High glucose levels inhibit focal adhesion kinase-mediated wound healing of rat peritoneal mesothelial cells

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Background. The peritoneum is progressively denuded of its mesothelial cell monolayer in patients on continuous ambulatory peritoneal dialysis (CAPD). These alterations of the mesothelium cause membrane dysfunction and progressive peritoneal fibrosis. Integrins regulate cell motility and play an important role in wound healing. We investigated the effects of high glucose on the regeneration process of the peritoneal mesothelial cell monolayer using cultured rat peritoneal mesothelial cells (RPMC).

Methods. The effects of glucose or mannitol on the regeneration of RPMC and formation of focal adhesions were examined by in vitro wound healing assay and immunocytochemistry, respectively. Activities of focal adhesion kinase (FAK) and its downstream p130Cas were examined by Western blotting. Effects of wild-type and dominant-negative FAK on RPMC migration were examined by a transient transfection assay.

Results. Cell migration over fibronectin (FN) was clearly inhibited in culture media containing high glucose (28 to 140 mmol/L). RPMC formed focal adhesions on FN in the presence of a regular glucose concentration (5.6 mmol/L); however, tyrosine phosphorylation of FAK and p130Cas and formation of focal adhesions observed by FAK and vinculin staining were substantially inhibited by high glucose. Mannitol also induced significant inhibitory effects, but these were milder than those of glucose. Transfection of dominant-negative FAK inhibited cell migration in a regular glucose concentration, whereas overexpression of wild-type FAK abrogated glucose-induced inhibition of cell migration.

Conclusions. Our results demonstrate that high glucose concentrations as well as high osmolarity inhibit FAK-mediated migration of mesothelial cells, and suggest that dialysates containing high glucose concentrations may cause peritoneal damage by inhibiting wound healing of the mesothelial cell monolayer.

Key words: continuous ambulatory peritoneal dialysis, dialysate, focal adhesion kinase, peritoneal mesothelial cells, integrins, glucose.

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It is important to maintain adequate peritoneal function for long-term continuous ambulatory peritoneal dialysis (CAPD) therapy. Loss of ultrafiltration is one of the most important reasons for discontinuation of CAPD [1, 2]. Furthermore, some patients develop sclerosing encapsulating peritonitis, a condition that hinders CAPD and is associated with high mortality [3–5]. The peritoneal membrane and the monolayer of mesothelial cells are the key structures involved in regulating permeability and ultrafiltration as well as biological and physical barriers in patients with CAPD. Morphological studies of the peritoneal membrane in patients chronically exposed to the dialysis fluid have shown loss of mesothelial cells and replacement of the membrane by collagen-based fibrous tissue [1, 6]. Furthermore, shedding and regeneration of peritoneal mesothelial cells continuously occur during CAPD in response to direct exposure of mesothelial membrane to non-physiological dialysis solution soon after initiation of CAPD [7, 8]. Peritonitis accelerates such damage of the peritoneal membrane, which is followed by shedding of mesothelial cells and peritoneal fibrosis. These changes in mesothelial cells are associated with high permeability of solutes and loss of ultrafiltration [1, 4, 9]. Therefore, there is a pressing need for a peritoneal dialysate that is less harmful to the peritoneal membrane and that does not inhibit regeneration of the mesothelial cell monolayer.

The peritoneal dialysate, containing non-physiological materials such as glucose, hypertonic, lactate-buffered, and acidic solution, is thought to be toxic and is implicated in long-term damage of the peritoneal membrane [6, 10]. In particular, there is a growing body of evidence indicating that high glucose concentration plays important roles in the progression of mesothelial fibrosis in patients with CAPD. Clinically, peritoneal sclerosis and increased membrane solute transport are associated with the use of hypertonic glucose dialysate [11]. These findings suggest that high glucose concentrations in perito-
neal dialysis solution may induce accumulation of ECM and eventual peritoneal fibrosis. On the other hand, there is limited information about the effect of high glucose concentration on the regeneration of mesothelial monolayer. Growth factors, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF) are known to induce chemotaxis of various cells [12–15]. However, to our knowledge there are no studies that have examined the effects of high glucose concentration per se on integrin-mediated cell migration or formation of focal adhesions.

