Effect of Glucose on Intercellular Junctions of Cultured Human Peritoneal Mesothelial Cells

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Abstract. During continuous ambulatory peritoneal dialysis, the peritoneum is directly and continuously exposed to unphysiologic peritoneal dialysis fluid; the resulting mesothelial damage has been suggested to cause loss of ultrafiltration and dialysis efficacy. The present study investigated the effect of a high glucose concentration on cultured human peritoneal mesothelial cells to clarify the cause of decreased dialysis efficacy during prolonged peritoneal dialysis. High glucose caused a concentration-dependent decrease in cell proliferation, damage to the intercellular junctions, and excess production of transforming growth factor-β (TGF-β). The levels of intercellular junctional proteins (ZO-1, E-cadherin, and β-catenin) were decreased, and immunostaining by anti–ZO-1 and anti–β-catenin antibodies became weaker and often discontinuous along the cell contour. Mannitol had similar but weaker effects at the same osmolality, and an anti–TGF-β neutralizing antibody reduced the effects of high glucose. Therefore, these effects were induced not only by glucose itself but also by hyperosmolality and by a glucose-induced increase of TGF-β. These findings suggest that the peritoneal mesothelium is damaged by prolonged peritoneal dialysis using high glucose dialysate and that impairment of the intercellular junctions of peritoneal mesothelial cells by high glucose dialysate induces peritoneal hyperpermeability and a progressive reduction in dialysis efficacy.

Continuous ambulatory peritoneal dialysis (CAPD) has been used for two decades as an adequate and effective treatment for end-stage renal disease. In patients with CAPD, the peritoneum functions as a dialyzer and forms the permeability barrier across which ultrafiltration and diffusion occur. Progressive loss of dialysis efficacy is a major complication of long-term CAPD. It has been attributed to the direct and continuous exposure of the peritoneum to peritoneal dialysis fluid, which is always unphysiologic because of its low pH, high glucose content, and hyperosmolality (1). Previous studies have shown that CAPD is associated with morphologic changes of mesothelial cells, such as loss of the microvilli, widening of the intercellular spaces, and exfoliation (2–5). Also, the mesothelium fails to regenerate properly after a long period of dialysis, so the peritoneum becomes denuded of mesothelial cells and undergoes fibrotic thickening (3,6,7). Damage to mesothelial cells is considered to be the primary cause of the decline in ultrafiltration and dialysis efficacy, which might be responsible for the relatively high failure rate of CAPD (8,9).

Mesothelial cells have the capacity to produce a variety of matrix macromolecules. Synthesis of the extracellular matrix is regulated by several growth factors, among which transforming growth factor-β (TGF-β) is the critical one (14). TGF-β has been detected in CAPD effluent (15), peritoneal mesothelial cells are known to synthesize this growth factor (16), and a high glucose concentration enhances TGF-β gene expression by cultured human peritoneal mesothelial cells (HPMC) (17–21). Moreover, it has been reported that TGF-β inhibits mesothelial cell growth and significantly increases the permeability of mesothelial cell monolayers for FITC-labeled albumin (22). Both of these effects of TGF-β are completely blocked by an anti–TGF-β neutralizing antibody (17,22).

Little is known about the effects of high glucose levels and TGF-β in peritoneal dialysis fluid on the intercellular junctions of mesothelial cells. Accordingly, this study investigated the effect of a high glucose concentration on the intercellular junctions and on TGF-β production by HPMC, as well as the effect of TGF-β and an anti–TGF-β neutralizing antibody.

