Differential proteomic characterization between normal peritoneal fluid and diabetic peritoneal dialysate

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Abstract

Background. Since the mechanism of comorbidity and mortality in peritoneal dialysis is unclear, a comparison of peritoneal dialysate and normal peritoneal fluid may provide clues to the biological and pathological processes involved in peritoneal damage.

Methods. Peritoneal dialysate and control samples were collected from five diabetes mellitus (DM) patients and two patients receiving laparoscopic cholecystectomy. Proteins were separated by two-dimensional gel electrophoresis (2D-GE). After image analysis, altered gel spots between these two sample groups were subjected to trypic digestion and mass spectrometry analysis. The results were searched against the NCBI database.

Results. A total of 26 protein spots were considered altered in 2D-GE between the two sample groups. After western blotting confirmation, vitamin D-binding protein, haptoglobin and α-2-microglobulin were at higher levels in the DM samples, while complement C4-A and IGK@ protein were at lower levels compared to the control samples.

Conclusion. The loss of vitamin D-binding protein, haptoglobin and α-2-microglobulin may be due to a change in the permeability of the peritoneal membrane to middle-sized proteins or leakage from peritoneal inflammation. Lower levels of complement C4-A in dialysate may shed light on the beginning of peritoneal membrane scleroses.

Keywords: diabetes mellitus; peritoneal dialysate; peritoneal dialysis; proteomic analysis; two-dimensional gel electrophoresis

Introduction

Peritoneal dialysis (PD) is one type of therapy for patients who have lost their kidney function. Although PD replaces the function of the kidney, pathologic damage of the peritoneum frequently results from PD, and it cannot be easily identified without invasive techniques. The most important contributing factor to mortality in patients receiving maintenance PD is fluid overload-related cardiovascular disease [1]. One proposed mechanism is glucotoxicity to the peritoneal membrane [2]. However, the details of the pathogenic mechanism remain unclear. Proteins whose abundance has changed due to PD can be observed in peritoneal dialysate. For example, it has been reported that increased production of daintain/allograft inflammatory factor-1 (AIF-1) is observed in the first spent dialysate from newly started continuous ambulatory peritoneal dialysis (CAPD) patients, and an early rise in daintain/AIF-1 might be used as a marker of CAPD peritonitis [3]. Therefore, protein changes between peritoneal dialysate and normal peritoneal fluids may provide clues for understanding the mechanism of peritoneal damage caused by PD. In addition, a non-invasive means of detecting peritoneal damage is urgently needed. The altered proteins in the peritoneum may function as biomarkers for monitoring or detection. The peritoneal cavity contains a small amount of fluid functioning as a lubricant under normal conditions. Normal peritoneal fluid, which is generally available when invasive procedures are conducted, represents an ideal control sample for diseases involving the peritoneum.

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In recent years, proteomic analyses of particular biological samples or clinical samples have drawn much interest and provided much information. Proteomic tools such as two-dimensional gel electrophoresis (2D-GE) and mass spectrometry analysis have been widely applied in the study of body fluids, e.g. cerebrospinal fluid [4], pleural and pericardial effusions [5–7] and urine [8–10], but few studies have focused on peritoneal dialysate [11–14]. Herein, a comparative proteomic analysis was carried out on diabetic peritoneal dialysate and normal peritoneal fluids of patients receiving laparoscopic cholecystectomy.

Materials and methods

Patient sample preparation

Peritoneal dialysate was collected from five diabetes mellitus (DM) patients. Normal peritoneal fluid samples from two patients receiving elective laparoscopic cholecystectomy were used as controls. This study was approved by the Institutional Research Board and carried out according to the Helsinki Declaration Principles. Written informed consent was collected from all participating subjects. Each sample was centrifuged at 1000 g at 4°C for 10 min and the supernatants were stored at −20°C until further use. An aliquot of each sample was used to assay protein concentration.

