The potential of matrix metalloproteinase-2 as a marker of peritoneal injury, increased solute transport, or progression to encapsulating peritoneal sclerosis during peritoneal dialysis—a multicentre study in Japan

Ichiro Hirahara, Makoto Inoue, Kousuke Okuda, Yasuhiro Ando, Shigeaki Muto and Eiji Kusano

Division of Nephrology, Department of Medicine, Jichi Medical School, 3311-1 Yakushiji, Shimotsuke-city, Tochigi 329-0498, Japan

Abstract

Background. Long-term peritoneal dialysis (PD) leads to peritoneal injury. At worst, peritoneal injury leads to encapsulating peritoneal sclerosis (EPS), which is a serious complication of PD. The mortality rate of EPS is extremely high. To perform PD safely, monitoring of peritoneal injury that leads to EPS is a necessity.

Methods. A total of 444 PD patients with end-stage renal disease at 60 centres in Japan were analysed (sex, 54% males; median age, 56 years; median PD duration, 55 months). Matrix metalloproteinase (MMP)-2 and MMP-9 in the peritoneal effluents were analysed with gelatin zymography or enzyme-linked immunosorbent assay. Cells expressing MMP-2 in the peritoneal tissue were investigated immunohistologically with anti-MMP-2 antibodies. Peritoneal solute transport was assessed with the peritoneal equilibration test (PET).

Results. The MMP-2 levels in peritoneal effluents obtained with the PET were significantly correlated with the D/P Cr ratio ($R = 0.69, P < 0.001$) and the D/D0 glucose ratio ($R = -0.59, P < 0.001$). The MMP-2 levels in patients with mild peritoneal injury, moderate peritoneal injury, severe peritoneal injury (EPS) and infectious peritonitis were significantly higher than those in control patients ($P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively). MMP-2 was produced by myofibroblast-like mesenchymal cells and macrophages in the peritoneum. The peritoneal effluents from patients with infectious peritonitis showed strong MMP-9 signals.

Conclusions. From these results, MMP-2 levels in peritoneal effluents reflect peritoneal solute transport and changes in MMP-2 levels are associated with peritoneal injury that leads to EPS. MMP-2 may be a useful marker of peritoneal injury, increased solute transport or progression to EPS.

Keywords: encapsulating peritoneal sclerosis (EPS); matrix metalloproteinases (MMP); peritoneal dialysis; peritoneal injury

Introduction

Peritoneal dialysis (PD) is a common treatment for patients with end-stage renal disease reduced or absent renal function. Long-term PD leads to peritoneal injury with functional decline, such as increased solute transport and ultrafiltration loss. At worst, peritoneal injury leads to encapsulating peritoneal sclerosis (EPS), which is the most serious complication in patients undergoing PD [1–9]. The cause of EPS has not been clarified, but it probably develops through multiple factors. In EPS, evidence of chronic inflammation and ascites is often seen [2–7]. The small intestine appears as a mass encapsulated with thick peritoneum rich in collagenous fibres forming an adhesion, called an abdominal cocoon. It has been reported that administration of steroid drugs is an effective therapeutic method in the early stage of EPS [2–7], but there is no effective treatment in advanced stages, when bowel adhesions have formed, except for surgically dissecting the encapsulated peritoneum using specialized techniques [3–8]. At present, the main diagnostic methods for EPS include abdominal palpation (palpation of the mass) and detecting symptoms of ileus. Some physicians use diagnostic imaging methods, such as radiography, computed tomography and ultrasonography [3–7], but these methods cannot detect early EPS. C-reactive protein (CRP) is often also used as a biochemical marker for
The potential of MMP-2 as an indicator of the progression to EPS inflammation [2–6,10]. However, CRP often does not increase in advanced EPS and cannot be used to differentiate between EPS and infectious peritonitis, because CRP levels increase in infectious peritonitis. As mentioned earlier, steroid therapy is effective in early EPS [2–7], but steroids, which suppress the immune system, may aggravate symptoms when administered to patients with infectious peritonitis. Therefore, a diagnostic method that can differentiate EPS from infectious peritonitis is required. Because of the lack of a reliable early diagnostic method for EPS and the consequent delay in starting appropriate treatment, EPS has a mortality rate of >50% [3–7]. To perform PD safely, it is important to monitor the peritoneal injury that leads to EPS and to diagnose EPS at an early stage.