Materials and Methods

Isolation and Culture of HPMC

HPMC were isolated according to the method of Stylianou et al. (23). Briefly, a piece of human omentum (approximately 3 to 5 cm²) obtained during abdominal surgery was washed three times with...
sterile phosphate-buffered saline (PBS) (pH 7.2; Nissui Pharmaceutical Co., Tokyo, Japan) and then incubated for 15 to 20 min at 37°C with shaking in PBS containing 0.125% (wt/vol) trypsin-0.01% (wt/vol) ethylenediaminetetraacetate solution (Life Technologies, Grand Island, NY). After incubation, the omental tissue was removed, the solution containing free mesothelial cells was centrifuged (100 g for 10 min at 4°C), and the supernatant was discarded. Then the cell pellet was washed once and suspended in M199 culture medium (Life Technologies) supplemented with 10% fetal calf serum (FCS; vol/vol) (Mitsubishi Kasei Corp., Tokyo, Japan), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and 2 mM L-glutamine (Life Technologies). After being seeded into 75-cm² tissue culture flasks coated with rat type I collagen (Becton Dickinson, Bedford, MA), the cells were grown in the same medium at 37°C under a 5% CO₂ atmosphere in a humidified incubator. The medium was changed at 24 h after seeding and then once every 3 d. Cells from the first to third passages were used.

Identification of Mesothelial Cells
The cultured cells were examined under an inverted phase contrast microscope. In addition, immunostaining was performed with monoclonal antibodies for human cytokeratin (Dako, Kyoto, Japan), vimentin (Dako), desmin (Dako), and human Factor VIII (Dako). Visualization was done with a Texas red-labeled secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA).

Culture Media
Fifteen different culture media were tested. Standard M199 medium containing 1.0% FCS (vol/vol), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine was used as the control (glucose concentration, 5.6 mM). In the experimental cultures, M199 medium was supplemented with D-glucose (Kanto Chemical Co., Inc., Tokyo, Japan), D-mannitol (Sigma Chemical Co., St. Louis, MO) at various concentrations (75 mM [1.35%], 140 mM [2.5%], and 222 mM [4.0%]), or with recombinant TGF-β1 (R&D Systems Inc., Minneapolis, MN) at various concentrations (100, 500, and 1000 pg/ml). In some experiments, the 222 mM D-glucose or 500 pg/ml TGF-β1 media also contained various concentrations of an anti-TGF-β neutralizing antibody (1, 4, and 10 μg/ml; R&D Systems). To assess the effect of blocking TGF-β activity, media containing TGF-β1 were preincubated with this neutralizing antibody for 2 h at room temperature. All media were sterilized by filtration (0.22 μm, Millex-GV, Millipore Corp., Bedford, MA) and then stored at 4°C for use within 3 d.

Assay of Cell Proliferation
Cells were grown to confluent monolayers in culture flasks, harvested with 0.25% (wt/vol) trypsin-0.02% (wt/vol) ethylenediaminetetraacetate in PBS, and resuspended in fresh M199 medium containing 10% FCS. Then the cells (2 × 10⁴/100 μl per well) were seeded into 96-well tissue culture plates coated with rat type I collagen (Becton Dickinson). After the cultures were confirmed to be subconfluent by microscopy, cells were washed twice with PBS and the medium was replaced with 100 μl of fresh standard medium. The cells were then cultured for an additional 48 h to allow them to become confluent and quiescent, washed twice with PBS, and used in

**Figure 1.** Standard curve of the cell counting kit (WST-1 colorimetric assay). The absorbance showed a good linear correlation with the number of cells at an excitation wavelength of 420 nm (n = 4, r = 0.966, P < 0.0001).

**Figure 2.** Effects of culture for 48 h with media containing various concentrations of glucose (G) or mannitol (M). A medium containing 5.6 mM glucose (pH 7.3) was used as the control. Results are mean ± SD of three experiments, each performed in duplicate. The number of cells decreased as the concentration of glucose or mannitol increased (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).
the following experiments. Cell viability was assessed using a cell counting kit (Dojindo, Kumamoto, Japan) that used a colorimetric assay based on cleavage of the water-soluble tetrazolium salt WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] to a formazan dye by mitochondrial dehydrogenase in viable cells (24,25). Test solutions (100 μl/well) were added to confluent HPMC and incubated for 6, 12, 24, or 48 h, with the WST-1 solution (5 mM WST-1, 20 mM HEPES, and 0.2 mM...
1-methoxy-5-methylphenazinium methylsulfate (being added at 10 μl/well for the last 4 h. The absorbance was determined using an enzyme-linked immunosorbent assay reader (SLT Lab Instruments, Salzburg, Austria) at an excitation wavelength of 420 nm and an emission wavelength of 630 nm.