Protein precipitation

The DM sample was pooled from peritoneal dialysates from the five DM patients, each containing 24 μg of protein. The control sample was pooled from the two normal peritoneal fluid samples, each with 60 μg of protein. Sample solutions were mixed with trichloroacetic acid (1:9) in cold acetone (−20°C) containing 0.1% dithiothreitol. The specimen was stored in a freezer overnight, followed by centrifugation at 8500 rpm for 30 min. The pellet was washed three times with acetone (−20°C) containing 0.1% dithiothreitol and air dried. The dry pellet was dissolved in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS (3-[[(3-Cholamidopropyl)Di-methyl-Ammonio]-1-Propanesulfonate), 2% ampholytes, 120 mM dithiothreitol) and carefully sonicated for 30 to 60 min.

2D-GE

The DM and control samples (each containing 120 μg protein) were loaded onto gel strips. IPG (immobilized pH gradients) cover oil (0.8 μl) was applied on top of the IPG gel strip to minimize evaporation and urea crystallization. IEF (Isoelectric focusing) was run following a stepwise incremental voltage program on an IECphor™ Isoelectric Focusing System: 30 V for 16 h, 500 V for 1 h, 500 V for 1 h and 8000 V for 4 h. After IEF, the strips were subjected to two-step equilibration in equilibration buffers with 1% dithiothreitol for the first step and 2.5% w/v iodoacetamide for the second step. The IPG strip was placed onto a 1.0-mm thick 12% polyacrylamide gel and run at 300 V for 4–5 h. Gels were visualized by silver staining. The stained gels were scanned using an ImageScanner operated by the software LabScan 3.00 with 300 DPI. Intensity calibration was carried out using an intensity step wedge prior to gel image capture. Image analysis was carried out using the software ImageMaster 2D (Amersham Biosciences). Image spots were detected and matched. The protein spots with altered intensity step wedge prior to gel image capture. Image analysis was carried out using the software ImageMaster 2D (Amersham Biosciences). Image spots were detected and matched. The protein spots with altered intensity were cut off from the gels and subjected to tryptic in-gel digestion.

Trypsin in-gel digestion

The protein spots were excised and transferred into siliconized 0.5-ml Eppendorf tubes. The gel pieces were washed twice with 50% acetonitrile and 50% acetonitrile/25 mM ammonium bicarbonate. For protein reduction and alkylation, the gel fragments were placed at 56°C for 45 min in 10 mM dithiothreitol and 55 mM iodoacetamide in 25 mM ammonium bicarbonate. Approximately 10 μL of 0.1 μg/μL of modified trypsin digestion buffer in 25 mM ammonium bicarbonate was added to the gel pieces and the samples were incubated overnight at 37°C. The
supernatant was transferred to an Eppendorf tube and the peptides were further extracted from the gel piece by incubation in 50% acetonitrile/5% formic acid. After acidification with 5% acetonitrile/0.1% formic acid, samples were subjected to mass spectrometry analysis for protein identification.

**Protein identification by liquid chromatography–mass spectrometry and database search**

RP-nano-HPLC-ESI-MS/MS (reverse phase-nano-High performance liquid chromatography-electrospray ionization-tandem mass spectrometry) was performed to identify digested protein spots. The protein tryptic digests were fractionated using a C18 microcapillary column (75 μm i.d. × 15 cm) at a flow rate of 200 nL/min with a nano-HPLC system (LC Packings, The Netherlands) coupled to an ion trap mass spectrometer equipped with an electrospray ionization source (LCQ DECA XP Plus, ThermoFinnigan, San Jose, CA). Chromatographic elution was performed using a 40-min gradient from 0 to 60% buffer B (80% acetonitrile/0.1% formic acid). As peptides eluted from the microcapillary column, they were electrosprayed into the ESI-MS/MS (electrospray ionization-tandem mass spectrometry) with the application of a distal 1.3 kV spraying voltage. Each cycle of one full scan mass spectrum (m/z 150–2000) was followed by three data-dependent tandem mass spectra on the three most intense peaks.