In cases of sclerosis or fibrosis in various organs, such as artery, lung, liver and kidney, tissue destruction and excessive remodelling occur [11]. In such events, matrix metalloproteinases (MMPs) play important roles. In particular, gelatinases, such as MMP-2 and MMP-9, degrade components of the extracellular matrix (ECM) such as type IV collagen and fibronectin, which comprise the basement membrane, and play important roles in angiogenesis and the migration of cells that promote fibroplasia. Because EPS is accompanied with marked fibrous thickening or sclerosis of the peritoneum, levels of gelatinases are likely increased in this complication. In the present study, we investigated whether gelatinases have a potential as an indicator of the progression to EPS.

**Patients and methods**

**Patients**

The 441 PD patients (54% males) with end-stage renal disease at 60 centres in Japan were analysed. Patients with appendicitis were excluded. The peritoneal effluents were prepared from April 1999 through July 2005. The patients were divided into five groups as shown in Table 1. The parameters for classification of patients were based on the criteria recommended by the Japanese Sclerosing Encapsulating Peritonitis Study Group [3] and by an International Society for Peritoneal Dialysis Ad Hoc committee [4]. The characteristics of patients are summarized in Table 2. The characteristics of age, sex and aetiology did not differ significantly between groups. The patients with infectious peritonitis were also compared with the patients without infectious peritonitis. The characteristics of patients are summarized in Table 3.

**Table 1.** The definitions of classification of patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control group (n = 311) PD patients without peritoneal injury</td>
</tr>
<tr>
<td>2</td>
<td>Mild peritoneal injury group (n = 78) Ultrafiltration loss [3]: less than 500 ml/day using less than 8 l of PD solution containing 2.5% glucose</td>
</tr>
<tr>
<td>3</td>
<td>CRP levels in plasma; more than 1.0 mg/dl [3–6]</td>
</tr>
<tr>
<td>4</td>
<td>Hyperleukocytosis; more than 10 000 cells/μl [3]</td>
</tr>
<tr>
<td>5</td>
<td>Deposition of fibrin in PD effluents frequently [5]</td>
</tr>
<tr>
<td>6</td>
<td>Peritoneal sclerosis [3]: the peritoneal sclerosis was defined as loss of mesothelial cells, peritoneal thickness and vasculopathy. Classification was as follows: Early stage: loss of mesothelial cells, fibrosis thickening of submesothelial compact zone, thickening of blood vessel wall Middle stage: loss of mesothelial cells, decrease of cells in submesothelial compact zone, luminal narrowing Late stage: loss of mesothelial cells, acellular submesothelial compact zone, luminal obliteration</td>
</tr>
<tr>
<td>7</td>
<td>Group 2: Mild peritoneal injury group (n = 78)</td>
</tr>
<tr>
<td>8</td>
<td>PD patients with ascites: more than 100 ml/day [2–7]</td>
</tr>
<tr>
<td>9</td>
<td>Group 3: Moderate peritoneal injury group (n = 14)</td>
</tr>
<tr>
<td>10</td>
<td>PD patients with edema</td>
</tr>
<tr>
<td>11</td>
<td>Group 4: Severe peritoneal injury group (EPS group) (n = 12)</td>
</tr>
<tr>
<td>12</td>
<td>EPS patients with adhesions of the small intestine and/or ileus [2–7]. The adhesions of the small intestine were confirmed by laparoscopic examination, macroscopy at laparotomy, or computed tomography analysis</td>
</tr>
<tr>
<td>13</td>
<td>Group 5: Infectious peritonitis group (n = 29)</td>
</tr>
<tr>
<td>14</td>
<td>PD patients with infectious peritonitis</td>
</tr>
</tbody>
</table>