Integrins are major adhesion receptors composed of pairs of α and β transmembrane subunits that mediate the specific association of cells with the extracellular matrix (ECM) and an assembly of cytoplasmic cytoskeletal and signaling complexes, including focal adhesion kinase (FAK), p130Cas, paxillin, vinculin, tensin, and localized phosphotyrosine, which lead to activation of various intracellular signal transduction pathways [16–18]. Of particular interest is FAK, which is a key molecule in integrin-mediated regulation of cell spreading and migration. FAK expression is enhanced in rapidly migrating keratinocytes [19] and in invasive human tumors [20], and migration of endothelial cells is also positively associated with FAK activity [21]. Cells from FAK-deficient mice [22] and cells in which FAK signaling is inhibited by dominant-negative FAK [23] or specific phosphatase exhibit reduced cell migration [15, 24–27], whereas overexpression of FAK increases migration toward FN [28]. However, involvement of integrin in peritoneal dialysis has not been thoroughly investigated. In particular, integrin-mediated signaling in peritoneal mesothelial cells is poorly understood. Various phenotypes of integrin subunits, such as β1 and α1, 2, 3, 5, and v, are constitutively expressed in human peritoneal mesothelial cells (HPMC) [29]. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) stimulates β1 integrin expression and promotes HPMC migration [30]. The present study was designed to investigate the effects of high glucose concentration as well as high osmolarity on wound healing of injured mesothelial cell monolayer in vitro, especially focusing on integrin-mediated cell adhesion mechanisms. Our studies analyzed the underlying mechanisms of dialysate-induced peritoneal injury.

METHODS

Cell culture

Rat peritoneal mesothelial cells (RPMC) were obtained using a standard trypsin/ethylenediaminetetraacetic acid (EDTA) digestion method from the peritoneal wall of male Wistar rats weighing about 150 g [31]. Briefly, the excised peritoneal flap was stretched on a sterile culture plate, and phosphate-buffered saline (PBS) containing 0.25% trypsin and 1 mmol/L EDTA was placed on the peritoneum for 15 minutes. The recovered fluid was centrifuged at 1000 rpm for five minutes after adding Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were cultured in DMEM supplemented with 10% FBS. Cells were identified as RPMC by its cobblestone appearance (Fig. 3) and positive staining for mon Nolanclonal anti-human mesothelial cell antibody (HBME-1; DAKO, Carpinteria, CA, USA), mon Nolanclonal anti-cytokeratin 19 antibody (RPN 1165; Amersham Life Science, Little Chalfont, UK), and mon Nolanclonal anti-cytokeratin 18 antibody (#10500, Cappel; ICN Biomedicals, Aurora, OH, USA) by immunocytochemistry using Cy-3-conjugated goat antibody to mouse IgG as a secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA). RPMC were maintained in DMEM containing 5.6 mmol/L glucose, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO2 at 37°C. RPMC were used between passages 3 and 6.

Materials

Monoclonal antibodies for paxillin, tensin, FAK, p130Cas, and phosphotyrosine (PY20) were obtained from Transduction Laboratories (Lexington, KY, USA). Monoclonal antibodies against vinculin (VIN-11-5) and HA (12CA5) were from Sigma Aldrich (St. Louis, MO, USA) and Boehringer Mannheim (Indianapolis, IN, USA), respectively. Rabbit polyclonal antibodies against Y397-phosphorylated FAK were from BioSource International (Camarillo, CA, USA). α-glucose and α-mannitol were purchased from Nacalai (Tokyo, Japan) and used for cell culture immediately after a 0.2 μm filter sterilization to avoid the effects of glucose degradation products (GDP). Human fibronectin (FN) was from Sigma. Cy-3-conjugated goat antibody to mouse IgG was from Jackson Immunoresearch Laboratories.

Immunoprecipitation and Western blotting

After incubating cells in 10% serum-containing DMEM supplemented with indicated amounts of either glucose or mannitol for 24 hours on 10 μg/mL FN-coated Petri dishes, cells were solubilized in RIPA buffer [50 mmol/L Tris, pH 7.4, 150 mmol/mL NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L egtazic acid (EGTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and a protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany)]. Homogenates were clarified by centrifugation at 15,000 × g for 15 minutes at 4°C. Immunoprecipitation was performed using anti-p130Cas (4 μg/mL) antibody as described previously [24]. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blotting with RC20 (1:2500)
or p130Cas (1:1000) antibodies using the enhanced chemiluminescence system (ECL; Amersham Life Science, Arlington Heights, IL, USA) [32]. Phosphorylation of FAK was analyzed by Western blotting using anti-phospho-FAK monoclonal antibody (1:2000) as described previously [33].