Immunostaining of Intercellular Junction Proteins

Cells were cultured on 24 × 24-mm glass coverslips coated with porcine type I collagen (Nitta Gelatin Co., Osaka, Japan) in 60-mm culture dishes (Becton Dickinson). After cultures were shown to be subconfluent by microscopy, cells were washed twice with PBS, the medium was replaced with a fresh standard medium, and the cells were incubated for another 48 h to reach confluence. After the cells were washed twice more with PBS, they were then cultured for 48 h in the test media. Next, the cells were fixed in cold acetone/methanol at −20°C for 5 min, rehydrated in PBS, and blocked for 1 h in PBS containing 20% Block Ace (Dainippon Seiyaku Co., Ltd., Tokyo, Japan). This was followed by overnight incubation with a primary antibody at 4°C. The primary antibodies used were a rabbit antibody for ZO-1 (a tight junction protein; Zymed Laboratories Inc., San Francisco, CA) at a dilution of 1:100 and a mouse antibody for β-catenin (an adherens junction–associated protein; Zymed Laboratories Inc.) at 1:100. After incubation with the primary antibody, cells were washed five times with PBS. Then an appropriate secondary antibody (1:100) labeled with FITC (Dako) or Texas red (Jackson Immuno Research Laboratories) was applied for 1 h at room temperature, and the cells were again washed five times with PBS. Stained specimens were examined under a confocal laser scanning microscope (TCS-NT, Leitz, Wetzlar, Germany).

Western Blot Analysis of Intercellular Junction Proteins

Cells were cultured in 100-mm dishes. After the cultures were confirmed to be subconfluent by microscopy, the cells were washed twice with PBS, the medium was replaced with a fresh standard medium, and the cells were incubated for an additional 48 h to reach confluence. After the cells were washed twice with PBS, they were cultured for 48 h in the test media. Next, the cells were again washed twice with PBS and were lysed in a culture dish by adding 900 μl of TRIZOL® Reagent (Life Technologies). Cellular protein was isolated, and 20 μg of total protein, as measured with the BCA protein assay (Pierce, Rockford, IL), was dissolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 3 min. Samples were subjected to SDS-PAGE on 8% gels and then transferred to PVDF membranes (Millipore Corp.). The membranes were blocked for 1 h at room temperature in PBS containing 1% Tween 20 (Katayama Chemical, Osaka, Japan) and 20% Block Ace (PBST-20% Block Ace) and were incubated for 1 h with a primary antibody: rabbit anti-ZO-1 (1:5000), mouse anti–E-cadherin (Transduction Laboratories, Lexington, KY; 1:5000), or mouse anti–β-catenin (1:10,000). Then the membranes were washed (once for 15 min and twice for 5 min) in PBST-20% Block Ace and incubated for 1 h at room temperature with an appropriate secondary antibody (1:5000) labeled with horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, England). After incubation, the membranes were washed again (once for 15 min and four times for 5 min), and the reaction products were detected by chemiluminescence (Amersham Pharmacia Biotech). The intensity of each band was estimated using NIH image software (version 1.61).

Assay of TGF-β1 Production by HPMC

Supernatants were collected after the cells were cultured for 48 h with various glucose or mannitol concentrations in 12-well tissue culture plates (Becton Dickinson). The TGF-β1 level in culture su-
pernents was determined with an enzyme-linked immunosorbent assay kit (R&D Systems Inc.) and was normalized for the number of cells. All experiments were performed in duplicate. We also determined the TGF-β1 level in each test medium without HPMC.

Statistical Analyses
Results are expressed as the mean ± SD of three experiments, each performed in duplicate. Data were examined using ANOVA (Fisher’s protected least significant difference test), and differences were considered significant at \( P < 0.05 \).

Results
Identification of HPMC
The cultured cells were identified as mesothelial cells by their typical cobblestone appearance at confluence, positive staining for cytokeratin and vimentin, and negative staining for desmin and Factor VIII.

