Epithelial-to-mesenchymal transition and slit function of mesothelial cells are regulated by the cross talk between mesothelial cells and endothelial cells

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Submitted 11 October 2013; accepted in final form 31 October 2013

PERITONEAL DYSFUNCTION is a major factor leading to treatment failure of peritoneal dialysis (PD) (5, 6). Dialysis works on the principle of both solute diffusion of dissolved substances and ultrafiltration of fluid across a semipermeable membrane, and PD uses the patient’s peritoneal membrane in the abdomen as the semipermeable membrane. Loss of diffusion capacity of the peritoneal membrane leads to a reduction in the clearance of solutes and causes insufficient efficiency of dialysis. This dysfunction is a common consequence of inadequate peritoneal permeability secondary to inappropriate regeneration of the peritoneal membrane (16). However, the precise relationship between peritoneal permeability and pathological morphology remains to be clarified.

The peritoneal membrane is divided into three components that each has a specific function, namely, the mesothelial layer, interstitial collagen, and small vessels (Fig. 1, A and B). These components have specific pores and a resistance that contributes to solute diffusion (12, 17, 23). It is known, however, that the small pores of endothelial cells are the most important slits for solute diffusion (13, 22), and the slit function of both the mesothelial layer and the interstitial collagen remains unexplained in vitro. The epithelial-mesenchymal transition (EMT) of peritoneal mesothelial cells is recognized to play an important role in denudation of the peritoneal membrane (11, 28). However, the role of EMT of mesothelial cells in the slit function of the peritoneal membrane remains uncertain. Interstitial tissues generally consist of bundles of collagen, and their influence on the diffusion coefficient of the peritoneal membrane has been poorly understood.

To clarify the changes in the slit function in the peritoneal membrane, it is necessary to establish an appropriate permeability system in which the three fundamental factors of mesothelial cells, collagen scaffold, and endothelial cells are combined and which replicates a mesothelial cell EMT state. It remained difficult to reproduce the EMT process in vitro until we established a fluid flow stress culture model that can replicate mesothelial hyperplasia and EMT under culture conditions (1, 2). It was also difficult to create collagen-based membrane chambers that allow permeability tests. Since most collagen reagents exist in sol and gel states, it is difficult to apply a chamber membrane created from commercial collagen products. Meanwhile, Takezawa et al. (25) developed a novel biomaterial consisting of high-density collagen fibrils equivalent to connective tissues in vivo, and named it “collagen vitrigel” because its preparation required a vitrification process. Recently, we succeeded in developing a collagen vitrigel membrane chamber useful for reconstructing culture models, such as “tissue sheets” composed of epithelial cells, mesenchymal cells, or endothelial cells alone, and “organoid plates” composed of more than two types of cells (24, 26, 27). This novel collagen material has enabled us to make a new culture chamber that replicates the microenvironment of organs specifically related to the extracellular matrix.

In this study, we have established a novel peritoneal diffusion model to clarify the precise relationship between peritoneal permeability and pathological changes of cell constituents of the peritoneum. In the model, mesothelial cells, collagen...
vitrigel membrane, and endothelial cells replicate an artificial peritoneal membrane, which we refer to as a vitrigel-PM model. Fluid flow stress and high-glucose stimulation induce a mesothelial EMT state of the vitrigel-PM model. Permeability tests of this artificial membrane in the EMT state revealed an unknown peritoneal slit factor and close cell-cell interactions between mesothelial cells and endothelial cells. This artificial peritoneum diffusion model will likely open up a new field for peritoneal cellular kinetics and specific slit function.

METHODS

Animals and cell line. All procedures involving animal materials were performed in accordance with the regulations laid down by Saga University as Ethical Guidelines.

Collagen vitrigel membrane chamber. A collagen vitrigel membrane was prepared by the following three processes as previously reported (25): 1) a gelation process in which 2.0 ml of 0.25% type I collagen sol was allowed to form an opaque soft gel in a culture dish with a diameter of 35 mm; 2) a vitrification process in which the gel became a rigid material through sufficient drying; and 3) a rehydration process in which the vitrified material was converted into a thin transparent gel membrane with enhanced gel strength by moisture supplementation. Subsequently, a collagen xerogel membrane defined as a dried collagen vitrigel membrane without free water was prepared by simply revitrifying a collagen vitrigel membrane on a separable sheet. The collagen xerogel membrane was pasted onto the bottom edge of a plastic cylinder with an inner-outer diameter of 11–13 mm and a length of 15 mm, and two hangers were connected to the top edge of the cylinder, resulting in the fabrication of a collagen xerogel membrane chamber that easily allowed conversion into a collagen vitrigel membrane chamber by taping the edges of the two cylinders together via the collagen xerogel membrane.

