Are \textit{ex vivo} mesothelial cells representative of the \textit{in vivo} transition from epithelial-to-mesenchymal cells in peritoneal membrane?

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Abstract

Background. We investigated whether \textit{ex vivo} mesothelial cells found in peritoneal dialysis (PD) effluents were representative of the \textit{in vivo} epithelial-to-mesenchymal transition (EMT) in peritoneal membrane.

Methods. Thirty-six male Sprague–Dawley rats were equally divided into three groups: Group C (control), no PD; Group D, infused with 4.25% Dianeal and Group P, infused with 4.25% Physioneal. PD infusions (25 mL) were given twice daily for 8 weeks. The \textit{in vivo} study included morphometric analyses performed on the peritoneal membranes of tissue specimens obtained at the end of the study. The \textit{ex vivo} study included peritoneal mesothelial cells collected from PD effluent and cultured to confluence. Cells were scored with light microscopy.

Results. PD for 8 weeks induced significant EMT. The \textit{in vivo} expression of EMT markers ($\alpha$-smooth muscle actin:E-cadherin ratio, matrix metalloproteinase-2 and Snail) was higher in Group D than in Group P. However, \textit{ex vivo} EMT marker expression was similar in cells derived from Groups D and P. A significant correlation was observed among \textit{in vivo} EMT markers. Moreover, the \textit{ex vivo} cell score increased with time on PD. However, changes in the \textit{ex vivo} cell score did not correlate with changes in the \textit{in vivo} EMT marker expression. Furthermore, we found no correlation between \textit{ex vivo} and \textit{in vivo} cells in the expression of EMT markers.

Conclusions. In this animal study, \textit{ex vivo} findings did not reflect the \textit{in vivo} EMT changes in the peritoneum. It may be necessary to improve the current methodology for \textit{ex vivo} studies.

Keywords: epithelial-to-mesenchymal transition; peritoneal dialysis; peritoneal membrane

Introduction

In the last decades, peritoneal dialysis (PD) has become an increasingly common alternative to hemodialysis for the treatment of end-stage renal disease. One of the most important challenges in PD is the long-term preservation of peritoneal membrane integrity [1]. The peritoneal membrane is lined with mesothelial cells (MCs), which secrete various substances involved in homeostasis of the peritoneum [2]. The peritoneum can be injured by long-term exposure to hyperosmotic (364–485 mOsm/L), hyperglycemic (1.36–3.86 g/dL) and acidic (pH 5.2–5.5) dialysis solutions and recurrent or severe episodes of peritonitis or hemoperitoneum. The injured peritoneum undergoes progressive fibrosis and angiogenesis [1, 3], which are considered the main
Mesothelial cells ex vivo and peritoneal EMT in vivo

causes of ultrafiltration failure and, potentially, of encapsulating peritoneal sclerosis [1, 4].

The epithelial-to-mesenchymal transition (EMT) is characterized by the disruption of intercellular junctions in epithelial cells and a loss of apical–basolateral polarity, followed by a transformation into fibroblast-like cells with increased migratory, invasive and fibrogenic features. EMT is an important mechanism involved in peritoneal membrane fibrosis [5, 6].

Historically, MCs have been considered the target of PD-related peritoneal injury. However, recent studies showed that, soon after PD is initiated, peritoneal effluent-derived MCs exhibit a progressive loss of the epithelial phenotype and acquire fibroblast-like characteristics; thus, the biochemical and morphological changes to MCs are reminiscent of those that take place during EMT [7]. It can be argued that effluent MCs are not representative of the mesothelium because they might be cells specifically programmed to detach from the peritoneum. Evidence is lacking among in vivo and ex vivo data [4, 7–10] that clearly show that MCs released into PD effluent are entirely representative of the MC population that remains attached to the peritoneum. Moreover, a confirmative diagnosis of EMT requires a peritoneal biopsy, which is not trivial, and it may not apply to all patients that receive PD.

This study evaluated the validity and the efficacy of a current ex vivo protocol for studying EMT in a rat model of PD. In addition, we investigated the improvements of current ex vivo methodology and the ability of ex vivo biomarkers to reflect the changes of in vivo EMT status.