Product ion scan data obtained from tandem mass spectrometry experiments were analysed by the MASCOT search engine, which is freely available at http://www.matrixscience.com (Matrix Science Ltd, UK). Peak lists were generated using the BioWorks program (version 3.3), and the resultant .DTA files were merged and used to search against the Homo sapiens (human) protein sequence database (obtained from Swiss-Prot/TrEMBL). For proteolytic cleavage, only tryptic cleavage was allowed and number of maximal missed cleavage sites was set to 2. Modifications at cysteine with carboxamidomethylation, methionine with oxidation and asparagines and glutamine with deamidation were allowed. The mass tolerance of the precursor peptide ion was set to 0.5 Da and that for fragment ion tolerance was set to 0.5 Da. Each of the protein identifications was based on MS/MS fragment. Proteins identified with more than two peptides were considered to be significant hits. Expected value smaller than 0.05 was acceptable for peptide identification. Protein functions were referenced from the SwissProt/TrEMBL database (http://www.expasy.ch).

**Western blotting**

Proteins were separated on commercial 1.0 mm × 10 well gradient gels (4 to 12%, NuPAGE Bis-Tris, Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membranes. The membranes were blocked in 5% non-fat milk solution and Tris-buffered saline with Tween 20 (TBST) (3.0 g/l Tris, 14.4 g/l glycine, 0.5% Tween 20, pH 8.3) at room temperature for 1 h. After being washed three times with TBST, the membranes were probed with antibodies at 4°C overnight. The primary antibodies and concentrations are as follows: anti-vitamin D-binding protein (1:5000, Epitomics, CA, USA), anti-IgG: chain (1:200, Santa Cruz Biotechnology, Inc., CA, USA), anti-haptoglobin (1:500, Santa Cruz Biotechnology, Inc., CA, USA), anti-α-2-macroglobulin (1:500, Santa Cruz Biotechnology, Inc., CA, USA), anti-fibrinogen (1:10000, Epitomics, CA, USA), anti-α-1-antitrypsin (1:500, Santa Cruz Biotechnology, Inc., CA, USA), anti-fibrinogen (1:500, Santa Cruz Biotechnology, Inc., CA, USA), anti-cytoplasmic actin (1:10 000, Epitomics, CA, USA) and anti-complement C4 (1:500, Santa Cruz Biotechnology, Inc., CA, USA). Before incubation with secondary antibodies, membranes were washed with TBST three times. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:5000 at room temperature for 1 h. After washing with TBST three times, membranes were developed with enhanced chemiluminescence detection. The volume densitometry of each band was quantified by using the software ImageMaster 2D (Amersham Biosciences).

**Results**

**Analysis of individual DM and non-DM samples by 2D-GE**

In this study, peritoneal fluid from non-DM patients was chosen as the control. To obtain the control samples, we collected normal peritoneal fluid from the cul-de-sac during elective laparoscopic cholecystectomy of non-inflamed gallbladders. A total of five peritoneal dialysate samples were collected from DM patients and two peritoneal fluid samples were collected from non-DM patients. The demographic data of these seven subjects is shown in Table 1.

To address differences among individual samples, dialysate from the five DM patients (DM1–DM5) and the two control peritoneal fluid samples (C1 and C2) were analysed by 2D-GE. After image analysis, a master gel was generated by combining all gels run. There were 181 spots on the master gel. To evaluate the variance within each group, the numbers of gels in which each spot appeared are depicted in Figure 1. As shown in Figure 1A, the presence of the...
Fig. 2. Representative 2D-GE gels for the DM samples (A) and the non-DM (B) samples: DM samples and non-DM samples were pooled separately for 2D-GE analysis (pH 4–7); a total of 120 μg was used; the analysis of each group was repeated six times and two representative gels are shown [an average of 200 protein spots was detected in both gels; among these, 17 spots were found with higher levels in the peritoneal dialysate (indicated in A by arrows) and nine spots were found with higher levels in the control samples (indicated in B by arrows)].
181 spots in the five DM gels was estimated. Among the 181 spots, most of the spots appeared in five or four gels (66.85 and 18.23%, respectively), reaching to 85% of all the spots. Only 11 (6.08%) and 5 (2.76%) spots were detected on two and one gels, respectively, and these may have resulted from individual differences. As for the control gels, 130 spots (71.82%) appeared on the two control gels, while 15 spots (8.28%) were absent from the control gels. The absence of these spots suggests some differences between peritoneal dialysate and normal peritoneal fluid. Although several proteins showed differential expression, no consistent, statistically valid ($P < 0.05$) differences within the sample groups were obtained. Further experiments were performed using pooled samples.