**Cell migration and spreading assays**

Cell migration was measured using an in vitro wound healing assay as described previously [24, 25]. Briefly, in vitro “scratch” wounds were created by scraping confluent cell monolayers plated on 10 μg/mL FN-coated Petri dishes with a sterile pipette tip. Numbers of migrating cells from the wound edge were quantified after 24-hours of incubation. Each determination represents the mean value (± SD) of eight individual experiments. Cells spreading on 10 μg/mL FN-coated glass cover slips were quantified as described previously [24] by scoring at least 100 cells per sample and pooling data of five experiments.

**Immunocytochemistry**

Immunocytochemistry was performed as described [24]. RPMC were plated on glass cover slides coated with FN (10 μg/mL). Cells were allowed to spread for the indicated times in DMEM containing 1% bovine serum albumin (BSA), fixed with 4% paraformaldehyde and 0.5% Triton X-100 in PBS for five minutes, and then incubated with 4% paraformaldehyde in PBS for another 20 minutes. Cytoskeletal proteins were visualized by incubating first with monoclonal antibodies to paxillin (0.5 μg/mL), tensin (5 μg/mL), FAK (5 μg/mL), phosphotyrosine (10 μg/mL), or vinculin (20 μg/mL), and then with Cy-3-conjugated goat antibody to mouse IgG (dilution 1/600) and assessed by using confocal microscopy (LSM410, Carl Zeiss, Oberkochen, Germany).

**Cell cycle analysis**

Fluorescence-activated cell sorter (FACS) analysis was carried out with 1 × 10⁶ cells plated on 6-cm plastic tissue culture dishes in 10% serum-containing medium [33, 34]. After 24 hours of exposure to DMEM containing indicated concentration of glucose, cells were collected, washed with PBS, and fixed in 70% ethanol for two hours at 4°C, and washed once with PBS. After treatment of cells with 10 μg/mL ribonuclease (Wako, Osaka, Japan) for 15 minutes at 37°C, fixed cells were stained with 50 μg/mL propidium iodide (Sigma Aldrich) for 15 minutes. DNA content was subsequently measured by FACSscan (Becton Dickinson, San Jose, CA, USA).

**Plasmids and transfection assay**

Hemagglutinin (HA)-tagged FAK was kindly provided by Kenneth M. Yamada (NIDCR, NIH, Bethesda, MD, USA) [24]. The point mutant Y397F was introduced into HA-FAK by site-directed mutagenesis using PCR (Stratagene, La Jolla, CA, USA) and confirmed by DNA sequencing [33]. Puromycin-resistant plasmid pHNA262pur was obtained from Dr. Heinte Riele (Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Netherlands). Either wild-type or Y397F HA-FAK (5 μg) was transfected into preconfluent RPMC plated at 10 μg/mL FN-coated 8-well chamber slides (Nalge Nunc International, Naperville, IL, USA) using the cationic liposome (lipofectamine; Gibco BRL, Gaithersburg, MD, USA)-mediated transfection method [35, 36]. To increase the expression of transfected genes, 5 mmol/L sodium butyrate was added to the culture medium for 24 hours [24]. At 24 hours after transfection, cells were used for in vitro wound healing assay as described above. Cells expressing HA-FAK were confirmed by immunocytochemistry using anti-HA monoclonal antibody.

**Statistical analysis**

Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) or the Student t test. A P value less than 0.05 denoted the presence of a statistically significant difference.