Vitrigel-PM model and permeability test. For the reconstructed peritoneum conditions, 5 × 10⁴ mesothelial cells of the cell line MeT-5A (CRL-9444; American Type Culture Collection, Manassas, VA) and 5 × 10⁴ endothelial cells of the cell line MS-1 (CRL-2279; American Type Culture Collection) were seeded onto the inner and outer surfaces of the collagen vitrigel membrane chamber, respectively. We prepared three types of vitrigel-PM models: 1) mesothelial cells with endothelial cells (vitrigel-ME); 2) mesothelial cells alone (vitrigel-M); and 3) endothelial cells alone (vitrigel-E). To mimic normal and fibrotic peritoneum conditions, the cell-seeded chambers were cultured for 2 or 28 days in complete medium, comprising low-glucose (LG) DMEM containing 1,000 mg/l of glucose (Sigma, St. Louis, MO) and high-glucose (HG) DMEM containing 4,500 mg/l of glucose, both supplemented with 10% FBS, 100 µg/ml of streptomycin, and 100 µg/ml of penicillin under fluid flow stress (1, 2).

To replicate the PD conditions, 0.5 ml of PD solution (Dianeal-N PD-2-1.5; Baxter, Tokyo, Japan) was poured into the inner side of the reconstructed chambers (Fig. 1C). The chambers were then settled in 12-well plates in 1.5 ml of complete medium described above containing 1.5 mg/ml of urea (Kanto Chemical, Tokyo, Japan), 0.15 mg/ml of creatinine (Kanto Chemical), 0.2 mg/ml of uric acid (Kanto Chemical), or 0.5 µg/ml of β2-microglobulin (β2MG; Sigma). Transport of low- and medium-molecular weight solutes was assessed by the dialysate-over-medium (D/M) ratio for urea, creatinine, uric acid, and β2MG. Transport of glucose was assessed by the end-to-initial dialysate concentration (D/D₀) ratio. Before the permeability assay, the chamber membranes for observation were fixed in neutral-buffered 10% formalin and vertically embedded in paraffin to allow confirmation of the phenotypic changes as well as

In the diffusion test, dialysate and culture medium samples were collected at 0, 0.5, 1.0, 2.0, and 4.0 h of dwell time. The concentrations of urea, creatinine, uric acid, and β2MG in the PD solution and culture medium were measured by a clinical chemistry analyzer.
showed high vimentin expression. The vimentin expression increased with time, and vitrigel-M in HG medium showed higher expression than that of vitrigel-ME at D2. The expression intensity of vimentin in HG medium was higher than that of vitrigel-M in LG medium. AE1/AE3 expression, respectively, at D28 (Fig. 3). The vimentin expression of vitrigel-M in LG and HG media was higher than that of vitrigel-ME at D2. At D28, all mesothelial cells expressed VEGF, and the HG concentration synergistically promoted the VEGF expression of mesothelial cells. The mesothelial cells of vitrigel-ME showed high expression of BMP-7, while faint BMP-7 expression was seen in the mesothelial cells of vitrigel-M at D2 and D28. The mesothelial cells of vitrigel-ME in both LG and HG media showed weak or faint expression of CTGF, while the mesothelial cells of vitrigel-M in LG and HG media showed distinct expression of CTGF at D2. At D28, the mesothelial cells of vitrigel-ME in HG medium and vitrigel-M showed stronger positivity for CTGF compared with those of vitrigel-ME in LG medium.

The vitrigel-PM models replicated the EMT process and reactivity to glucose of mesothelial cells, and the cellular dysfunction of endothelial cells. The models showed a clear inverse correlation between BMP-7 and CTGF expressions in mesothelial cells, which depended on the presence of endothelial cells.

Vitrigel-PM-based peritoneal diffusion model. To evaluate the permeability of the vitrigel-PM, we performed permeability tests using a PD solution. The three types of vitrigel-PM models were precultured for 2 or 28 days (D2, D28) in LG or HG medium under fluid flow stress before the permeability tests. To replicate PD conditions, 0.5 ml of PD solution was poured into the inner side of the reconstructed chambers. Transport of low- and medium-molecular weight solutes was assessed by the D/M ratio for urea, creatinine, uric acid, and β2MG. Transport of glucose was assessed by the D/D0 ratio. The control vitrigel membrane without a cell component showed predominantly higher permeability for the tested items under all D2 conditions examined. The line plots for D/M and D/D0 are shown in Fig. 4.