Materials and methods

In vivo experiments

Laboratory animals and study protocol. We used 36 Sprague–Dawley male rats (B&K Universal, Hull, UK) that weighed 270–320 g at the start of the experiment. Initially, peritoneal catheters were implanted (Day 0), as previously described [11]. Catheter-implanted rats were randomly divided into three groups (n = 12 each) with the following treatments: (D) infusions of a conventional dialysis solution (Dianeal®, 4.25% dextrose, pH 5.2, lactate buffer; Baxter Healthcare, Woodlands, Singapore); (P) infusions of a low glucose degradation product solution (PhysioNeal®, 4.25% dextrose, pH 7.0–7.4, bicarbonate/lactate buffer; Baxter Healthcare) and (C) catheters, but no PD fluid (PDF) infusions. On the day after catheter insertion, PDF infusions (twice daily at 0900 and 1800 h) were given for 8 weeks. The volume infused was gradually increased from 15 to 25 mL over the first 3 days; thereafter, 25 mL was infused. All PDF infusions contained prophylactic antibiotics, Amikacin, 100 mg/L (Boryn Pharm, Seoul, Korea) and Cefazolin, 1 g/L (Chong Kun Dang Pharm, Seoul, Korea). PDFs were allowed to dwell and were absorbed gradually from the peritoneal cavity. To diagnose peritonitis, the dialyzate effluent was cultured at 0, 4 and 8 weeks.

Peritoneal membrane function was assessed in all animals on Days 0, 28 and 55 with a 4-h peritoneal transport test that required 25 mL of 4.25% dextrose solution (Dianeal®). Dialyze samples were taken at 0 and 4 h. Dialyze glucose concentrations were determined with the glucose oxidase method. To evaluate peritoneal small solute transfer, the glucose mass transfer from the peritoneal cavity to the circulation was calculated with the Fick method. To evaluate peritoneal small solute transfer, the glucose mass transfer from the peritoneal cavity to the circulation was calculated with the Fick method.

RNA isolation and complementary DNA synthesis. Total RNA was extracted from tissue samples with TRI Reagent (Molecular Research Center, Cincinnati, OH), according to manufacturer instructions. RNA purity and concentration were determined with the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). One microgram of total RNA was reverse transcribed with the Prime Script cDNA Synthesis kit (Takara Shuzo Co., Otsu, Japan).

Real-time polymerase chain reaction for expression analysis of EMT markers. All polymerase chain reaction (PCR) primers (Table 1) were designed with Primer Express V1.5 software (Applied Biosystems, Foster City, CA). Real-time PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the SYBR Green PCR master mix (Qiagen GmbH, Hilden, Germany). All reactions were performed in duplicate, beginning with a pre-denaturation at 50°C for 2 min, denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Results were normalized to the corresponding ΔCt values of β-actin messenger RNA (mRNA). Relative changes in gene expression were calculated as:

\[ \Delta \Delta C_t = \Delta C_t (\text{assayed gene}) - \Delta C_t (\beta\text{-actin}) \]

Ex vivo experiments

Isolating, culturing and scoring rat peritoneal MCs. During each peritoneal transport test, on Days 0, 28 and 55, cellular components in the effluents were collected after a 4-h dwell with 4.25% glucose dialyze. Cells were isolated by centrifugation [2500 r.p.m. (640 g), 10 min, 4°C]
and washed twice with phosphate-buffered saline (PBS, GIBCO, Grand Island, NY) and re-suspended in 10 mL of M199 (GIBCO) supplemented with 20% fetal bovine serum (GIBCO), 1% penicillin-streptomycin (GIBCO), 2 mM t-glutamine (GIBCO), 0.4 μg/mL hydrocortisone (Sigma), 50 μg/mL apo-transferrin (Sigma) and 2.5 μg/mL bovine insulin (Sigma). Cells were then seeded into 100-mm plastic culture dishes (Nunc Plate Delta; Nunc, Roskilde, Denmark) and incubated at 37°C in a humidified 5%-CO2 atmosphere. Culture medium was replaced every 3 days. When cells were near confluence (5–10 days), blinded cultures were scored by the same person using light microscopy. Scoring was based on morphological classifications, as previously defined (1 = cobblestone-shaped MCs, 2 = mixed and 3 = fibroblastoid cell dominant) [12]. Then, confluent cells were washed once with PBS and treated with 0.25% trypsin-EDTA (GIBCO) for up to 3 min at RT. Culture medium was added, and detached cells were separated into two groups; one for protein extraction and the other for RNA isolation.