Figure 6. Transforming growth factor-β1 (TGF-β1) concentration in the culture supernatant after incubation of HPMC with various concentrations of glucose (G) or mannitol (M). Medium containing 5.6 mM glucose (pH 7.3) was used as the control. Results are mean ± SD of three experiments, each performed in duplicate. Glucose caused TGF-β1 to increase markedly, whereas mannitol increased TGF-β1 only moderately (***P < 0.01, ****P < 0.0001). In media without HPMC, the TGF-β1 concentration was always under the detection limit of the assay.

Figure 7. Effect of culture of HPMC for 48 h in media containing various concentrations of recombinant TGF-β1. Medium containing 5.6 mM glucose (pH 7.3) was used as the control. Results are mean ± SD of three experiments, each performed in duplicate. The number of cells decreased with an increasing concentration of recombinant TGF-β1 (**P < 0.01, ****P < 0.0001).

Standard Curve of WST-1 Colorimetric Assay
The absorbance showed a linear correlation with the number of cells (from 5.0 × 10^3 to 3.0 × 10^4 per well) in a 96-well culture plate (\( r = 0.966, P < 0.0001 \)). Therefore, the number of cells was calculated from the absorbance (Figure 1).

Proliferation of HPMC
There was a concentration-dependent decrease in cell proliferation after culture for 48 h as the glucose concentration increased from 5.6 mM to 222 mM (Figure 2). High glucose (222 mM) significantly depressed cell proliferation after 48 h when compared with the lowest glucose concentration tested (5.6 mM). Similar results were also seen after 6, 12, and 24 h of incubation. Mannitol also decreased cell proliferation in a concentration-dependent manner, but the extent of inhibition was less than that caused by glucose at the same osmolality. There was a significant difference in cell proliferation after 48 h of incubation with glucose or mannitol at 222 mM (\( P < 0.01 \)).
Immunostaining
In control cultures, the cell contour was clearly outlined by fluorescence immunostaining for ZO-1 and β-catenin. The contour immunofluorescence of both ZO-1 and β-catenin decreased and became discontinuous after incubation with glucose, and this effect was concentration-dependent (Figures 3 and 4). In addition, immunofluorescence for β-catenin was markedly increased in the perinuclear region. However, mannitol caused only a slight decrease in the expression of these proteins, and there was no discontinuity of staining along the cell contour. All of these changes were more marked after 48 h of culture.

Western Blot Analysis
High glucose (222 mM) decreased the levels of ZO-1, E-cadherin, and β-catenin (Figure 5). Mannitol also decreased ZO-1 and β-catenin, but the extent of the change was smaller than with glucose at the same osmolality. These changes all were more marked after 48 h of culture.

TGF-β1 Production
In control cultures, confluent HPMC were shown to constitutively release TGF-β1, and the mean (± SD) TGF-β1 level was 15.6 ± 0.4 pg/ml per 10^4 cells. The highest TGF-β1 concentration detected was 51.9 ± 2.6 pg/ml per 10^4 cells after culture with high glucose (222 mM). However, the TGF-β1 concentration was under the detection limit of the assay in media without HPMC. Induction of TGF-β1 secretion by glucose or mannitol was concentration dependent and was greater with glucose than with mannitol at the same osmolality (140 mM; P < 0.01, 222 mM; P < 0.0001) (Figure 6).

Effect of Recombinant TGF-β1
There was a concentration-dependent decrease of cell proliferation after culture for 48 h with recombinant TGF-β1 at concentrations from 100 to 1000 pg/ml (Figure 7). Contour immunofluorescence for both ZO-1 and β-catenin also decreased and became irregular in a concentration-dependent manner (Figure 8). Furthermore, culture with recombinant TGF-β1 decreased the levels of ZO-1, E-cadherin, and β-catenin, as shown by Western blotting (Figure 9).

Effect of the Anti–TGF-β Neutralizing Antibody
Addition of the anti–TGF-β neutralizing antibody to cultures increased the proliferation of HPMC in the presence of 222 mM D-glucose or 500 pg/ml recombinant TGF-β1 (Figure 10). We tested the neutralizing antibody at 1, 4, and 10 μg/ml and found that it was most effective at 4 μg/ml. It also increased the contour immunostaining for ZO-1 and β-catenin in cells incubated with 222 mM D-glucose (Figure 11) or 500 pg/ml recombinant TGF-β1 (Figure 12). Western blotting confirmed that the levels of ZO-1, E-cadherin, and β-catenin were increased by the neutralizing antibody (Figures 13 and 14).

