Activation of matrix metalloproteinase-2 causes peritoneal injury during peritoneal dialysis in rats

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Abstract

Background. Sclerosing peritonitis (SP) and encapsulating peritoneal sclerosis (EPS) are serious complications of continuous ambulatory peritoneal dialysis. Although we have shown previously that matrix metalloproteinase-2 (MMP-2) is increased in peritoneal injury leading to SP/EPS, most of the MMP-2 in the dialysate drained from the peritoneal cavity was the latent form that was lacking activity. In the present study, we investigated whether MMP-2 causes peritoneal injury.

Methods. To create an animal model of peritoneal injury, we administered intraperitoneally chlorhexidine gluconate to rats. Dialysate drained from these rats was analysed by gelatin zymography and MMP-2 activity was analysed by an in situ film zymography method.

Results. Zymographic analysis revealed that latent form MMP-2 levels were high in the dialysate from peritoneal injury rats, whereas the active form was barely detectable. MMP-2 activity in the peritoneal tissue of the peritoneal injury rats was strongly detected by in situ film zymography. In vitro myofibroblasts were cultured in collagen three-dimensional culture and then MMP-2 in conditioned medium from the culture was analysed by gelatin zymography.

Conclusions. In the present model, most of the MMP-2 was in the latent form, but activation of MMP-2 was promoted in the peritoneum during peritoneal injury. Activated MMP-2 may be associated with the progression of peritoneal injury.

Keywords: matrix metalloproteinase-2; myofibroblasts; peritoneal injury; encapsulating peritoneal sclerosis; sclerosing peritonitis; peritoneal dialysis

Introduction

Continuous ambulatory peritoneal dialysis (CAPD) provides a common treatment for patients with reduced or absent renal function. However, long-term CAPD leads to peritoneal injury with ultrafiltration loss and insufficient elimination of solutes. At worst, peritoneal injury leads to sclerosing peritonitis (SP) and encapsulating peritoneal sclerosis (EPS), which are serious complications of CAPD [1–5]. The causes of SP/EPS have not been clarified and are probably multifactorial [1–6]. During development of these complications, evident diffuse fibrous thickening and/or oedema of the peritoneum occurs and evidence of chronic inflammation is often seen [3]. EPS is an especially serious complication. It brings about not only reduced peritoneal function, but also clinical symptoms, such as ileus. The small intestine appears as a mass that is encapsulated with thick peritoneum rich in collagenous fibres forming an adhesion, called an abdominal cocoon. At present, the lack of a reliable diagnostic method for SP/EPS and consequent delays in initiating appropriate treatment produce mortality in more than half of patients who develop SP/EPS [2,4]. Thus, in order to improve the safety of peritoneal dialysis, it will be necessary to clarify the mechanism of SP/EPS progression.

During both sclerosis and fibrosis there is tissue destruction and excessive remodelling [7]. In these cases, matrix metalloproteinases (MMPs) play important roles. Among these, gelatinases, such as MMP-2 and MMP-9, have activities that degrade the extracellular matrix (ECM) such as type IV collagen and fibronectin [8]. These gelatinases play a critical role in angiogenesis as well as in the migration of cells that promote fibroplasia. We have shown previously that peritoneal injury leading to SP/EPS causes higher MMP-2 levels in drained dialysate is increased in peritoneal injury leading to SP/EPS [9,10].

Most of the MMP-2 in the dialysate was of the latent type, whereas the active type was barely detected [9,10]. Therefore, in the present study, we investigated whether
the active form of MMP-2 could be detected in the peritoneum and whether it is associated with peritoneal injury that leads to SP/EPS.

Subjects and methods

Animals

We used male Sprague–Dawley rats [Crj (CD) SD-IGS rat] that were 8 weeks old and weighed ~300 g (n = 6 rats per group; Charles River Japan, Kanagawa, Japan). The animals were housed in an air-conditioned room at a constant temperature of 23 ± 2°C and relative humidity of 50 ± 10%, under a 12 h light/dark cycle. They were given free access to sufficient pelleted food and water. We performed our experiments in accordance with the ethical guidelines for care and use of animals issued by the ethical committee of Terumo Corporation.