Immunoblotting and real-time PCR. EMT-associated molecules were detected by western blot analysis and real-time PCR in cultured effluent cells at 0, 4 and 8 weeks. Unlike the in vivo study, PRO-PREP (iNtRON Biotechnology, Seongnam-Si, Korea) was used for protein extraction, and TRIzol Reagent (Invitrogen Life Technology, Carlsbad, CA) was used for total RNA isolation.

Additional ex vivo experiments on the effluent-derived cells at the 0 time point

The effluent-derived cells from extra Sprague–Dawley male rats that weighed 270–320 g at 0 day (abbreviated as ‘0-day sample’) were used for additional analysis of their MC character. 

Immunofluorescence. PD effluent cells were collected after a 4-h dwell with 4.25% dextrose dialyze at 0 day. The cells were seeded in 8-well glass chamber slides (Lab-Tek; Nunc) in M199, supplemented with 20% fetal bovine serum until confluent (5–10 days). The confluent cells were washed with ice-cold PBS and fixed with 3.7% paraformaldehyde for 30 min at RT, followed by 0.3% Triton X-100 for 15 min at RT. After rinsing in PBS and blocking with 1% bovine serum albumin at RT for 30 min, monolayers were incubated with the appropriate primary antibodies with against α-SMA (1:100 dilution; Abcam), cytokeratin (1:50 dilution; Abcam) and fibroblast-specific protein-1 (FSP-1; 1:200 dilution; Abcam) overnight at 4°C and then incubated for 2 h with fluorescein-conjugated secondary antibodies (Alexa Fluor 488 and Alexa Fluor 594; Molecular Probes). The nucleus was counterstained with DAPI and the slides were mounted with anti-fade mounting reagent (Molecular Probes). The slides were viewed with a Zeiss confocal scanning laser microscope using LSM 5 EXCITER (Carl Zeiss).

Cell culture using plastic- and collagen-coated culture dishes. Differential culture, immunoblotting of EMT markers and cell scoring. Each 0-day sample was split at 1:1 ratio, and each half (or the half of it) was cultured on either 100-mm collagen-coated (Iwaki, Tokyo, Japan) or original plastic culture dishes, respectively, as described above. To increase the purity of the cells at the time of culturing, a ‘differential’ subculture was carried out as suggested in the previous review [13]. Briefly, the cells of 0-day sample were initially incubated for 10 min. Then, because fibroblasts tend to attach to culture vessels more rapidly than MCs, the medium containing the suspended cells was immediately removed and plated onto new plastic- and collagen-coated culture dishes.

When the cells were near confluence, expression levels of α-SMA, E-cadherin, α-actin and P did not differ significantly (Figure 1).

Table 1. Rat-specific primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5’ to 3’)</th>
<th>Product size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>ACCACAGTACCAGGGCATT</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACACAGGACTCTGCTGA</td>
<td></td>
</tr>
<tr>
<td>α-SMA</td>
<td>Forward</td>
<td>GCACTACCATGTACCACAGCAT</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGGTCTGGAAGGCAAATAA</td>
<td></td>
</tr>
<tr>
<td>E-cad</td>
<td>Forward</td>
<td>CAGATTAAAGTCCTCCGCAA</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACCGTCTCTCCGGTAGAA</td>
<td></td>
</tr>
<tr>
<td>Snail</td>
<td>Forward</td>
<td>CTTGTGCTCAGATGGCTGAT</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TAGTGGCTCAAGCGACTGTG</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>Forward</td>
<td>TCAGTTATTGCGGGAACGT</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTACACGGCATCAATCTTTTC</td>
<td></td>
</tr>
</tbody>
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*aα-SMA, α-smooth muscle actin; E-cad, E-cadherin; MMP-2, Matrix metalloproteinase-2.*
In vivo study

Morphometry of the peritoneum. At 8 weeks, the mean peritoneal thickness of the abdominal wall samples in Group D (18.6 ± 1.0 μm) was significantly higher compared with Groups C (12.4 ± 0.8 μm, P < 0.001) and P (14.0 ± 0.7 μm, P < 0.01). The mean thickness of the liver samples was higher in Groups D (4.9 ± 0.3 μm) and P (4.7 ± 0.2 μm) compared with Group C (3.1 ± 0.1 μm) (P < 0.001, Figure 2).