**Table 2.** Characteristics of patients with various degrees of peritoneal injury

<table>
<thead>
<tr>
<th>Group</th>
<th>1 (n = 311)</th>
<th>2 (n = 78)</th>
<th>3 (n = 14)</th>
<th>4 (n = 12)</th>
<th>5 (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>55 (45, 63)</td>
<td>58 (51, 64)</td>
<td>62 (58, 67)</td>
<td>61 (52, 69)</td>
<td>57 (37, 65)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>57</td>
<td>65</td>
<td>71</td>
<td>75</td>
<td>59</td>
</tr>
<tr>
<td>Aetiology (% diabetes)</td>
<td>17</td>
<td>17</td>
<td>23</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>PD duration (months)</td>
<td>48 (24, 78)</td>
<td>79 (51, 107)</td>
<td>92 (75, 106)</td>
<td>115 (87, 152)</td>
<td>42 (13, 86)</td>
</tr>
<tr>
<td>Peritonitis episode (times)</td>
<td>0 (0, 1)</td>
<td>1 (0, 2)</td>
<td>1 (0, 2)</td>
<td>1 (1, 2)</td>
<td>3 (1, 4)</td>
</tr>
</tbody>
</table>

Data except sex and aetiology of renal failure are expressed as medians with interquartile ranges (25th and 75th). Group 1: control group; Group 2: mild peritoneal injury group; Group 3: moderate peritoneal injury group; Group 4: severe peritoneal injury group (EPS group); Group 5: infectious peritonitis group.
The characteristics of age, PD duration sex and aetiology did not differ significantly between the two groups. The Ethics Committee of Jichi Medical School approved this study protocol, and informed consent was obtained from each patient.

**Peritoneal equilibration test (PET)**

Peritoneal solute transport was assessed with the PET [12]. A total of 222 patients (sex: 57% males; aetiology of renal failure: 15% diabetic) with a median (interquartile range) age of 56 years (46–65 years) who had undergone PD for a median of 49 months (25–69 months) were analysed with the PET. Intra-abdominal fluid was drained, and the 21 of PD fluid containing 2.27–2.5% glucose was injected intraperitoneally. The creatinine (Cr) level of peritoneal effluents obtained 4 h after the injection (D) was divided by that of plasma (P) to obtain the D/P Cr ratio. The glucose level of peritoneal effluents obtained 4 h after the injection (D) was divided by that obtained immediately after the injection (D0) to obtain the D/D0 glucose ratio.

**Analysis of gelatinases**

The gelatinases in peritoneal effluents (0.25 μl) were diluted to 16 times and analysed with gelatin zymography, as described before [13]. MMP-2 or MMP-9 concentration was measured by enzyme-linked immunosorbent assay (ELISA) system (Amersham Bioscience, NJ, USA).

**Western blotting analysis**

Peritoneal effluents (1 μl) were electrophoresed under non-reducing conditions on 8% polyacrylamide gel and then separated proteins were transferred onto nitrocellulose membranes at room temperature. Membranes were blocked with Block Ace (Snow Brand Co., Hokkaido, Japan) overnight at 7°C and subsequently incubated with a monoclonal antibody against MMP-2 (1:100 dilution, clone No. 42-5D11, Fuji Chemical Industries Ltd., Toyama, Japan) or a monoclonal antibody against MMP-9 (1:50 dilution, clone No. 56-2A4, Fuji Chemical Industries Ltd.) for 2 h at room temperature. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween-20, membranes were incubated with peroxidase-conjugated goat affinity-purified antibody to mouse immunoglobulins (1:1000 dilution; ICN Pharmaceuticals Inc., Cappel Products, Costa Mesa, CA, USA) for 1 h at room temperature. The immunoreactive proteins were visualized with a tetramethyl-benzidine membrane peroxidase substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA).