Urea has a molecular weight of 60.06, and its D/M ratio in vitrigel-E was significantly higher than that in vitrigel-M and vitrigel-ME in both LG and HG media for 4 h in the D2 group. Vitrigel-ME in LG medium showed a significantly higher urea D/M ratio than vitrigel-ME in HG medium and vitrigel-M in LG and HG media at 0.5 h. In the D28 group, the D/M ratio of urea in vitrigel-E was significantly higher than that in vitrigel-M and vitrigel-ME in LG and HG media for 4 h. Vitrigel-ME in LG medium showed a significantly higher urea D/M ratio than vitrigel-ME in HG medium and vitrigel-M in both LG and HG media for 4 h.

Creatinine has a molecular weight of 113.12, and its D/M ratio in vitrigel-E was significantly higher than that in vitrigel-ME and vitrigel-M in both LG and HG media until 2 h in the D2 group. Vitrigel-ME showed a significantly higher creatinine D/M ratio than vitrigel-M in LG medium at 0.5, 2.0, and 4.0 h. In the D28 group, the D/M ratio of creatinine in vitrigel-E was significantly higher than that in vitrigel-ME and vitrigel-M in LG and HG media for 4 h. Vitrigel-ME in LG medium showed a significantly higher creatinine permeability than vitrigel-ME in HG medium at 2.0 and 4.0 h. Vitrigel-M in LG medium also showed a higher creatinine permeability than vitrigel-M in HG medium at 2.0 and 4.0 h.
Uric acid has a molecular weight of 168.11, and its D/M ratio in vitrigel-E was significantly higher than that in vitrigel-ME and vitrigel-M in both LG and HG media until 2 h in the D2 group. Vitrigel-ME showed a significantly higher uric acid D/M ratio than vitrigel-M in both LG and HG media at 0.5 h. At 2.0 and 4.0 h, the uric acid permeability in vitrigel-ME was higher than that in vitrigel-M in HG medium. In the D28 group, the permeability of uric acid in vitrigel-E was significantly higher than that in vitrigel-ME in HG medium and vitrigel-M in both LG and HG media for 4 h. In the D28 group, the permeability of \( \beta \)2MG in vitrigel-ME in HG medium was significantly lower than that in vitrigel-ME in LG medium and vitrigel-M in both LG and HG media at 1.0 and 2.0 h. The D/M ratio of \( \beta \)2MG in vitrigel-E in LG medium was significantly higher than that in vitrigel-M in HG medium at 1.0 h.

Glucose has a molecular weight of 180.16, and its D/D0 ratio in vitrigel-E was significantly lower than that in vitrigel-M in both LG and HG media for 4 h in the D2 group. There was no difference between vitrigel-ME and vitrigel-E in both LG and HG media. In the D28 group, the permeability of \( \beta \)2MG in vitrigel-ME in HG medium was significantly lower than that in vitrigel-ME in LG medium and vitrigel-M in both LG and HG media at 1.0 and 2.0 h. The D/M ratio of \( \beta \)2MG in vitrigel-E in LG medium was significantly higher than that in vitrigel-M in HG medium at 1.0 h.

\( \beta \)2MG has a molecular weight of 11,800, and its D/M ratio in vitrigel-E was significantly higher than that in the other models for 4 h in the D2 group. There was no difference between vitrigel-ME and vitrigel-E in both LG and HG media. In the D28 group, the permeability of \( \beta \)2MG in vitrigel-ME and endothelial cells of vitrigel-M in LG medium show a monolayer structure, while the mesothelial cells of vitrigel-M in HG medium show mild hypertrophy and a multilayer structure at day 2 (D2). The mesothelial cells of vitrigel-M show an augmented hyperplastic change in LG and HG media at day 28 (D28). On the other hand, the mesothelial hyperplasia is significantly inhibited in vitrigel-ME, even in HG medium, at D28. The endothelial cells of vitrigel-ME and endothelial cells of vitrigel-E do not show any obvious phenotypic changes under all conditions examined. B: endothelial cells inhibit the hyperplastic change in mesothelial cells. Bar = 50 \( \mu \)m. Values are means ± SE. **P < 0.001.

Fig. 2. A: mesothelial cells cultured with endothelial cells of vitrigel-mesothelial cells with endothelial cells (ME) in both low-glucose (LG) and high-glucose (HG) media and mesothelial cells of vitrigel-M in LG medium show a monolayer structure, while the mesothelial cells of vitrigel-M in HG medium show mild hypertrophy and a multilayer structure at day 2 (D2). The mesothelial cells of vitrigel-M show an augmented hyperplastic change in LG and HG media at day 28 (D28). On the other hand, the mesothelial hyperplasia is significantly inhibited in vitrigel-ME, even in HG medium, at D28. The endothelial cells of vitrigel-ME and endothelial cells of vitrigel-E do not show any obvious phenotypic changes under all conditions examined. B: endothelial cells inhibit the hyperplastic change in mesothelial cells. Bar = 50 \( \mu \)m. Values are means ± SE. **P < 0.001.
DISCUSSION

In the present study, we have established a novel peritoneal diffusion model in vitro and demonstrated for the first time that mesothelial cells and endothelial cells each have a specific slit function. In addition, the mesothelial slit function is not only regulated by the EMT process induced by high glucose but is also affected by an endothelial paracrine effect.