Histology of abdominal wall. At 8 weeks, cytokeratin-positive cells were predominantly harbored in the mesothelial layer of Group C. Group D exhibited a higher number of α-SMA-positive cells compared with Group P. In dual stains, submesothelial cytokeratin- and α-SMA-positive myofibroblasts were most prominent in Group D among the three groups; this was associated with a decrease in cytokeratin and an increase in α-SMA expression (Figure 3).

Expression of EMT markers in the peritoneum. After 8 weeks of experimental PD, western blot analysis showed that the ratios of α-SMA:E-cad expression in the abdominal wall were significantly higher in Groups D (P < 0.001) and P (P < 0.05) compared to controls, and the ratio in the omentum was higher in Group D (P < 0.05, Figure 4A) compared to controls. In addition, real-time PCR of peritoneal tissue from the abdominal wall showed that the ratio of α-SMA:E-cad mRNA expression was significantly

![Fig. 1. Changes in the levels of glucose mass transfer normalized for body weight. Data are expressed as means ± SEM. C, control; D, Dianeal® infusion; P, Physioneal® infusion. **P < 0.01, ***P < 0.001.](image1)

![Fig. 2. Morphologic changes in the peritoneum at 8 weeks. Peritoneum sections of the (A) abdominal (ABD) wall (magnification ×200; white scale bar, 20 μm) and (B) liver cell surface (magnification ×630; white scale bar, 10 μm) were stained with Masson’s trichrome (C). Data are expressed as means ± SEM. C, control; D, Dianeal® infusion; P, Physioneal® infusion. **P < 0.01; ***P < 0.001.](image2)
higher in Group D compared with Groups C and P (data not shown); the mRNA expression of matrix metalloproteinase-2 (MMP-2) (Figure 4B) and Snail (Figure 4C) was also significantly higher in Group D ($P < 0.01$ and $P < 0.05$, respectively) compared with controls. On the other hand, the omentum did not show any significant differences in mRNA expression of these markers among the three groups.

Correlations between peritoneal morphometry and EMT marker expression. Analyses of data pooled from all three groups showed that the peritoneal thickness of the abdominal wall was correlated with that of liver ($r = 0.502$, $P = 0.002$, Figure 5A), and the $\alpha$-SMA:E-cad ratio of the abdominal wall was correlated with that of the omentum ($r = 0.398$, $P = 0.02$, Figure 5B). Moreover, the $\alpha$-SMA:E-cad ratios were positively correlated with the peritoneal thicknesses of the abdominal wall ($r = 0.444$, $P = 0.008$, Figure 5C) and liver ($r = 0.53$, $P = 0.001$, Figure 5D).

**Ex vivo study**

**Cell score.** In all groups, the prevalence of cell score 1 decreased progressively, and the prevalence of cell score 2 or 3 increased with increasing study duration (linear associations, Group C, $P = 0.007$; Group D, $P = 0.005$; Group P, $P = 0.001$; Figure 6). However, no significant ex vivo morphologic differences were found among the three groups at 0, 4 and 8 weeks.

**Expression of EMT markers in cultured effluent cells.** At 0, 4 and 8 weeks, western blot analyses showed that E-cad was not expressed in cultured effluent cells (data not shown). At 8 weeks, $\alpha$-SMA protein expression was higher in Groups D and P compared with Group C ($P < 0.05$, Figure 7A). Real-time PCR showed that the mRNA expression of $\alpha$-SMA:E-cad ratio (data not shown) and MMP-2 (Figure 7B) was not significantly different among the groups during the 8-week PDF infusion. At 4 weeks, Snail mRNA expression in Groups D and P was significantly

Fig. 3. Immunofluorescence of abdominal wall sections stained for $\alpha$-SMA (red) and cytokeratin (green) with nuclear counterstain (blue, DAPI) at 8 weeks; images were superimposed (merge) to visualize dually stained cells. (A through C) control; (D through F) Dianeal® infusion and (G through I) Physioneal® infusion; an increase in dual staining was observed in Dianeal-treated submesothelial myofibroblasts (arrows, F). Magnification $\times640$. Scale bars (white): 10 µm.
lower than in Group C ($P < 0.001$, Figure 7C). At 8 weeks, however, there was no significant difference in Snail expression among the three groups.