**Comparative proteome analysis by 2D-GE**

The peritoneal dialysates from the five DM patients and the two non-DM patients were pooled separately and subjected to 2D-GE analysis. A total of 120 μg protein was loaded onto pH 4–7 strips for 2D-GE and the resulting gels were visualized with silver staining. To reduce gel-to-gel variations in the proteome pattern, two gels from each of the DM and control samples were analysed simultaneously in a back-to-back arrangement. The analysis was repeated six times to get statistical results. Only gels with well-resolved proteome patterns were used for the following quantitative analysis. Figure 2A shows a representative gel of a DM sample pooled from the five DM patients, while a representative control gel is shown as Figure 2B. Each 2D gel replicate contained an average of 200 protein spots. Eighty percent of the spots could be matched across the six replicates. The arrows in Figure 2A indicate spots with significantly higher protein expression in DM peritoneal dialysate than in the control peritoneal fluids, while the spots marked in Figure 2B appeared with higher protein levels in the control samples. A total of 17 spots were found with higher levels in peritoneal dialysate. Nine spots were found with higher levels in the control samples than in the DM group. Examples of protein spots (P01, P07, P18 and P26) with higher or lower intensities with respect to the control gels are shown in Figure 3.

**Protein identification of the 26 altered spots by liquid chromatography–tandem mass spectrometry**

These 26 altered protein spots were then excised and subjected to in-gel digestion and LC-MS/MS (liquid chromatography-tandem mass spectrometry) analysis. After database searching, 26 spots were identified and the detailed information is listed in Table 2. The 24 spots were identified into nine proteins. Among them, vitamin D-binding protein, haptoglobin, α-2-microglobulin and fibronectin showed higher levels in peritoneal dialysate, while α-1-antitrypsin, fibrinogen gamma chain, actin cytoplasmic 1, complement C4-A and IGK@ protein showed higher levels in the control samples. The number of identified peptides for each protein, the expected values of individual peptides and the sequence coverage are also described in Table 2.

Most of the expected values of individual peptides were much smaller than 0.05. Most proteins were identified with more than three peptides. Similar peptide sequences were found in spots identified as the same protein. It is interesting that some spots were identified as the same proteins but with slight differences in molecular weight and isoelectric point. For example, spots P03–P10 were all identified as haptoglobin. Since the experimental molecular weights of these spots (41–45 kDa) were close to the theoretical molecular weight (45.2 kDa), we speculated that the different isoelectric points may result from different modifications of the proteins. Spots P12–P15 were identified as α-2-macroglobulin. Their molecular weight measured from 2D-GE image (31 kDa) was much smaller than the theoretical weight (163.2 kDa). Therefore, these proteins may exist as α-2-macroglobulin degradation fragments with different modifications or sequences.

**Confirmation of the identified proteins by western blot**

To further confirm the results of the 2D-GE analysis, western blots were performed to evaluate the expression levels of all the nine identified proteins in individual DM and control samples. The fold changes of the identified proteins was obtained by dividing the mean band density of the DM samples by that of the control samples, adjusting the mean band density of the control sample as 1.

As shown in Figure 4A, vitamin D-binding protein (287.76-fold), haptoglobin (183.25-fold) and α-2-macroglobulin (323.45-fold) were detectable in all five DM sam-
Table 2. Identification of 26 altered proteins, 17 of which were at higher levels in the DM samples and nine of which were at lower levels