Preparation of the animal model of peritoneal injury

Rats in the chlorhexidine gluconate (CHX; Wako Chemicals, Osaka, Japan) group were given daily intraperitoneal injections, through a 22-G needle-syringe (Terumo Co., Tokyo, Japan), of 3 ml aseptically prepared 0.1% CHX/15% ethanol/physiological saline [9,10]. If there was solution remaining in the peritoneal cavity from the previous injection, it was drained using a 22-G needle before the subsequent injection. Negative control rats were treated with consecutive administrations of 0 ml physiological saline (Terumo Co.). All rats received daily administrations for 21 days and none of the rats dropped out of the study. On day 22 after study onset, intra-abdominal fluid was drained out and 50 ml/kg 2.5% glucose containing peritoneal dialysis fluid (Peritoliq P250; Terumo Co.) was injected intraperitoneally. This fluid was then collected as the drained dialysate under the same conditions in the control and CHX-treated groups. The drained fluid was collected 90 min after the administration and was analysed for MMP-2 by gelatin zymography. On day 23, rats were exsanguinated under ether anaesthesia to obtain parietal peritoneum from the left abdomen or visceral peritoneum on liver for analysis of peritoneal tissue injury. Samples were obtained from two corresponding sites from each rat. The peritoneal samples were fixed with 10% formaldehyde/0.1 M phosphate buffer (pH 7.2), embedded in paraffin and sectioned at a thickness of 2–3 μm. Each section was stained with haematoxylin–eosin (HE) to analyse cell type and then was analysed by photomicroscopy. Peritoneal thickness was measured using image analysis software (Win ROOF; Mitani Co., Fukui, Japan). Thicknesses were measured at 30 points/site × 2 sites (0.5 mm intervals within the range of 15 mm) and averages were calculated. Peritoneal samples were also fresh-frozen for in situ zymography analysis.

In this study, care was taken to maintain a hygienic environment and to prevent infectious peritonitis. Furthermore, on day 22, a sterility test was performed using the drained dialysate to check for the presence of aerobic bacteria, anaerobic bacteria or fungi in the drained dialysate. This test confirmed the absence of infection in our dialysate samples.

Gelatin zymography

Drained dialysate was electrophoresed under non-reducing conditions on 8% polyacrylamide gels containing 1 mg/ml gelatin (Sigma Chemical Co., St Louis, MO, USA). After electrophoresis, the gel was washed with 2.5% Triton-100/200 μl M NaCl/50 mM Tris–HCl (pH 7.5) for 2 h to remove sodium dodecyl sulphate and incubated for 18 h at 37°C in 50 mM Tris–HCl (pH 7.5)/10 mM CaCl2. The gel was stained with 0.1% Coomassie brilliant blue. Gelatinases were detected as unstained proteolytic bands in the stained gel. The relative concentrations of gelatinases in dialysate were quantified by scanning proteolytic bands on the zymograms using a scanner (CanoScanFB 620S; Canon, Tokyo, Japan) and densitometry software (NIH Image Quantitation Program; National Institutes of Health, Bethesda, MD, USA).

We also analysed MMP-2 to determine whether it was the latent form or the active form. Before zymographic analysis, samples were incubated for 3 h at 37°C with 1 mM 3-aminophenyl mercuric acetate (APMA; Nacalai Tesque, Kyoto, Japan) to induce the active form [10].

