0.01$). Cytokeratin⁺/FSP-1⁺ cells were reported to cause

FIG. 3. The effects of Epi and Fib cell therapy were compared in a mouse model of peritoneal scratching. Light microscopy images depict parietal (A–D) and visceral (E–H) peritoneum. Compared with the sham-operated group (A, E), increased peritoneal thickness was observed in the PC group (B, F). Compared with the PC group, Epi cell therapy (C, G) ameliorated peritoneal thickness. In contrast, cell therapy with fibroblast-like cells (D, H) failed to prevent thickening of both the parietal and visceral peritoneum compared with Epi cell therapy. Scale bar: 100 μ m.

the PF.¹¹ These data suggested that Fib cells may have a relation for PF. We then examined the cell proliferation and attachment ability of the Epi and Fib cells using the ECIS method, where high resistance reflects the increased strength of intracellular junctions and the connection strength between a cell and its scaffold. Low capacitance reflects active cell proliferation. The capacitance of Fib cells was significantly lower than that of Epi cells between 2.5 and 30 h in culture (Fig. 1I). At that time, the Fib cells exhibited higher proliferation than the Epi cells, although the proliferation differences between the Epi and Fib cells were not statistically significant. However, the resistances of these two cell populations were significantly different (Fig. 1J). These results suggest that Fib cells and Epi cells possessed different characteristics. The attachment of the Fib cells to the ECM is stronger than that of the Epi cells. However, the Epi cell-cell contact is stronger than that of the Fib cells.

Cell therapy for injured peritoneum using PMCs

To determine whether the two types of cells could treat injured peritoneum, we implanted the two types of mesothelial cells, namely Epi cells and Fib cells, into the peritoneal cavity of a mouse model of PF. The implanted Epi cells, labeled with DiO, attached to the surface of the parietal (Fig. 2A: light microscopy image; 2B: FITC; 2C: merge) and visceral (Fig. 2D: light microscopy image; 2E: FITC; 2F: merge) peritoneum. Fib cells exhibited a similar migration and attachment pattern (Fig. 2K). These results demonstrated that implanted PMCs could migrate and attach to parietal and visceral peritoneum. Next, we examined whether the adherent cells were viable and proliferated at the attachment point by investigating the staining pattern for PCNA. With

regard to Epi cells, several implanted DiO-positive cells were observed in the injured parietal peritoneum (Fig. 2G), and many PCNA-positive cells were observed in the injured peritoneum (Fig. 2H). PCNA and DiO double-positive cells were observed in the parietal peritoneum (Fig. 2J). Fib cells behaved in a similar manner (Fig. 2K–N). These results suggested that implanted PMCs could attach to and survive and proliferate within the injured peritoneum.

Evaluation of the two PMC subtypes in peritoneal injury therapy

To determine whether PMC therapy could ameliorate peritoneal injury, we implanted Epi and Fib cells into the abdominal cavity of PF model mice. We measured the peritoneal thickness and intra-abdominal adhesions after treatment with or without cell therapy in mice whose parietal and visceral peritoneum had been scratched. As expected, we observed an increased peritoneal thickness and adhesions in the positive control group (Fig. 3B and F vs. Fig. 3A and E). However, the increased thickness and adhesions of the parietal and visceral peritoneum were significantly ameliorated in the Epi cell therapy group (Fig. 3C, G). In contrast, the treatment with Fib cells exacerbated the thickness and number of adhesions (Fig. 3D, H). The number of intra-abdominal parietal-to-bowel (parietal adhesion) adhesions was decreased in the EPI cell therapy group compared with the positive control group (Fig. 4A). Interestingly, the number of adhesions was increased in the Fib cell group compared with the Epi cell group (Fig. 4A, B). The thickening of the peritoneum in the Epi cell therapy group was ameliorated (Fig. 4C, D), whereas the peritoneal thickness in the Fib cell therapy group was increased (Fig. 4C, D).

FIG. 4. Evaluation of peritoneal thickness and intra-abdominal adhesion in Epi cell therapy (Epi C) and Fib cell therapy (Fib C) in a mouse model of peritoneal scratching. (A) Epi cell therapy significantly decreased the number of both parietal and visceral adhesions compared with the PC and Fib cell therapy groups. (B) Epi cell therapy could decrease the visceral adhesion compared with Fob cell therapy. (C, D) Epi cell therapy significantly prevented PF, and significant differences were observed between Epi and Fib cell therapy. Fib cell therapy failed to prevent PF. Sham-operated model (negative control: NC); $n=6$; PC: $n=6$; Epi C: $n=5$; Fib C: $n=4$. * $p < 0.05$, ** $p < 0.01$.

Epi cells contributed to an improvement in peritoneal injury. However, Fib cells promoted PF. These results suggest that the two types of PMCs from the same PD effluent play different roles in peritoneal injury.