<table>
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<tr>
<th>Serial code</th>
<th>2D-GE spot number</th>
<th>Protein name</th>
<th>NCBI accession number</th>
<th>Mr (E/T)c (kDa)</th>
<th>pl (E/T)d</th>
<th>No. of identified peptides</th>
<th>Sequence coverage (%)</th>
<th>Alteration in DM sample</th>
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<td>P01</td>
<td>60</td>
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<td>P02774</td>
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<td>7</td>
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<td>92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P03</td>
<td>101</td>
<td>Haptoglobin</td>
<td>P00738</td>
<td>44/45.2</td>
<td>4.8/6.13</td>
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<td>16.3</td>
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<td>17.8</td>
<td>Up</td>
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<td>P05</td>
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<td>Haptoglobin</td>
<td>P00738</td>
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<td>5.0/6.13</td>
<td>7</td>
<td>14.34</td>
<td>Up</td>
</tr>
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<td>105</td>
<td>Haptoglobin</td>
<td>P00738</td>
<td>44/45.2</td>
<td>5.1/6.13</td>
<td>5</td>
<td>12.39</td>
<td>Up</td>
</tr>
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<td>5.3/6.13</td>
<td>6</td>
<td>16.52</td>
<td>Up</td>
</tr>
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<td>10.43</td>
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<td>5</td>
<td>12.6</td>
<td>Up</td>
</tr>
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<td>Haptoglobin</td>
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<td>5.6/6.1</td>
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<td>6.95</td>
<td>Up</td>
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<td>α-2-macroglobulin</td>
<td>P01023</td>
<td>31/163.2</td>
<td>5.9/5.9</td>
<td>6</td>
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<td>Up</td>
</tr>
<tr>
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<td>α-2-macroglobulin</td>
<td>P01023</td>
<td>31/163.2</td>
<td>5.9/5.9</td>
<td>4</td>
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<td>Up</td>
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<td>Up</td>
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<td>Up</td>
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<td>P02751</td>
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<td>Actin cytoplasmic 1</td>
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<td>5.8/5.4</td>
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<td>5.8</td>
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<td>Complement C4-A</td>
<td>P0COL4</td>
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<td>IGK@ protein</td>
<td>Q6GMV9</td>
<td>26/23.1</td>
<td>5.2/5.8</td>
<td>4</td>
<td>20</td>
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</table>

*These protein serial codes are for use in this table.

The spot numbers are designated in Figure 1A and B.

Mr (E/T)c is the experimental observation of apparent molecular weight in 2D-GE/theoretical molecular weight calculated from the protein sequence database.

pl (E/T)d is the experimental observation of pl in 2D-GE/theoretical pl calculated from the protein sequence database.

Discussion

Functional description of the five confirmed proteins

The fact that the expression levels of the five proteins (vitamin D-binding protein, haptoglobin, α-2-macroglobulin and fibronectin) were consistent between the 2D-GE and western blot analyses lends confidence to the results. Therefore, protein functions are discussed here to suggest potential mechanisms and/or applications. Vitamin D-binding protein, haptoglobin and α-2-macroglobulin were found to lose due to PD. After inspecting their molecular weights by 2D PAGE, we speculated that their loss may be due to a change in the permeability of the peritoneal membrane to middle-sized proteins or leakage from peritoneal inflammation. Interestingly, the loss of vitamin D-binding protein after CAPD was also observed in Joffe and Heafs’ work in 1989. They concluded that CAPD leads to losses of 25-OH-D3 and DBP [15]. Vitamin D-binding protein (DBP) is a multifunctional protein found in plasma, ascitic fluid, cerebrospinal fluid, urine and on the surfaces of many cell types. It is a specific transporter of circulating vitamin D metabolites [16,17]. DBP also plays important roles in vitamin D endocytosis and metabolism [18]. Complexes formed
between DBP and vitamin D ensure the delivery of circulating vitamin D to target tissues [19]. As a consequence, alterations in serum DBP concentration usually coincide with changes in the total concentration of vitamin D [20]. It is reported that PD leads to loss of DBP, and this causes loss of vitamin D. Low levels of 25-OH-D in plasma may aggravate the symptoms of osteodystrophy and renal failure in patients treated with CAPD [21]. Also, vitamin D deficiency causes reduced insulin secretion in rats and humans, and its replenishment improves B cell function and glucose tolerance [22–25]. Therefore, vitamin D levels in serum should be monitored after long-term PD. The other protein lost due to PD is haptoglobin. Haptoglobin combines with free plasma haemoglobin and thereby prevents loss of iron through the kidneys, protects the kidneys from damage by haemoglobin and makes the haemoglobin accessible to degradative enzymes. Haemoglobin initiates or catalyzes free radical formation, activates macrophages, enhances endotoxin effects, causes neurotoxicity, vasoconstriction and platelet adhesion [26–29]. It has