**Immunohistochemistry**

Peritoneal biopsy specimens were obtained from the parietal peritoneum. Peritoneal tissue samples embedded in paraffin were sectioned at a thickness of 2–3 μm. The sections were dewaxed with xylene and ethanol, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide/methanol for 10 min, followed by rinsing with PBS. Sections were then incubated for 4 h at room temperature with a monoclonal antibody against MMP-2 (Fuji Chemical Industries Ltd.) at a dilution of 1:100. Following rinsing with PBS, sections were incubated with peroxidase-conjugated anti-mouse Ig [Histfine Simple-stain PO (MULTI), Nichirei Co., Tokyo, Japan] for 30 min. After washing with PBS, peroxidase activity on the sections was developed with aminoethylcarbazole (Nichirei Co.) as the substrate and then sections were counterstained with Meyer’s haematoxylin for 10 s.

**Statistical analysis**

Data are expressed as medians with the spread from 25th to 75th percentiles. Comparisons between two groups were performed with the Mann-Whitney U-test. Multiple comparisons were performed with the Kruskal-Wallis test, followed with the Steel-Dwass test. Relationships between clinical variables and MMP-2 levels were analysed with Spearman’s correlation coefficient test. Statistical analyses were performed with the SAS system for Windows, version 8.2 (SAS Institute Inc., Cary, NC, USA).

**Results**

**Analysis of gelatinases in the peritoneal effluents**

The gelatinases in peritoneal effluents were analysed with gelatin zymography (Figure 1A). On gelatin zymograms, strong signals of 64 kDa gelatinase were observed in patients with moderate peritoneal injury, but faint signals of 64 kDa gelatinase were detected in control patients and in the patient with infectious peritonitis. The signal of 90 kDa gelatinase was strong in the patient with infectious peritonitis. Western blotting analysis with an anti-MMP-2 or anti-MMP-9 antibody shows positive signals at 64 or 90 kDa, respectively (Figure 1B and C). The 64 and 90 kDa gelatinases were identified as the latent type of MMP-2 (proMMP-2) and the latent type of MMP-9 (proMMP-9), respectively. High molecular weight bands that reacted with the anti-MMP-2 or anti-MMP-9 antibody might be complexes of MMP and other proteins (Figure 1B, lane 2 and Figure 1C, lane 2).

**Table 3.** Characteristics of patients without or with infectious peritonitis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median</th>
<th>Interquartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>56/57</td>
<td>46/37, 64/65</td>
</tr>
<tr>
<td>PD duration (months)</td>
<td>55/42</td>
<td>27/13, 92/86</td>
</tr>
<tr>
<td>Peritonitis episode (times)</td>
<td>0/3</td>
<td>0/2, 1/4</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>59/59</td>
<td></td>
</tr>
<tr>
<td>Aetiology (% diabetes)</td>
<td>15/10</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as the value of patient without/with infectious peritonitis. Data except sex and aetiology of renal failure are expressed as medians with interquartile ranges (25th and 75th).
Relationship between MMP-2 concentration and patient characteristics

Peritoneal solute transport determined with the PET was correlated with the MMP-2 concentrations in peritoneal effluents obtained with the PET (D/P Cr vs MMP-2 level; \( P < 0.001 \), \( R = 0.69 \). D/D0 glucose vs MMP-2 level; \( P < 0.001 \), \( R = -0.59 \) (Figure 2).

No correlations were observed between MMP-2 concentration and age, PD duration, peritonitis episodes, sex and aetiology of renal failure (non-diabetic/diabetic) (Tables 4 and 5).