Alteration of ECM accumulation in peritoneal injury by cell therapy

We then evaluated the effect of cell therapy on ECM accumulation using immunohistochemistry analysis. In the mouse model of peritoneal injury, we observed the accumulation of type I collagen and fibronectin in the parietal peritoneum (Fig. 5A vs. B and Fig. 5E vs. F). Type

I collagen accumulation was inhibited in the Epi cell therapy group compared with the peritoneal injury group (Fig. 5C vs. B). However, type I collagen accumulation was not inhibited in the group receiving Fib cell therapy (Fig. 5D). Similar to type I collagen accumulation, fibronectin accumulation was inhibited by Epi cell therapy but not by Fib cell therapy (Fig. 5E–H). Epi cells suppressed the accumulation of ECM by the residual PMCs, whereas Fib cells were unable to do so. These results suggest that the grafted and residual mesothelial cells were responsible for the ECM accumulation and that the type of grafted PMCs influenced the ECM accumulation by affecting these residual PMCs.

FIG. 5. Alteration of extracellular matrix (ECM) accumulation in peritoneal injury due to cell therapy. We evaluated the ECM components type I collagen (green: A–D) and fibronectin (green: E–H). In the peritoneal injury mouse model (B, F), we observed a greater accumulation of type I collagen and fibronectin compared with the negative control (A, E). Epi cell therapy prevented both type I collagen and fibronectin accumulation (C, G). However, Fib cell therapy failed to prevent the accumulation of either ECM component (D, H). Scale bar: 50 μm. Color images available online at www.liebertpub.com/tea

Growth factor expression in Epi and Fib cells alters the ECM and PF in vitro

Many growth factors have been reported to ameliorate PF. One of these candidate factors, namely HGF,¹² and one permeability factor, namely VEGF,¹³ were examined in our PMCs *in vitro*. Immunocytochemistry revealed that Epi cells expressed HGF (Fig. 6A), whereas Fib cells expressed HGF only weakly (Fig. 6B). Epi cells produced higher levels of HGF than Fib cells, as detected by Western blot analysis (Fig. 6E, F). In contrast, VEGF expression was strongly detected in Fib cells (Fig. 6D) and only weakly detected in Epi cells (Fig. 6C). The difference in VEGF protein levels between Fib cells and Epi cells was statistically significant (Fig. 6E). In addition, we investigated other secreted factors, TGF- β 1 and BMP-7. TGF- β 1 was reported to enhance the PF.¹⁴ BMP-7 was reported to ameliorate the PF.¹⁵ We could not observe the significance difference between Epi-cell and Fib cell on TGF- β 1 and BMP-7 production. These results suggest that Epi cells can produce HGF to ameliorate PF and ECM accumulation, whereas Fib cells produce large

amounts of VEGF, which may raise the vascular permeability of the peritoneum and lead to increased ECM production and PF.

The role of implanted Epi and Fib cells in injured peritoneum in vivo

We examined the growth factor secretion of grafted Epi and Fib cells in mouse models of PF. When we injured the parietal peritoneum, we observed HGF expression by the residual PMCs. In the Epi cell therapy group, HGF expression was observed in implanted DiO-positive cells (Fig. 7C, arrowhead), whereas only weak HGF expression was observed in the Fib cell therapy group (Fig. 7D). These results suggest that some residual PMCs expressed HGF to prevent ECM accumulation and PF. Implanted Epi cells may contribute additional HGF to ameliorate peritoneal injury. VEGF expression was observed in residual PMCs in the peritoneal injury group (Fig. 7F). Interestingly, the implanted Epi-Dio cells did not express VEGF (Fig. 7G, arrow), although the residual PMCs were observed to express VEGF (Fig. 7G,

FIG. 6. Expression of hepatocyte growth factor (HGF) (A, B) and vascular endothelial growth factor (VEGF) (C, D) in Epi cells (A, C) and Fib cells (B, D) *in vitro*. In Epi cells, HGF was strongly expressed, but it was only slightly expressed in Fib cells. In contrast, VEGF expression was stronger in Fib cells than in Epi cells. Scale bar: 50 μ m. (E) Difference in production of growth factors between Epi and Fib cells. Representative western blotting demonstrated HGF protein (~83-kDa band), VEGF protein (45-kDa band), bone morphogenic protein 7 (BMP-7) protein (16-kDa band), transforming growth factor- β 1 (TGF- β 1) protein (48-kDa band), and b-actin protein (45-kDa band) production. (F) Evaluation of secreted factor production in Epi cell and Fib cell (* p < 0.05, ** p < 0.01).