A limitation of this study may lie in the discrepancy between solute transport in our artificial model and clinical findings for long-term PD patients. Over time, PD patients develop increased transport of low-molecular-weight solutes across the peritoneal membrane (18). The enhanced diffusion is recognized by an increase in the number of peritoneal vessels, which frequently leads to an increased vascular surface area in long-term PD. This pathological change in the peritoneum causes an elevation of the D/P ratio (14, 19). On the other hand, the surface area of our vitrigel-PM model is fixed, and it can only be used to evaluate changes in solute diffusion with respect to the identical surface area. In other words, our vitrigel-PM model cannot increase its surface area in a manner that mimics the increase in vascular surface area in vivo. This study has demonstrated that high glucose stimulation slightly affects the endothelial slit function. However, our findings are consistent with the common recognition that diffusion coefficients are largely dependent on the vascular surface area.

Endothelial cells have been widely recognized as having specific pores and are supposed to play a key role in solute transport (12, 17, 23). In contrast, the mesothelial layer was not regarded as making a major contribution to the barrier function of the peritoneum (9, 10). Flessner (8) reported that the parietal peritoneum, including the mesothelial layers, which usually serves as a barrier, did not function against small solutes. Several reports have described mesothelial cells undergoing EMT and suggested that this may be responsible for high peritoneal transport rates (4, 7). Despite speculation that a phenotypic change, especially EMT, of mesothelial cells may affect peritoneal permeability, the precise mechanism has remained unclear. Our simplified in vitro system revealed that endothelial cells inhibited the EMT process and the permeability loss of mesothelial cells in LG medium and that this effect was abolished by HG medium in a short time period. With the abundant neovascular vessels present in peritoneal fibrosis, some research into the cell-cell interactions between endothelial cells and mesothelial cells has been carried out (21), but their cross talk has not been demonstrated. Both BMP-7 and CTGF play major roles in peritoneal fibrosis and have opposite effects and expression patterns (15, 20, 29). In this study, endothelial cells were shown to promote BMP-7 expression and inhibit CTGF expression in mesothelial cells. In addition, these expression patterns were strongly correlated with the morphological EMT change in mesothelial cells. These findings suggest that the high glucose concentrations in dialysates would not only lead to mesothelial dysfunction brought on by EMT but also reduce the protective effect afforded by endothelial cells. This breakdown of the homeostasis of the microenvironment between endothelial cells and mesothelial cells and the mesothelial EMT would synergistically promote peritoneal fibrosis, leading to ultrafiltration failure in long-term PD.

In conclusion, we have established a peritoneal diffusion model. Permeability tests of the vitrigel-PM model demonstrated for the first time that mesothelial cells and endothelial cells each play a role in the slit function related to peritoneal permeability. In addition, the mesothelial slit function is not

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Fig. 3. Immunostaining of vitrigel-PM models. All mesothelial cells show cytokeratin (CK) AE1/AE3 expression at D2, while those of vitrigel-M in LG and HG media show weak and absent CK AE1/AE3 expression, respectively, at D28. The intensity of vimentin expression increases with time, and vitrigel-M in HG medium shows high expression of vimentin. The mesothelial cells of vitrigel-ME in HG medium and vitrigel-M in LG and HG media show VEGF expression at D2. At D28, all mesothelial cells express VEGF, and a HG concentration synergistically promotes VEGF expression in mesothelial cells. Bone morphogenic protein (BMP-7) expression levels are high in the mesothelial cells of vitrigel-ME and low in those of vitrigel-M. The CTGF expression pattern shows an inverse correlation with that of BMP-7 expression. Bar = 50 μm.
only regulated by the EMT process induced by high glucose but is also affected by an endothelial paracrine effect. This alternative peritoneal model could become a promising tool for further PD development. More specifically, focusing on the dysfunctional peritoneal homeostasis in PD patients can help one to realize that the distinct cross talk between endothelial dysfunction and cellular endothelial-mesothelial cross talk in peritoneal fibrosis may represent a novel therapeutic target for peritoneal fibrosis.

ACKNOWLEDGMENTS

We thank H. Ideguchi, M. Nishida, F. Mutoh, S. Nakahara, and I. Nanbu for excellent technical assistance. We are grateful to K. Tokaichi for refining the English of the manuscript.

GRANTS

This work was supported in part by an Agri-Health Translational Research Project (No. 6110) from the Ministry of Agriculture, Forestry and Fisheries of Japan and a Grant-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology for Scientific Research (No. 25461701 to S. Aoki).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