Three-dimensional culture of myofibroblasts

Rat mesothelial cells were isolated from the parietal peritoneum of male Sprague–Dawley rats. The methods of isolation and cell culture have been reported previously [13]. The mesothelial cells were cultured with 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium (10% FBS/DMEM; Sigma-Aldrich, St Louis, MO, USA). Cultured mesothelial cells were identified based on their positive reaction, as well as their negative and negative reactions against anti-human mesothelial cell IgM (clone HBME-1; Dako, Carpinteria, CA, USA), mouse monoclonal anti-α smooth muscle actin (clone 1A4; Sigma-Aldrich) and rabbit anti-human von Willebrand factor (Dako), respectively. Rat peritoneal myofibroblasts were prepared from the inner surface of the parietal peritoneum by digestion with 0.5 mg/ml collagenase at pH 7.4 after the mesothelial cells had been removed by mild collagenase treatment. The peritoneal myofibroblasts were cultured using the same methods that were used for the mesothelial cells. Cultured peritoneal myofibroblasts were identified by their negative, positive and negative reaction against anti-human mesothelial cell IgM, mouse monoclonal anti-α smooth muscle actin and rabbit anti-human von Willebrand Factor, respectively. After
these cells had been subcultured for 4–7 passages, 80–85% confluent cells were cultured in three-dimensional collagen gel, on collagen-coated plates, in three-dimensional fibrin gel, or on fibrin-coated plates in 12-well culture plates (Becton Dickinson, NJ, Franklin Lakes, USA) with DMEM (FBS-free). The collagen gel was prepared with Kokencellagen I-PC (0.5% pepsin-solubilized type I collagen from calf skin; Koken, Tokyo, Japan). To prepare the fibrin gel, thrombin was added to 7.7 mg/ml fibrinogen (Sigma-Aldrich)/DMEM to a final concentration of 1.4 unit/ml and this incubated for > 10 min at room temperature. After culturing for 2 days, 3 μl conditioned medium was analysed by gelatin zymography and MMP-2 gene expression of these cells was examined by reverse transcription–polymerase chain reaction (RT–PCR). The experiment was repeated three times. We confirmed that the collagen and fibrin used in this study were free of endotoxin contamination (< 0.001 EU/ml; Limulus ES-II single test Wako kit; Wako Chemicals).

Analysis of MMP-2 gene expression by RT–PCR
MMP-2 gene expression was analysed by RT–PCR. Total RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The prepared total RNA concentration was determined spectrophotometrically using a GeneQuant DNA/RNA calculator (Amersham Pharmacia Biotech Ltd, Little Chalfont, UK). Total RNA (0.5 μg) from the peritoneum was reverse-transcribed with 200 units Superscript II reverse transcriptase (Gibco BRL, Rockville, MD, USA). The resulting cDNA was amplified with Taq DNA polymerase (Takara Shuzo Co., Ltd, Tokyo, Japan) using a thermal cycler (GeneAmp PCR System 2400; PE Biosystems, Foster, CA, USA). After initial denaturation at 94°C for 5 min, PCR was performed under the following cycle conditions: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. The PCR primer sequences and the cycle numbers were as shown in Table 1.

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a constitutive control to allow comparison of RNA levels among different samples. The PCR products were analysed by electrophoresis on 1.5% agarose gels in TAE buffer (0.04 M Tris, 0.04 M acetic acid, 0.001 M EDTA). After electrophoresis, the gels were stained with ethidium bromide and destained with distilled water for visualization of PCR products. The resulting gels were photographed and the signal intensities of the PCR products were analysed using NIH Image.

Statistical analysis
Data are expressed as means ± SD. Comparisons between the two groups were performed using unpaired Student’s t-tests. Analyses of multiple comparisons between experimental and control values were made using Dunnett’s test. A P-value of < 0.05 was accepted as significant.

Results
MMPs in the drained dialysate from CHX-treated rats
The dialysate obtained from peritoneal injury model rats was subjected to gelatin zymography for analysis of gelatinases (Figures 1A and 1B).

Table 1.