FIG. 7. The role of implanted Epi and Fib cells in injured peritoneum *in vivo*. Implanted Epi and Fib cells were labeled with DiO (green). In injured peritoneum, we observed HGF expression in residual PMCs (**B**) compared with the negative control (**A**). Implanted Epi cells were observed to be DiO-HGF double positive (**C**, arrowhead), but the expression of HGF was very weak in Fib cells (**D**). In the injured peritoneum, VEGF was also expressed in residual PMCs (**F**) compared with the negative control (**E**). In implanted Epi cells, we did not observe VEGF expression (**G**, arrow), although residual PMCs expressed VEGF (**G**, arrowhead). In contrast, we observed VEGF expression in Fib cells as Dio-VEGF double-positive cells (**H**, arrowhead). Scale bar: 50 μ m.

arrowhead). Fib-DiO cells that were implanted at sites of peritoneal injury expressed VEGF (Fig. 7H). These results suggest that implanted Epi cells may supply HGF to ameliorate peritoneal injury and that Fib cells may promote PF through the production of VEGF.

Discussion

The long-term stimulation increases vascularization and decreases the PMC population, leading to PF or sclerosis.¹⁶ In recent years, several studies have demonstrated the

possible use of cell therapy for the treatment of chronic peritoneal damage in animal models. We could demonstrate the possibility of human mesothelial cell therapy that were harvested from effluent of PD patients. The mesothelial cells could attach to the injured peritonea. However, the effect to injured peritonea is different from the kinds of cells that were separated based on morphological characteristics, Epi cells, and Fib cells. It is suggested that the PMCs showed different characteristics on morphological appearance, and it is important to select the characteristics to investigate the system for peritoneal regeneration and cell therapy.

For peritoneal regeneration, there are many reports that many factors are involved for the system to PF. HGF has been reported to prevent the fibrosis of and the angiogenesis within the peritoneum that are associated with enhanced peritoneal cell proliferation and viability.^{6,17} BMP-7 has also been demonstrated to improve PF in a rat PD model.¹⁵ In our study, Epi cells produced HGF strongly.

The first autologous mesothelial cell transplantation was performed in 1989.³ Several studies on mesothelial cell transplantation have followed this initial report. Bertram *et al.* reported that the intra-peritoneal transplantation of isologous mesothelial cells prevents adhesion.⁵ However, there is little knowledge regarding the effect of PMC transplantation on peritoneal damage on the point of the kinds of PMCs. We have demonstrated that the selection of PMCs is important for cell therapy. However, we did not identify specific markers for Epi cells. We have considered two mechanisms for the prevention of fibrosis and adhesions in injured peritoneum by cell therapy. One is the direct prevention by the migration and attachment of implanted cells, which might obscure the space for the production of ECM components, such as collagen and fibronectin, over the transplanted cell layer. For example, Septrafilm reduces postoperative adhesions, and the prevention of peritoneal adhesion by Septrafilm is thought to be due to its acting as a physical barrier.¹⁸ In our experiment, the ability of this mechanism to prevent PF may be limited because engrafted cells were not distributed evenly at the damaged peritoneal surface. Instead, engrafted cells were scattered randomly at the peritoneal membrane. However, it is possible that this mechanism would exert more of an effect if the number of grafted cells were increased.

A second potential mechanism by which cell therapy may prevent fibrosis and adhesion formation is through the secretion of growth factors by implanted cells. Mesothelial cells are known to release growth factors that initiate cell proliferation, differentiation, and mesothelial cell migration. TGF- β ,¹⁹ platelet-derived growth factor,¹⁶ FGF,¹⁹ HGF,²⁰ keratinocyte growth factor,²¹ and members of the epidermal growth factor family²² are some of the factors that are likely to regulate these processes. In our study, HGF and VEGF production by engrafted cells was examined. HGF has been reported to inhibit PF²³ by blocking the EMT. VEGF plays an important role in the regulation of the proliferation and migration of endothelial cells during angiogenesis.²⁴ Several studies have demonstrated that human PMCs can produce VEGF in response to varied stimuli present in PD fluids.²⁵ It has also been reported that treatment with an anti-VEGF antibody reduces the thickness of the compact zone and decreases vasculopathy.¹² We determined that Fib cells strongly expressed VEGF. On the other hands, Epi cell did

not express BMP-7 stronger than Fib cells, and TGF- β 1 was not observed to express any significant differences between Epi cells and Fib cells. There are some possibilities that BMP-7 or TGF- β 1 is secreted by other cells.

Our results suggest that therapy with selected PMC subtypes is an effective way to prevent PF and adhesion, although its efficacy may depend on the growth factor production of engrafted cells. As previously described, there is a possibility that HGF may induce EMT and facilitate peritoneal sclerosis. Thus, we cannot say for sure that HGF is the most important factor in the prevention of PF and adhesion, but it may represent one of the most important factors in preventing PF.

Another limitation is that this mechanical scraped peritoneal injury model may not reflect the peritoneal injury in PD patients, because the mechanism of peritoneal injury is not the same between the scraped model and long-term PD fluid stimulation.

In summary, there are some differences between Epi and Fib cells that were harvested from same PD effluent. The selection of mesothelial cells may be important for not only cell therapy but also the mechanism to peritoneal regeneration.

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Disclosure Statement

The authors declare that the establishment and selection of PMCs and their therapeutic application to peritoneal disorders were filed and submitted to the Japan Patent Office (Issue No. 2009-104704) in April 2009.

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