**RESULTS**

**Effects of high glucose concentration on cell viability**

First, the effects of high glucose on viability of RPMC were examined, since high glucose is known to induce apoptosis and growth inhibition [37], which might influence the results of the cell migration assay. The effect of glucose on cell cycle of RPMC was examined in 0.5 or 10% FBS. By FACS analysis, in culture medium with regular glucose concentration (5.6 mmol/L) supplemented with 10% serum, there was a decrease in sub-G₁ population and an increase in G₂/M population compared with those of serum-starved regular glucose medium after 24 hours of incubation (Fig. 1A). Higher concentrations of glucose resulted in a decrease in G₂/M population and an increase in sub-G₁ population. These changes were statistically significant at 222 mmol/L glucose, indicating that high glucose concentration in the culture medium increases growth-arrested cells and inhibits cell cycle progression. Furthermore, the increase in lactate dehydrogenase (LDH) released into the culture medium by RPMC was similar in media containing 222 mmol/L glucose or mannitol (Fig. 1B). Thus, 222 mmol/L glucose and mannitol exhibited significant cytotoxicity, and for this reason we selected glucose or mannitol at concentrations less than 140 mmol/L in subsequent experiments to assess cell migration. Interestingly, acidic culture medium had no significant effects on the cell cycle (Fig. 1A). We also examined the effects of 140 mmol/L glucose, 140 mmol/L mannitol, and acidosis (pH
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**Fig. 2. Dose response of glucose-induced inhibition of mesothelial cell migration.** In vitro “scratch” wounds were created by scraping confluent monolayers of cultured rat peritoneal mesothelial cells in FN-coated dishes. After 24 hours of incubation in 10% serum-containing DMEM supplemented with the indicated glucose concentration, cell numbers migrating into the wounded area from the wound edge were counted. The values are expressed as migrating cell number per 1 mm wound width. Data are mean ± SD values of four independent experiments.

**Effects of high glucose concentration on cell migration**

Next, RPMC migration was examined using the in vitro wound healing assay by creating scratch wounds in confluent monolayers of RPMC. After a 24-hour incubation in 10% serum-containing DMEM supplemented with the indicated glucose concentrations, the number of cells migrating from the wound edge decreased in a glucose concentration-dependent manner (Fig. 2). At 140 mmol/L, respectively. The cell cycle was analyzed by FACScan after a 24 hour incubation in each medium as described in the Methods section. The numbers in each panel represent the percentage of cells exhibiting mean channel fluorescence (FL2-H) below the G1 phase or in the G2/M phase of the cell. Symbols are: (○) cells below the G1 phase; (■) cells in the G2/M phase. Data are mean ± SD values of four independent experiments. *P < 0.05 vs. control with 10% FBS. (B) Effects of glucose (■) and mannitol (○) on LDH release in RPMC. RPMC were incubated for 24 hours in 10% FBS-containing culture medium supplemented with indicated amount of either glucose or mannitol. Results are relative LDH activity in culture supernatant versus total cellular LDH activity. Values are adjusted to the control (100). Data are mean ± SD values of five independent experiments (*P < 0.01 vs. control). (C) Effects of glucose, mannitol, and acidosis on cell growth. RPMC were incubated for 24 hours in 10% FBS-containing regular culture medium, culture medium supplemented with either 140 mmol/L glucose or mannitol, or acidic culture medium (pH 6.0) with regular glucose concentration. Cell numbers were counted before the incubation (baseline) and 24 hours after exposure and expressed as ratios against the value of baseline (100). Data are mean ± SD values of six independent experiments.
Fig. 3. Effects of various dialysate ingredients on cell migration. In vitro wound healing assay was performed as described in the legend to Figure 2 in 10% serum-containing DMEM supplemented with either regular glucose concentration, indicated concentration of glucose or mannitol, or acidic medium with regular glucose concentration. After 24-hours of incubation, cells migrating from the wound edge were photographed (A) and counted (B). Dotted lines in panel A indicate the wound edge. Data are mean ± SD values of five independent experiments (*P < 0.05 vs. 75.6 mmol/L glucose).

glucose reduced cell migration by 76.6% (23.4% of control, P < 0.001). Figure 3A shows representative photographs of cells migrating into the wound. Both 140 mmol/L glucose and mannitol showed substantial inhibition of cell migration on FN, and cell migration was reduced in glucose and mannitol concentration-dependent manner. However, the effect of 75.6 mmol/L mannitol on cell migration was less than that of 75.6 mmol/L glucose (glucose: 40.3% of control, mannitol: 69.9% of control, P < 0.05, Fig. 3B). On the other hand, there was no significant inhibition of RPMC migration under acidic conditions compared with cells grown at normal pH.