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Cycle(s)</th>
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<tbody>
<tr>
<td>MMP-2 sense primer</td>
<td>5′ACAGCCTGGCATGGGGCAGT 3′</td>
<td>(27 cycles)</td>
</tr>
<tr>
<td>MMP-2 antisense primer</td>
<td>5′TTCTCCTCCATCCAGTGGAG 3′</td>
<td></td>
</tr>
<tr>
<td>MTI-MMP sense primer</td>
<td>5′CTCACCCCAGCTCACCTCAG 3′</td>
<td>(26 cycles)</td>
</tr>
<tr>
<td>MTI-MMP antisense primer</td>
<td>5′CTGGGTGGAGGGGCATCTTT 3′</td>
<td></td>
</tr>
<tr>
<td>TIMP-2 sense primer</td>
<td>5′TCTGTGCGAGGGAGTGGAGAA 3′</td>
<td>(23 cycles)</td>
</tr>
<tr>
<td>TIMP-2 antisense primer</td>
<td>5′GGAAGGATGTCAAGGGCTGGA 3′</td>
<td></td>
</tr>
<tr>
<td>G3PDH sense primer</td>
<td>5′TCTTCACCACCATGGAGAACGCTGGA 3′</td>
<td>(25 cycles)</td>
</tr>
<tr>
<td>G3PDH antisense primer</td>
<td>5′TCCAGGGGGTCTTACTCCTTGGAGG 3′</td>
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Fig. 1. Analysis of drained dialysate using gelatin zymography. (A) Analysis of MMP type in dialysate. Lane 1: drained dialysate from control rat. Lane 2: drained dialysate from CHX-treated rat. Lane 3: to analyse the form of MMP-2, dialysate from CHX-treated rats was incubated for 3 h at 37°C with 1 mM APMA before zymographic analysis. Lane 4: MMP marker (Yagai, Yamagata, Japan). (B) Zymogram signals were quantified by densitometer analysis. Data are expressed as intensity relative to the control group. Each column represents the means ± SD of six rats. **P < 0.01 compared with control. Open column, active form of MMP-2; closed column, latent form of MMP-2 (proMMP-2); ND, not detected.
On the gelatin zymogram, a 64 kDa gelatinase was observed as a main band in the CHX-treated group (Figure 1A, lane 2). The bands in the CHX-treated group were 12-fold denser than in the control group (Figure 1A, lane 2 vs lane 1 and Figure 1B: \( P < 0.01 \)). A 57 kDa band was present but barely detectable. After the drained dialysate was incubated with APMA, the 64 kDa gelatinase level decreased, whereas two additional lytic bands appeared at 57 and 59 kDa (Figure 1A, lane 3). On this zymogram, other gelatinases, such as MMP-9, were not detected. The detection sensitivity of MMP-9 with the present gelatin zymography method was >10 \( \mu \)g/ml, as determined by analysing a set of serial dilutions of standard human MMP-9, measured using the MMP-9 human ELISA Biotrak system (Amersham Pharmacia Biotech Ltd) (data not shown).

Peritoneal injury in CHX-treated rats

In CHX-treated rats, we observed a fist-like round liver edge and extensive adhesion of the bowel and stomach. In two rats, the bowel adhesion appearing as an abdominal cocoon was a mass surrounded by thickened peritoneum. As a result of peritoneal injury, the parietal and visceral peritoneum in CHX-treated rats had a 10-fold greater thickness than in the control group (Figure 2; \( P < 0.01 \) vs control). In the parietal and visceral peritoneum of CHX-treated rats, infiltration consisted mainly of fibroblasts/myofibroblasts and macrophages, as well as of lymphocytes. Representative samples of the parietal and visceral peritoneum are shown in Figures 3B and 4B, respectively.

Gelatinolytic activity in peritoneal tissue

Gelatinolytic activity in the peritoneal tissues was investigated by \textit{in situ} film zymography. Findings from this analysis are shown in Figures 3 and 4. Strong gelatinolytic activity was detected in both the parietal and visceral peritoneum from peritoneal injury rats (Figures 3D and 4D). However, on gelatin film with 1,10-phenanthroline, an inhibitor of MMP, there was no activity. Specimens from control rats also showed no activity on normal gelatin film (Figures 3C, 3E, 4C and 4E).

**MMP-2 production in three-dimensional culture of myofibroblasts**

Myofibroblasts and mesothelial cells were cultured in three-dimensional gel. The morphology of myofibroblasts changed to a star shape in three-dimensional collagen gel (Figure 5C) and to a round shape in fibrin gel (Figure 5E). The morphology of mesothelial cells also changed to a round shape in three-dimensional collagen gel (Figure 5H). MMP-2 production was enhanced in three-dimensional fibrin and collagen gel cultures of myofibroblasts, but not in three-dimensional fibrin gel culture. MMP-2 production in mesothelial cells was undetectable during all of the study conditions. MMP-2 activation was enhanced in the three-dimensional collagen gel culture of myofibroblasts, but not in three-dimensional fibrin gel culture. MMP-2 gene expression in mesothelial cells was not detected. The production and gene expression of MMP-2 are shown in Figures 6 and 7, respectively.