Although cell proliferation and the expression of intercellular junctional proteins both were increased in cultures with high glucose (222 mM), they did not reach control levels. Thus, the blocking effect of the neutralizing antibody on the actions of 222 mM glucose was weaker than that against 500 pg/ml of recombinant TGF-β1.

Figure 8. Immunocytochemistry for ZO-1 (a through d) and β-catenin (e through h) after culture with TGF-β1. (a, e) Control, (b, f) 100 pg/ml recombinant TGF-β1, (c, g) 500 pg/ml recombinant TGF-β1, (d, h) 1000 pg/ml recombinant TGF-β1. Bar, 10 μm. The cell contour is outlined by continuous fluorescence in the control (a, e). Fluorescence decreases and becomes irregular as the TGF-β1 concentration increases.
CAPD is a well-accepted treatment for end-stage renal disease, but the long-term success of peritoneal dialysis is limited. Both long-term CAPD and episodes of peritonitis cause significant abnormalities in the morphology and function of the peritoneal membrane; the predominant changes are the development of peritoneal fibrosis and sclerosis. Previous studies have demonstrated that high glucose and hyperosmolar peritoneal dialysates can damage mesothelial cells both in vivo and in vitro (1–7,12,26–29). Thus, damage to mesothelial cells, which act as a selective permeability barrier to regulate the passage of water and solutes between the intravascular compartment and the peritoneal cavity, is considered to be the primary cause of peritoneal hyperpermeability and/or peritoneal fibrosis. However, the changes of intercellular junctions, which play an essential role in controlling permeability and in maintaining tissue organization, are poorly understood.

In the present study, we assessed the effects on HPMC of culture with glucose at 75 mM (1.35%), 140 mM (2.5%), and 222 mM (4.0%), which were the same concentrations as those in commercial peritoneal dialysates. In the presence of high glucose, we confirmed that there was a concentration-dependent decrease in cell proliferation, as has also been demonstrated in previous studies (17–21,29). We also assessed the effect of mannitol on cultured HPMC as an osmotic control. Even though a concentration-dependent decrease in cell proliferation was induced by mannitol, its effect was significantly weaker than that of glucose.

It has been reported that chronic exposure to dialysis fluid induces widening of the intercellular spaces between HPMC and detachment of these cells from the peritoneum (2–5). In the present study, we investigated the effects of high glucose on the intercellular junctions of HPMC. Immunostaining for ZO-1 and β-catenin was linear and continuous along the cell contour in control cultures but became weaker and discontinuous in a concentration-dependent manner after culture with high glucose. Conversely, mannitol caused only a slight decrease of staining, with no discontinuity. Western blotting confirmed that high glucose decreased the levels of ZO-1, E-cadherin, and β-catenin. Mannitol also decreased ZO-1 and β-catenin, but the extent of the change was smaller.

It is generally believed that tight junctions can dynamically alter their structural and functional properties under different conditions and are subject to modulation by a variety of cellular and metabolic regulators (13,30–34). It has also been shown that the cadherin/catenin complex plays an important role in cell adhesion and signal transduction, as well as in initiation and maintenance of the structural and functional organization of cells and tissues (35–39). In the absence of functional catenins, adherens junctions do not form (37). The findings of the present study suggest that high glucose peritoneal dialysis fluid decreases the intercellular junctions of mesothelial cells and impairs their function, thus causing peritoneal hyperpermeability. The results of our cell proliferation, immunostaining, and Western blotting experiments demonstrated that the inhibitory effect of high glucose was not solely related to hyperosmolality but also to some property of glucose itself or its metabolic effects. Mannitol was less toxic than glucose in the present study, but the effects of metabolites of mannitol in humans are not well understood. In contrast, glu-