### Relationship between in vivo and ex vivo results

**Peritoneal thickness and EMT marker expression** (in vivo) compared to cell scores (ex vivo). At 8 weeks, cells were scored in tissues from 33 of the 36 rats that initially participated in the study (Group C, $n = 10$; Group D, $n = 12$ and Group P, $n = 11$). Rats were divided into two new groups: those with cell scores of 1 ($n = 2$) or 2 ($n = 13$) (total $n = 15$) and those with a cell score of 3 ($n = 18$). No significant differences between groups were found in peritoneal thickness or in the $\alpha$-SMA/E-cad ratio (Figure 8).

**Peritoneal thickness and EMT marker expression** (in vivo) compared to EMT marker expression in cultured effluent cells (ex vivo). At 8 weeks, $\alpha$-SMA protein expression in cultured effluent-derived cells (ex vivo) was not significantly
correlated with in vivo measurements of peritoneal thickness, α-SMA:E-cad protein expression ratio or α-SMA:E-cad mRNA expression ratio (Figure 9). Moreover, no significant correlation was found between the ex vivo α-SMA:E-cad mRNA expression ratio and any of these in vivo parameters (Figure 10).

Additional ex vivo experiments on the effluent-derived cells at the 0 time point

Immunofluorescence. Confocal microscopy showed that cytokeratin was expressed in the great majority of confluent cells in 8-well chamber slides, while FSP-1-positive cells were apparently scarce (data not shown).

Cell culture using plastic- and collagen-coated culture dish, differential culture, immunoblotting of EMT markers and cell scoring. Western blot analysis showed that E-cad, especially, was newly expressed and use of either collagen-coated dish or differential culture method did not affect markedly the level of E-cad expression (Figure 11A). In addition, there were no significant differences in mean cell score either between plastic- and collagen-coated dishes or between differential subculture and no subculture (Figure 11B).
Cell staining for SA-β-gal. A minority of the cells showed positive (blue) for our SA-β-gal staining (Figure 12).

Discussion

Long-term use of bioincompatible PD solutions and recurrent episodes of peritonitis can stimulate tumor growth factor-β (TGF-β) production in MCs [14]. TGF-β activates Smad proteins, which induce the transcription factor Snail. Snail regulates EMT by repressing cell–cell adhesion molecule expression (i.e. E-cad) and incurring the loss of epithelial cell polarization [15, 16]. Finally, cells acquire migratory/invasive and fibrogenic properties with expression of α-SMA [6, 7, 17]. In addition, TGF-β upregulates MMP-2 expression, which causes degradation and disruption of the underlying basal lamina of epithelial cells [18]. Therefore, the molecular markers for EMT include a reduction in E-cad and increases in α-SMA, MMP-2 and Snail expression.

Fig. 9. Correlations between ex vivo α-SMA protein (normalized to GAPDH) and in vivo parameters. Data represent 8-week expression in cultured effluent cells (ex vivo, X-axes) and measurements from peritoneal samples from animals treated for 8 weeks (in vivo, Y-axes). Ex vivo EMT marker expression did not correlate with (A and B) peritoneal thicknesses, (C and D) α-SMA/E-cad ratios measured by western blot or (E and F) the mRNA ratios measured by real-time PCR. C, control; D, Dianeal® infusion; P, Physioneal® infusion.
As far as we know, peritoneal biopsy is now the only method to evaluate human peritoneal MCs in vivo, but it is difficult to perform due to the requirement of open surgery and very experienced interpretation. Therefore, it is very difficult to collect both in vivo and ex vivo samples from the same PD patient at the same time point. The previous study, using effluent-derived MCs from PD patients, showed that morphological changes in the cells appeared to be related to the duration of PD [7]. However, strictly speaking, it was not a one-to-one match between the morphological feature and PD duration of the same patient. These are why we designed and conducted the present animal study. The strength of our study is that it is the first trial to directly compare ex vivo results with corresponding in vivo data of peritoneal EMT at the same time point.