**Discussion**

It has been proposed that vigorous peritoneal tissue remodelling develops in SP/EPS, because sclerosis or excessive fibrous thickening of the peritoneum occurs during these complications [1–3]. In scleroses and fibroses, the production of gelatinases, such as MMP-2 or MMP-9, is known to be induced [7]. We, therefore, investigated whether these gelatinases are associated with the peritoneal injury that leads to SP/EPS.

We reported previously that intraperitoneal administration of CHX creates a peritoneal injury model that reflects the pathology observed in SP/EPS [9,10]. CHX is known as a chemical irritant that induces inflammation [3,6,14]. In our rat model, CHX caused cocoon-like bowel adhesions that were similar to those seen in human EPS patients [10]. We also observed fibrous thickening of the peritoneum, accompanied by oedema and inflammatory cellular infiltration mainly with mononuclear cells [9,10]. The surface of the fibrous thickened peritoneum was covered with a thick fibrin layer. In addition to showing a very similar pathological picture as that observed in SP or EPS patients,
our rats also had enhanced peritoneal permeability to glucose [1–4,9,10].

In the present zymographic studies, peritoneal injury induced by CHX was associated with a gelatinase in the drained dialysate, which had a molecular weight of ~64 kDa as a main band and was present at remarkably higher amounts than in control rats. After incubation of the drained dialysate with APMA, the 64 kDa gelatinase decreased in quantity and two additional lytic bands appeared at 57 and 59 kDa. Since these two bands were thought to be the active form and intermediate form, respectively, we suspected that the

**Fig. 3. In situ film zymography of parietal peritoneum.** (A) HE stain of parietal peritoneum from control rat (magnification: ×40). (B) HE stain of parietal peritoneum from CHX-treated rat. (C) In situ film zymography of parietal peritoneum from control rat. (D) In situ film zymography of parietal peritoneum from CHX-treated rat. (E) In situ film zymography of parietal peritoneum from CHX-treated rat with 1,10-phenanthroline, an inhibitor of MMPs.
64 kDa gelatinase was the latent form of MMP-2 (proMMP-2) [10]. These results indicated that most of MMP-2 in the drained dialysate was the latent form, whereas the active form of MMP-2 was barely detectable. However, the in situ studies revealed a strong matrix degradation activity of MMP-2 in both the parietal and visceral peritoneum from the peritoneal injury model rats. We found previously that MMP-2 expression was correlated with changes in inflammation, D/D0 glucose levels and net ultrafiltration and that rats with excessive expression of MMP-2 formed abdominal cocoons [9,10]. In the current study, the

Fig. 4. In situ film zymography of visceral peritoneum. (A) HE stain of visceral peritoneum with liver from control rat. (B) HE stain of visceral peritoneum from CHX-treated rat. (C) In situ film zymography of visceral peritoneum from control rat. (D) In situ film zymography of visceral peritoneum from CHX-treated rat. (E) In situ film zymography of visceral peritoneum from CHX-treated rat with 1,10-phenanthroline.
Fig. 5. Myofibroblasts and mesothelial cells in three-dimensional culture. Myofibroblasts and mesothelial cells were cultured under three-dimensional conditions. Changes in cell morphology were analysed with a microscope (magnification: ×100). (A) Myofibroblasts on normal plate. (B) Myofibroblasts on collagen-coated plate. (C) Myofibroblasts in three-dimensional collagen gel culture. (D) Myofibroblasts on fibrin-coated plate. (E) Myofibroblasts in three-dimensional fibrin gel culture. (F) Mesothelial cells on normal plate. (G) Mesothelial cells on collagen-coated plate. (H) Mesothelial cells in three-dimensional collagen gel culture.
concentration of MMP-2 in drained dialysate was correlated with peritoneal injury, as assessed by peritoneal thickness (Figures 1B and 2). In addition to the production of MMP-2, we also observed increases in MMP-1 and MMP-3 in human SP or EPS patients (data not shown). The excessive levels of MMPs, including MMP-2, might play an important role in peritoneal injury by causing degradation of ECM or tissue destruction, which might induce abdominal cocoon formation.