MMP-2 concentration in various stages of peritoneal injury

The MMP-2 levels in the mild peritoneal injury group (group 2), the moderate peritoneal injury group (group 3) and the severe peritoneal injury group (EPS group) (group 4) were significantly increased. In particular, the MMP-2 level was higher in the moderate peritoneal injury group (group 3) than in any other group. These results are shown in Figure 3. In patients with infectious peritonitis, MMP-9 levels in peritoneal effluents increased markedly with a slight increase in MMP-2 levels. In only four out of 415 patients without infectious peritonitis, slight MMP-9 levels were detected. These results are shown in Figure 4.
Long-term outcomes in patients with peritoneal injury

Patient outcomes were examined after analysis of MMP-2. In the mild peritoneal injury group (group 2), three patients with MMP-2 concentrations of 623, 1642 and 3541 ng/ml had EPS. In the moderate peritoneal injury group (group 3), five patients with MMP-2 concentrations of 460, 900, 933, 1934 and 2760 ng/ml had EPS. In this group, 36% of patients developed to EPS although 67% of patients had been treated with steroids.

Of the 15 patients with MMP-2 concentration >600 ng/ml except the patients who had presented with EPS when MMP-2 levels were analysed, seven patients (47%) had EPS, though 53% of patients had been treated with steroid. Of the patients with MMP-2 concentration <600 ng/ml, only one patient with 460 ng/ml MMP-2 had EPS.

Production of MMP-2 in the peritoneum

Positive signals for the anti-MMP-2 antibody were detected in myofibroblast-like mesenchymal cells (α-smooth muscle actin, positive; data not shown) and macrophages in peritoneum specimens obtained from the patient with moderate peritoneal injury (Figure 5).
Discussion

EPS is the most serious complication of PD, and there is no effective treatment in the advanced stage of EPS except for surgery with specialized techniques. To perform PD safely, it is important to monitor the peritoneal injury that leads to EPS and to diagnose EPS at an early stage.

Peritoneal injury can be inferred on the basis of peritoneal solute transport assessed with the PET. In the present study, peritoneal solute transport correlated with the MMP-2 level in peritoneal effluents, as in a previous study in rats [14]. As peritoneal solute transport correlates with vascular surface area, neovascularization may promote solute transport. Vessel basement membrane degradation by MMP-2 may be an important stage in new blood vessel sprouting, but the mechanism has not been clear yet. In the present study, levels of MMP-2 in effluents were markedly increased in patients with moderate peritoneal injury with ascites, which is present in many cases of early EPS [2]. In addition, EPS developed in half of patients with MMP-2 concentration >600 ng/ml, although half of the patients had been treated with steroids. These results suggest that the MMP-2 might be used as a marker of peritoneal injury, increased solute transport or progression to EPS.

Peritoneal cells, such as macrophages, myofibroblasts, endothelial cells and mesothelial cells are activated by various stimuli, such as peritonitis due to infection with bacteria or fungi, exogenous materials such as particulates, antiseptics, advanced glycosylated end products, glucose degradation products in the PD fluid and the pH of the PD fluid. These activated cells produce various cytokines, growth factors and other mediators that induce peritoneal injury. Macrophages may infiltrate or migrate in the peritoneum while the ECM is being degraded by MMP-2 produced by these cells [15,16]. Activated mesothelial cells may transform to mesenchymal cells (epithelial mesenchymal transition) and infiltrate or migrate in the peritoneum, which is digested by MMP-2 [16,17]. Activated myofibroblasts synthesize ECM proteins or migrate while disassembling the ECM of the peritoneum with MMP-2 or other proteinases [13,15,16]. Excessive ECM proteins, such as collagen, lead to peritoneal fibrosis with peritoneal thickening and promote the production of MMP-2 by myofibroblasts [13]. Fibrin, often deposited in the peritoneum in EPS, also induces the production of MMP-2 by myofibroblasts [13]. In addition, neomicrovascularization may occur while the ECM is being degraded by MMP-2 produced by activated endothelial cells of the microvasculature [16]. As a result of tissue destruction and remodelling with ECM production or degradation by MMP-2, fibrous thickening of the peritoneum may occur and an abdominal cocoon may be formed.