We showed that the peritoneal solute transport rate of glucose increased over time with PD. In addition, expression of the α-SMA:E-cad ratio, MMP-2 and Snail significantly increased in vivo with conventional PDF (Group D). Moreover, these increases in EMT markers were accompanied by peritoneal fibrosis, an increase in

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**Fig. 10.** Correlations between ex vivo α-SMA/E-cad mRNA ratio (normalized to β-actin) and in vivo data parameters. Data represent 8-week expression in cultured effluent cells (ex vivo, X-axes) and measurements from peritoneal samples from animals treated for 8 weeks (in vivo, Y-axes). Ex vivo EMT marker expression did not correlate with (A and B) peritoneal thicknesses, (C and D) α-SMA/E-cad ratios measured by western blot or (E and F) mRNA ratios measured by real-time PCR. C, control; D, Dianeal® infusion; P, Physioneal® infusion.
cytokeratin- and α-SMA-positive cells and a thickened submesothelial matrix. These findings are consistent with results from previous studies in this animal model [19–21]. Furthermore, these in vivo EMT parameters, including the degree of peritoneal fibrosis, were significantly correlated. Our results confirmed that 8-week PDF infusion induced significant EMT of MCs in vivo in our chronic animal PD model.

Our data also showed that these in vivo changes were significantly attenuated in Group P. This was consistent with a recent study that showed that a PDF with low glucose degradation products reduced EMT and peritoneal fibrosis [19]. However, the potential effects of different buffers and pH changes were not evaluated in that study. The present immunofluorescence results called attention to the possibility that Physioneal attenuates Dianeal-induced submesothelial EMT-like changes. This seems potentially important in terms of biocompatibility of the relative PD solutions.

In addition, we found that the expression of EMT markers was more prominent in the abdominal wall than in omentum; this differential mesothelial response to the same environment suggested the presence of different mesothelial phenotypes [22]. Previous biopsy registry data from patients that underwent PD revealed that the parietal membrane had a significantly greater tendency to increase in thickness in the submesothelial compact zone, and it exhibited a significantly greater prevalence of vasculopathy than the corresponding visceral membrane. This suggested that PD-related changes in the visceral membrane were significantly less pronounced than those from the corresponding parietal membrane [23].

We performed the ex vivo study to confirm that EMT had taken place. Contrary to our expectations, our data showed that the prevalence of the epitheloid MC phenotype (Cell score 1) in Group C had dropped to <50% at baseline, and it significantly decreased over time. Groups D and P showed similar results. Our analysis of the relationship between in vivo and ex vivo results showed that the expression levels of EMT markers in ex vivo cells did not correlate with those found in vivo. Taking into consideration that our in vivo study showed significant signs of EMT, these ex vivo findings raised the question of whether effluent cells were truly representative of the in vivo surface MCs.

As illustrated in Figure 6, it seems curious that a substantial number of fibroblast-like cells ex vivo were observed even at the 0 time point. Furthermore, E-Cad was not expressed in these cells by western blot. From this perspective, one might argue that these were not MCs to start with, which was particularly troubling with respect to the control group. The cells can undergo transition rapidly on plastic but preserve epithelial characteristics when grown on a collagen-coated dish. In addition, there also remains the possibility that peritoneal fibroblasts might contaminate the ex vivo cell cultures during the cell isolation procedure. To overcome the issues discussed above, we performed additional ex vivo experiments using effluent cells derived from extra rats at 0 day. Our immunofluorescence analysis showed that cytokeratin, as an epithelial marker, was expressed in the great majority of

![Figure 11](image_url1)

**Fig. 11.** Effect of cell culture using collagen-coated culture dish and/or differential culture method on (A) expression of EMT markers and (B) cell scores in cultured effluent-derived cells at 0 day. (A, top) Representative western blots of α-SMA, E-cad and GAPDH (loading control); (A, bottom) quantification of α-SMA and E-cad, both normalized to GAPDH, and representation of the ratio of α-SMA to E-cad expression (α-SMA/E-cad). Data are expressed as raw data or means ± SEM. DC, differential culture; plastic, plastic culture dish; collagen, collagen-coated culture dish. Cell score 1 = cobblestone-shaped rat peritoneal MCs; cell score 2 = mixed; cell score 3 = fibroblastoid-type cells dominant.