Effects of high glucose concentration on focal adhesion formation

To explore the mechanism underlying the effect of high glucose on RPMC migration, we next examined the effect of high glucose on focal adhesion formation. After two hours of adhesion to FN, RPMC formed numerous focal adhesions, which contained tensin, paxillin, vinculin, FAK, and localized phosphotyrosine, as observed in many other cells (Fig. 4). In cells incubated with 140 mmol/L glucose, the total number of focal adhesions stained by anti-vinculin and FAK antibodies was substantially reduced in comparison with the control after one hour of spreading onto FN-coated cover slips (Fig. 5). Similarly, mannitol inhibited focal adhesion formation. High glucose also down-regulated formation of tensin-, paxillin-, and localized phosphotyrosine-containing focal adhesions and actin microfilament (stress fiber) as determined by staining with rhodamine-labeled phallloidin (data not shown).

Effects of high glucose concentration on cell spreading

To further assess glucose inhibition of focal adhesions, cell spreading onto FN was examined. Both glucose and mannitol showed marked reduction of cell spreading on FN at 0.5 to four hours (Fig. 6). In a two hour assay, only 21.7 ± 3.5% of cells in 75.6 mmol/L glucose had spread, whereas 59.0 ± 3.6% of cells in regular culture medium had spread (P < 0.001). Mannitol (75.6 mmol/L) also reduced cell spreading (35.0 ± 7.2%) but was less effective compared to 75.6 mmol/L glucose (P < 0.05; Fig. 6). An acidic condition (pH 6.0) had no effect on cell spreading, while excessive acidosis (pH 5.0) showed slight but significant reduction in cell spreading at one to four hours (Fig. 6).

Inhibition of FAK and p130Cas by high glucose concentration

Cell interactions with extracellular matrix proteins through integrin receptors can mediate transmembrane signal transduction, including integrin-mediated tyrosine phosphorylation of FAK and p130Cas [24, 25]. High concentrations of glucose and mannitol suppressed tyrosine phosphorylation of FAK after adhesion to FN, whereas
Fig. 4. Formation of focal adhesions in cultured peritoneal mesothelial cells. Cultured peritoneal mesothelial cells were allowed to spread on cover slips coated with FN (10 μg/mL) for two hours and were then immunostained with antibodies to tensin, phosphotyrosine, paxillin, vinculin, or FAK. Arrowheads indicate representative focal adhesions (scale bar, 50 μm).

Fig. 5. Inhibition of focal adhesion formation by glucose and mannitol. Cultured peritoneal mesothelial cells were immunostained with antibody to vinculin (upper panels) or FAK (lower panels) one hour after plating on FN (10 μg/mL)-coated cover slips. Arrows and arrowheads are samples of cells spread and cells not spread, respectively (scale bar, 50 μm).
an acidic condition (pH 6.0) had no effects on phosphorylation of these molecules (Fig. 7A). The adapter protein p130Cas is a downstream effector of FAK, and it is characteristically tyrosine phosphorylated after increases in FAK and c-Src kinase activity in response to attachment to the extracellular matrix [38]. Therefore, we investigated whether p130Cas also is implicated in high glucose-induced inhibition of cell migration as a downstream event of FAK dephosphorylation. Again, tyrosine phosphorylation level of p130Cas was repressed by both high glucose and mannitol concentrations (Fig. 7B), suggesting that high osmolarity also inhibits integrin-mediated intracellular signaling.

Effects of wild-type and dominant-negative FAK on glucose inhibition of mesothelial cell migration