In the present study, MMP-2 was produced by the peritoneal cells as in previous studies in rats [13,15,16]. In addition, there is a possibility that MMP-2 in effluents is transported from the plasma. But in our preliminary study, the measured D/P ratios of MMP-2 were higher than expected when MMP-2 would have been transported from the circulation only by diffusion (data not shown) [18]. In some patients in the moderate peritoneal injury group (group 3), MMP-2 levels in effluents were higher than that in plasmas (data not shown). Levels of MMP-2 in effluents may be strongly affected by MMP-2 produced from the peritoneal cells. The MMP-2 levels in the effluents of patients with severe peritoneal injury (EPS) tended to be lower than those of patients with moderate peritoneal injury. In end-stage EPS, the peritoneum is often acellular [4,9]. The loss of peritoneal cells producing MMP-2 may decrease levels of MMP-2 in the peritoneal effluents.

In the present study, levels of MMP-9, a gelatinase-like MMP-2, were hardly detected in peritoneal effluents of patients without infectious peritonitis. Interestingly, in patients with infectious peritonitis, MMP-9 levels in peritoneal effluents increased markedly with a slight increase in MMP-2 levels. Our data are consistent with most of a previous report [19,20]. These results suggest that peritoneal injury that might
lead to EPS can be clearly distinguished from infectious peritonitis by analysing gelatinases in the peritoneal effluents.

This diagnostic method employing peritoneal effluents as samples enables easy sampling, which is noninvasive to patients without pain. An MMP-9 test kit, consisting of an anti-MMP-9 antibody conjugated to a dye in a nitrocellulose membrane, has been developed to diagnose infectious peritonitis [20]. If such test kit for MMP-2 was developed, peritoneal injury could be diagnosed at home.

Our results suggest that MMP-2 may be useful as a marker of peritoneal injury, increased solute transport or progression to EPS that can differentiate from infectious peritonitis. However, it is not concluded that MMP-2 is useful as a diagnostic marker for early EPS, because most patients with moderate peritoneal injury are treated with steroids or discontinue PD to prevent the progression of EPS. Future studies should examine serial changes in MMP-2 levels in relation to the progression of peritoneal injury leading to EPS.

Acknowledgements. This study was supported by Terumo Co. (Tokyo, Japan). We would like to thank Dr Kiyoshi Sakata Mr Takehon Ishizuka, and Mr Toyohisa Shiono (Terumo Co.) for their help in statistical analysis, and Dr Ken-ichi Shoufuda (Terumo Co.) for his technical assistance.

Conflict of interest statement. None declared.

References


Received for publication: 26.12.05
Accepted in revised form: 29.8.06

Appendix

Collaborators were as follows; Akira Suzuki (Department of Urology, Morioka Yuai Hospital), Akiyasu Tsuchida (Department of Nephrology, Toho Hospital), Eiji Ishikawa (Hemo Dialysis Center, Mie University), Fujio Hamada (Department of Internal Medicine, Saiseikai Sendai Hospital), Harumichi Higashi (Department of Nephrology, St. Mary’s Hospital), Hideki Takizawa (Department of Nephrology, Teine Keijinkai Hospital), Hideaki Oda (Director, Oda Clinic), Hidenori Sugawara (The Second Department of Internal Medicine, Toyama Medical and Pharmaceutical University), Hiromi Hidaka (Kidney Internal Medicine, Shiraishi Hospital), Hironobu Kawai (Department of Nephrology, Saiseikai Maebashi Hospital), Hiroshi Miyazaki (Kurume University School of Medicine), Hiroshi Nagae (Department of Urology, Seirei Mikatahara General Hospital), Hiroshi Tanaka.
The potential of MMP-2 as an indicator of the progression to EPS