![Figure 12](image_url2)

**Fig. 12.** The presence of SA-β-gal in a representative culture of effluent-derived cells at 0 day. A minority of the cells showed positive (blue). Magnification ×200.
the confluent cells. Our western blot analysis showed that E-cad was newly expressed and use of either a collagen-coated dish or differential culture method did not markedly affect the level of E-cad expression. In addition, there were no significant differences in cell score either between plastic- and collagen-coated dishes or between differential subculture and no subculture. Nevertheless, we frankly do not know exactly the origin of a substantial number of fibroblast-like cells found even at the 0 time point. It has been proposed that the loss of cells from the surface of the peritoneal membrane is likely to be due to a change in their phenotype. This change allows them to detach and be washed out in the PD effluent, instead of migrating and invading the stroma [23]. The finding of ex vivo study also allows us to speculate that there may initially be differences in cell morphology and characteristics between rat and human. Additionally, previous findings have indicated that some human MCs isolated from PD effluent were senescent [24]; moreover, enlarged senescent-like cells were also detected in the mesothelium of rats exposed to high-glucose PDF [25]. We attempted to clarify the presence of SA-β-gal-positive cells (senescent cells), showing that a minority of the cells were positive for our SA-β-gal staining of effluent cells. Thus, it might be possible that a portion of the fibroblast-like cells is a senescent cell induced by our culture conditions, which may partially explain the heterogeneous nature of our cell populations. Furthermore, we did not rule out the possibilities that the nature of effluent cells might be changed or that some effluent cells might not survive our ex vivo culture conditions. Nevertheless, other data obtained in vivo and in vitro indicated that the MCs released into PD effluent were representative of the MC population that remained attached to the peritoneum [4, 7–10].

The reason why Snail mRNA expression, which serves as a robust marker of EMT, went down in the treatment groups is still quite unclear. As mentioned above, however, the additional data showed that a certain small number of senescent cells was observed in ex vivo cultures, cautiously suggesting that Snail expression in ex vivo might certainly not be correlated with its in vivo expression.

A potential limitation of our ex vivo methodology was the limited number of MCs released into the PD effluent during nocturnal exchanges. In addition, rats have relatively small body sizes; thus, tissue availability was limited. Therefore, the number of effluent-derived rodent MCs was expected to be much smaller than that derived from humans (normally, ~25000 ± 3000) [26, 27].

A recent animal study suggested that the effluent cells collected in PD solutions from patients may require further interpretation in light of the possibility that the studied cells may originate from the implanted catheter rather than the peritoneum [28]. One speculation was that the catheter provides a platform for the activation of MCs that are chronically shed from their basement membranes; the transition from an epithelial phenotype to a fibroblastic type is induced by the amplification of cytokine production within the cavity [29]. It is therefore plausible that the catheter themselves, at least in rodents, may have affected not only our data of glucose transport but also ex vivo results, including the cellular morphology assessments and MMP-2 expression, leading to the discrepancy between ex vivo and in vivo results. In fact, there is always concern whether the findings in animal experiments are applicable to the human. In the present study using experimental rat model of chronic PD, the implantation of custom-made miniature peritoneal catheters allowed us to utilize a permanent PD system with easy instillation and removal of PDFs, thus imitating more closely the clinical situation. No consensus, however, still exists on the optimal levels of the volume, frequency and other mechanical aspects of instillation of PDFs, which may affect the histopathology of the peritoneum [30]. Thus, we think that extrapolation of our results from rats to humans must be made with caution.

Finally, it is possible that our ex vivo EMT assessment was limited by our reliance on the expression of α-SMA, which is not specific for fibroblasts. Furthermore, α-SMA is only expressed by some subpopulations of fibroblasts, and it is not present in newly transitioning epithelial cells [31]. Thus, a definitive identification of EMT might have been lacking.

In conclusion, our results demonstrate that ex vivo findings, including cell scores and the expression of EMT markers, did not reflect in vivo EMT changes in a chronic PD animal model. Therefore, we suggest that it may be necessary to improve current methodologies for ex vivo studies.

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Conflict of interest statement. None declared.

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