![Fig. 4](https://academic.oup.com/ndt/article-abstract/25/6/1955/1889540/fig-4)

Fig. 4. Western blotting of the identified proteins in five individual DM samples and two individual control samples: the mean band density of five DM samples and two control samples were calculated and fold change between the two groups was calculated by dividing the mean band density of the DM samples by that of the control samples; the mean density of the control group was adjusted as 1, and the fold change was put in the bracket below each set of bands. (A) proteins, vitamin D-binding protein, haptoglobin and α-2-macroglobulin show higher expression levels in the DM samples than in the control samples; (B) proteins, complement C4-A and IGK protein show higher expression levels in the two control samples than in the five DM samples.)
been suggested that haptoglobin may be important in the physiological defence against haemoglobin toxicity, particularly renal toxicity [30]. As for α-2-microglobulin, it is expressed by the liver and secreted into plasma. α-2-microglobulin occurs in many physiological fluids including plasma, urine and cerebrospinal fluid. According to Argiles' work, β-2-microglobulin appeared in haemodialysis-associated amyloidosis and α-2-microglobulin may protect β-2-microglobulin from proteolytic digestion, leading to its accumulation in intact form and to amyloid fibril formation [31]. The observation of α-2-microglobulin in dialysate may be indicative of high levels of α-2-microglobulin in serum and a potential for amyloidosis.

Previous studies have suggested that long-term PD is associated with structural changes in the peritoneal membrane that may be involved in peritoneal fibrosis and loss of function [32]. Chronic peritoneal inflammation is one common feature that precedes the development of peritoneal fibrosis. A variety of mediators including proinflammatory and profibrotic cytokines, adhesion molecules and growth factors can trigger peritoneal inflammation. The complement cascade has been known to be one major mediator of inflammation and plays an important role in developing an effective inflammatory response. The complement cascade generates important peptides, of which C3a, C4a and C5a are the most proinflammatory [33]. Owing to complement activation in the peritoneal cavity in patients on chronic PD [34,35], local production of complement may be a possible inflammatory injury effector in the causation of chronic peritoneal damage. Other studies also found that complement components are synthesized by peritoneal mesothelial cells during PD [36], indicating that complement may play a role in immunoregulation in the peritoneal cavity during PD. C4-A is one among several forms of human complement component C4, and it plays a crucial role in the early steps of the classical pathway of complement activation [37]. One study has suggested that hypertonic glucose, lactate and possibly the bicarbonate components of commercial PD dialysates as well as AGEs (advanced glycation end products) are all toxic to human peritoneal mesothelial cells [38]. In that condition, resident macrophages and neutrophils, major components of the peritoneal defence system, are recruited from the systemic circulation, mesothelial cells and fibroblasts [39–41]. In addition, there are links between complement fragments and the oposnic and phagocytic property of macrophages [33,42]. Down-regulation of C3 and C4 expression are found after high-concentration glucose treatment [38], which concurs with our finding. The down-regulation of C4-A by PD solutions may imply the defence system of peritoneal mesothelium and the increased susceptibility to infection. Also, a complement defect was observed in sclerosis and the defect correlates with low levels of classical pathway components, particularly complement C4-A [43]. Thus, its lower levels in dialysate may shed light on the beginning of peritoneal membrane scleroses. Since there are limited studies of the role of IGKα@ protein in the peritoneum, its defect in dialysate may provide a novel aspect for peritoneal change after PD.

Conclusion

This is the first study showing proteomic differences between diabetic peritoneal dialysate and normal peritoneal fluid. The proteins identified may provide insights into the loss of proteins from peritoneal dialysate and may facilitate the non-invasive discovery of potential biomarkers for monitoring peritoneal damage and changes in transport. Further studies of these potential marker proteins are warranted to assess their utility for clinical monitoring and basic research.

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Conflict of interest statement. None declared.

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