**Figure 9.** (A) Western blot analysis of intercellular junction–associated proteins (ZO-1, E-cadherin, and β-catenin) after HPMC were cultured with 100 pg/ml (T100), 500 pg/ml (T500), or 1000 pg/ml (T1000) of recombinant TGF-β1. (B) ZO-1 and E-cadherin were reduced in a concentration-dependent manner. β-catenin was also reduced, but no significant difference was observed between each concentration of TGF-β1. Data show the mean values of three experiments. The control (5.6 mM glucose) value was assumed to be 100% (*P < 0.0001 versus control).
Figure 10. (A) Effect on the proliferation of HPMC after culture for 48 h in media containing high concentration of glucose (G 222 mM) with or without an anti–TGF-β neutralizing antibody (ATA, the numbers show the concentration in μg/ml). Medium containing 5.6 mM glucose (pH 7.3) was used as the normal control. Results are mean ± SD of three experiments, each performed in duplicate (*p < 0.05, **p < 0.01, ****p < 0.0001). The neutralizing antibody significantly increased the number of cells compared with culture with 222 mM glucose (G 222 mM) alone. (B) Effect on the proliferation of HPMC after culture for 48 h in media containing 500 pg/ml recombinant TGF-β1 with or without an anti-TGF-β neutralizing antibody (ATA, the numbers show the concentration in μg/ml). Medium containing 5.6 mM glucose (pH 7.3) was used as the normal control. Results are mean ± SD of three experiments, each performed in duplicate (**p < 0.01, ***p < 0.001, ****p < 0.0001). The neutralizing antibody, especially at 4 μg/ml, significantly increased the number of cells compared with culture with 500 pg/ml of recombinant TGF-β1 (T500) alone.

Figure 11. Immunocytochemistry for ZO-1 (a, b) and β-catenin (c, d) after culture of HPMC in medium containing 222 mM glucose alone (a, c) or 222 mM glucose plus an anti–TGF-β neutralizing antibody (4 μg/ml; b, d). Bar, 10 μm. The neutralizing antibody increased immunostaining of the cell contour.

Figure 12. Immunocytochemistry for ZO-1 (a, b) and β-catenin (c, d) after culture in medium containing 500 pg/ml recombinant TGF-β1 alone (a, c) or TGF-β1 plus an anti-TGF-β neutralizing antibody (4 μg/ml) (b, d). Bar, 10 μm. The neutralizing antibody increased immunostaining of the cell contour.
Glucose is known to be safe, effective, cheap, and readily metabolized, so glucose is used clinically in dialysis fluids (40).

TGF-β is a classical cytokine that plays a central role in regulating tissue repair and remodeling after injury, along with platelet-derived growth factor and basic fibroblast growth factor. Production and deposition of the extracellular matrix is an essential part of wound repair. TGF-β is unique among the
cytokines in stimulating matrix synthesis, inhibiting matrix degradation by proteases, and modulating matrix receptors to increase cell adhesion (41). In the present study, to investigate the mechanism behind the differing effects on cell proliferation and intercellular junctions of glucose and mannitol, we measured TGF-β1 levels in the culture supernatant. A concentration-dependent increase of TGF-β1 was observed with exposure of HPMC to glucose, so we also investigated the effects of TGF-β1 on HPMC. We showed that cell proliferation, immunofluorescence for ZO-1 and β-catenin, and the expression of ZO-1, β-catenin, and E-cadherin were decreased in a TGF-β1 concentration-dependent manner. Moreover, the present study showed that an anti–TGF-β neutralizing antibody could inhibit the effects of both high glucose and TGF-β1 on HPMC, although its inhibition of the actions of high glucose was weaker than its blocking effect on TGF-β1.

These findings suggest that TGF-β-induced by high glucose inhibits mesothelial cell proliferation and causes damage to intercellular junctions. The difference in cytotoxicity between glucose and mannitol may be largely related to TGF-β1.

In conclusion, the present study first clarified that (1) high glucose and TGF-β caused damage to the intercellular junctions of HPMC (2), an anti–TGF-β neutralizing antibody increased the expression of intercellular junctions, and (3) the effects of high glucose were caused not only by glucose itself but also by hyperosmolality and by glucose-induced TGF-β but also as a result of hyperosmolality per se.

Acknowledgment
This work was supported by the Baxter PD Fund.

References