The correlation of FAK dephosphorylation with inhibition of cell migration suggests that the inhibition of migration may be a downstream event of FAK dephosphorylation by glucose. Next, we tested whether the dominant-negative form of FAK could inhibit cell migration, and whether FAK overexpression, in turn, could attenuate the effects of high glucose on cell migration. Cells were transfected either wild-type or Y397F HA-FAK. After 24 hours of transfection, cells expressing either wild-type or Y397F HA-FAK were incubated with culture medium with regular glucose concentration or 140 mmol/L glucose for another 24 hours. Transfected cells were identified by positive staining for HA. We examined the effects of wild-type or Y397F HA-FAK on cell number to rule out possible effects of these constructs on glucose regulation.
Fig. 8. Effects of wild-type and dominant-negative FAK on glucose inhibition of mesothelial cell migration. Mesothelial cells were transfected without or with either wild-type or Y397F FAK and used for in vitro migration assay after 24 hours. In vitro “scratch” wounds were created by scraping confluent cell monolayers into 10 μg/mL FN-coated dishes. After 24 hours, cells expressing HA-FAK were identified by immunocytochemistry using anti-HA antibody. Cell numbers (A), cell spreading (B), and numbers of cells migrating from the wound edge (C) were counted. The transfection efficiencies for wild-type and Y397F FAK were 13 ± 7% and 15 ± 5%, respectively; control = 5.6 mmol/L glucose and glucose = 140 mmol/L glucose, while baseline represents the cells numbers counted before the incubation. Data are mean ± SD values of five independent experiments (*P < 0.001 vs. control cells without FAK transfection in regular glucose concentration, **P < 0.01 vs. cells without FAK transfection in 140 mmol/L glucose).

on cell growth that may affect the results of cell migration. Both wild-type and Y397F FAK showed a minimal effect on cell number during the experiment (Fig. 8A). As shown in Figure 8B and C, although overexpression of wild-type FAK had minimal to only very slightly positive effects on cell spreading and migration on FN, expression of Y397F-FAK, which results in inhibition of phosphorylation of endogenous FAK [33], clearly reduced cell spreading and migration even in the presence of regular glucose concentration. Furthermore, overexpression of wild-type FAK abrogated high glucose-induced down-regulation of cell spreading (101.2% of control) and migration (86.6% of control), suggesting that FAK is an important mediator of high glucose-induced inhibition of cell migration.

We also confirmed the effect of overexpression of wild-type or Y397F FAK on FAK phosphorylation levels as described previously [27]. RPMC were cotransfected with either wild-type or Y397F FAK and a puromycin-resistant plasmid, pHA262pur, and selected using puromycin. This selection for transient transfecants resulted in ~90% positive cells expressing HA, as determined by fluorescence microscopy. Transfection of Y397F FAK resulted in decrease in FAK phosphorylation levels (12 ± 4% of non-transfected cells) even in regular glucose concentration. Furthermore, overexpression of wild-type FAK rescued high glucose-induced decrease in FAK phosphorylation levels (101 ± 13% of non-transfected cells in regular glucose concentration). Thus, we confirmed that overexpression of wild-type or dominant-negative FAK indeed led to higher or lower levels of FAK phosphorylation. These data support our model in which high glucose levels affect cell spreading through interference with FAK signaling.

DISCUSSION

The major findings of the present study were: (1) glucose inhibited mesothelial cell migration and spreading in a dose-dependent manner (Fig. 2). (2) These alterations were associated with inhibition of focal adhesion formation and phosphorylation of FAK and its downstream p130Cas (Figs. 4 to 7). (3) The dominant-negative form of FAK inhibited cell migration even in regular glucose medium as effectively as in high glucose medium (Fig. 8). (4) Overexpression of wild-type FAK reversed the inhibitory action of high glucose on cell migration (Fig. 8). To our knowledge, our studies provide the first evidence that high glucose can restrain the capacity of peritoneal mesothelial cells to migrate over one of the basement membrane matrix component. These results suggest that high glucose concentrations in peritoneal dialysates might suppress peritoneal cell regeneration by inhibiting integrin-mediated cell migration, which results in a loss of the mesothelial cell monolayer, as seen in many patients undergoing long-term CAPD therapy.

Our results also showed that mannitol had similar, though lesser inhibitory effects on cell migration and spreading compared to those of glucose at equivalent osmolarity, suggesting that not only high glucose concentration but also hyperosmolarity per se might play a role in these inhibitory effects. Because it has been reported
that high glucose and hyperosmolarity inhibit cell proliferation and induce apoptosis after long-term exposure [37, 39, 40], these inhibitory effects of glucose and manni-tol on cell migration might stem from reduced cell viability. Therefore, we examined the effects of glucose and manniitol on cell cycle, LDH release, and cell growth less than 24 hours after exposure to exclude any influence of cell viability on cell migration. No significant changes were observed in cell viability compared to regular medium up to 140 mmol/L at 24 hours, whereas 222 mmol/L glucose and manniitol induced significant cytotoxicity. Therefore, glucose and manniitol concentrations below 140 mmol/L were used for evaluating cell migration and spreading. Under these conditions, we observed significant inhibition of cell migration and spreading of cells incubated with either glucose or manniitol. Furthermore, inhibition of cell spreading and migration by high glucose concentration could be reversed by intracellular over-expression of FAK, indicating that these alterations result from the specific inhibition of intracellular signaling involving FAK, not from non-specific effects of reduced cell viability. Interestingly, acidic culture medium had minimal effects on both cell viability and migration. Effects of extracellular acidosis on cell viability are poorly understood and controversial. Acidic pH has no effects on neutrophil apoptosis [41], while acidosis induces expression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), and inhibits endothelial cell apoptosis [42]. In the present study, we could not observe any significant effects of acidic culture medium on cell viability after 24-hours of exposure. Further studies are needed to investigate the effects of acidosis on mesothelial cell viability.