We performed in vitro studies to clarify how MMP-2 is activated in peritoneal tissue. In previous immunohistochemical studies, we found that MMP-2 was produced by stromal cells, such as by myofibroblasts in the peritoneum [10]. In the present in vivo study, cell infiltration consisting mainly of fibroblasts/myofibroblasts was observed in the peritoneum of peritoneal injury model rats. Thus, we believe that MMP-2 was produced from myofibroblasts. In the present in vitro study, the production and activation of MMP-2 was induced along with enhanced gene expression of MMP-2, TIMP-2 and MT1-MMP in the three-dimensional collagen culture of myofibroblasts. However, in the three-dimensional fibrin culture, the production of MMP-2 was induced along with enhanced gene expression of MMP-2 and TIMP-2, but activation of MMP-2 was not induced. In previous studies, MMP-2 was both trapped via the MT-1MMP/TIMP complex on the cell membrane and was activated by MT1-MMP on the cell surface [15,16]. These observations suggest

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**Fig. 6.** MMP-2 production in three-dimensional culture of myofibroblasts and mesothelial cells. (A) Gelatin zymogram of conditioned medium from myofibroblasts and mesothelial cells. Four samples of the same group were mixed and analysed. (B) Signals of the zymogram of myofibroblasts were quantified by densitometer analysis. Each sample was analysed by gelatin zymography for statistical analysis. Data are expressed as intensity relative to the normal plate culture. Each column represents the means±SD of four samples. **P<0.01, *P<0.05 compared with the normal plate culture.
that the activation of MMP-2 might be induced by the MT1-MMP/TIMP-2 system in peritoneal myofibroblasts.

We observed that the production and activation of MMP-2 was regulated by the three-dimensional structure of the ECM component and that the morphology of the myofibroblasts changed. To explain this, molecular mechanisms in the regulation of production and activation of MMP-2 by ECM structure might involve cell surface integrin-binding to ECM components followed by signal transduction processes and then cytoskeleton assembly [17]. It has been reported that endothelial cells stimulated by three-dimensional collagen culture cause increased production of the transcription factor Egr-1, which mediates transcription of MT1-MMP [18]. Rac1 was also reported to mediate MMP-2 activation by MT1-MMP during three-dimensional collagen condition [19]. These signal systems might induce an increase in the active form of MMP-2 in peritoneal myofibroblasts during three-dimensional collagen and fibrin rich conditions, such as in SP/EPS. Although we did not detect MMP-2 production in mesothelial cells derived from rat peritoneum, it was reported that human mesothelial cells produce MMP-2 in vitro [20,21]. Differences in signal transduction between humans and rats might explain this disparity, since human mesothelial cells may produce MMP-2 whereas rat mesothelial cells do not.

In our in vitro studies, MT1-MMP and TIMP-2 were highly expressed under conditions in which myofibroblasts were surrounded by collagen, such as occurs during SP/EPS. In addition, we have reported previously that MT1-MMP and TIMP-2 expression was induced in the peritoneum of CHX-treated rats [10]. Normally, only small amounts of MMP-2 are trapped by the MT1-MMP/TIMP-2 complex on the cell membrane and these are locally activated by MT1-MMP near the cell surface in order to keep tissue injury to a minimum. In this way, MMP-2 acts only where needed during cell migration or moderate matrix degradation. However, when excess amounts of the active type of MMP-2 are present due to excess expression of MT1-MMP and TIMP-2, vigorous tissue destruction and excessive remodelling occurs followed by the possible formation of an abdominal cocoon.

In summary, we showed that strong MMP-2 activity was present in a peritoneum of the peritoneal injury animal model. MMP-2 may play a role in the progression of SP/EPS.

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Conflict of interest statement. None declared.

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