Our results also showed that high glucose concentrations inhibited FN-induced FAK phosphorylation. FAK is thought to be a key molecule implicated in integrin signaling pathways. Activation of integrins by cell binding to extracellular matrix leads to increases in FAK tyrosine phosphorylation and enhances kinase activity [16, 18]. Among the proposed functions of FAK, focal adhesion assembly, cell adhesion, and cell migration are the central events. Overexpression of dominant-negative FAK causes a transient reduction in cell spreading and migration [23]. We also showed that inhibition of FAK phosphorylation by specific phosphatase results in reduction of cell migration and spreading [24, 25]. Thus, FAK is considered as an important regulator of integrin-mediated cell migration events. In fact, FAK expression is enhanced in rapidly migrating cells during wound healing [21], and FAK overexpression stimulates cell migration [28]. Cells in which FAK is inhibited by a tyrosine kinase inhibitor [21] and cells from FAK-deficient (gene knock-out) mice [22] exhibit decreased cell migration. Our findings, that high glucose concentration-induced inhibition of cell migration is associated with reduction of FAK phosphorylation levels, are consistent with these reports, and suggest that FAK plays an important role in cell migration even in peritoneal mesothelial cells. That glucose inhibition of cell migration could be reversed by intracellular overexpression of wild-type FAK further confirms the importance of FAK in glucose-induced inhibition of cell migration.

p130Cas has been reported to be a mediator of FAK-promoted cell migration in several cell lines [43]. In the present study, we showed that phosphorylation of p130Cas is also inhibited, similar to FAK, by high glucose and manni-tol. p130Cas is a signal transduction protein that binds directly to FAK and is phosphorylated upon cell adhe-sion to extracellular matrix proteins in a FAK- and Src-dependent manner [38, 44]. We have reported that de-phosphorylation of p130Cas and FAK by a phosphatase results in suppression of cell spreading and migration [24, 25]. Both p130Cas and FAK were phosphorylated after overexpression of FAK, but that p130Cas over-expression resulted in only its own phosphorylation without affecting the phosphorylation level of FAK [25]. These findings indicate that p130Cas is a downstream ef-fector in a signal transduction pathway leading to cell migration and is also implicated in glucose-induced inhibition of mesothelial cell migration.

In conclusion, our in vitro study showed that high glucose concentrations suppressed wound healing of peritoneal cell monolayer associated with inhibition of focal adhesion formation and phosphorylation of FAK and its downstream p130Cas. Mannitol also exhibited cell migration inhibitory effects but they were less than those of glucose. Our results suggest that high glucose dialysate causes peritoneal damage by inhibiting wound repair of the mesothelial monolayer, and that hypertonic dialysates should be used as less as possible for preserving the ability of mesothelial cells to regenerate wounded mesothelial cell monolayer of the peritoneum.

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APPENDIX

Abbreviations used in this article are: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CAPD, continuous ambulatory peritoneal dialysis; ECM, extracellular matrix; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorter; FAK, focal adhesion kinase; FBS, fetal bovine serum; FGF, fibroblast growth factor; FN,
fibronectin; GDP, glucose degradation products; HA, hemagglutinin; HB, heparin binding; HPMC, human peritoneal mesothelial cells; LDH, lactate dehydrogenase; PDGF, platelet-derived growth factor; PKC, protein kinase C; RPMC, rat peritoneal mesothelial cells; TGF-β, transforming growth factor-β.

REFERENCES