Patterns of matrix metalloproteinases and transforming growth factor-beta 1 expression during peritoneal repair in chlorhexidine induced peritoneal fibrosis mice

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Available online 21 February 2012

KEYWORDS
Chlorhexidine gluconate; Matrix metalloproteinase; Peritoneal fibrosis; Tissue repair; Transforming growth factor-beta 1

Summary Background/Purpose: Recovery from peritoneal fibrosis (PF) involves the digestion of accumulated collagens and remodeling. Matrix metalloproteinases (MMPs) may play an important role in repair. The role of MMP-13, an important component in the MMP cascade, in PF is still unclear. We examined the sequential expression of MMP synthesis during repair in a PF mouse. Methods: Forty-eight mice at 8 weeks of age were given an intraperitoneal injection of 0.1% chlorhexidine gluconate (CG) for 3 weeks. Control mice were injected with the same dosages of 15% ethanol dissolved in saline. These mice were sacrificed, and anterior abdominal walls were obtained on days 21, 28, 35, 42, 49, and 56. Gene expressions of MMP-2 and -13, tissue inhibition of metalloproteinase-1 (TIMP-1) and -2, MT1-MMP, transforming growth factor-beta 1, and collagen types I and III were analyzed by real-time polymerase chain reaction. MMP-13 enzyme activity was also measured. In immunohistological evaluation, MMP-13 expressing cells were examined. Results: Thickening of the peritoneum and marked infiltration of monocytes were induced by CG, and the alterations remained until 7 days after cessation of CG. Then tissue repair rapidly advanced. Synthesis of collagen types I and III, MMP-2, TIMP-1 and -2, and transforming growth factor-beta 1 in the injured peritoneum was significantly increased until day 28. These increments were preceded by an increase of MMP-13 synthesis and activity after cessation of CG. Some infiltrating macrophages in the thickened peritoneum showed MMP-13 expression early after cessation of CG.

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Deceased.

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Conclusion: MMP-13 was synthesized by infiltrating monocytes early in the repair process in the CG-induced PF mouse. After cessation of stimulant, increase of MMP-13 synthesis may act as an inducer of an efficient degradation cascade in collagen-rich peritoneal tissue.

Introduction

Marked thickening of the peritoneum and vasculopathy in the submesothelial compact zone (SMC) were reported in long-term peritoneal dialysis (PD) patients. The key factors of peritoneal regeneration after cessation of PD are still unclear. Recovery from peritoneal fibrosis (PF) to a normal peritoneum involved the destruction of accumulated collagens and excess matrix and tissue remodeling in the peritoneum. Matrix metalloproteinases (MMPs) may play important roles in such events.

Elevated expression of collagen type III was observed in early or active lesions of pulmonary fibrosis, and expression of collagen type I increased in chronic pulmonary fibrosis. In scar formation in the healing process of full-thickness excisional wounds, the synthesis of collagen types I and III increased during the later phase of wound healing and peaked at around 2 weeks after surgery. Suga et al. and Fukuda et al. reported that idiopathic pulmonary fibrosis cases showed a predominant expression of MMP-9, whereas nonspecific interstitial pneumonia and bronchiolitis obliterans organizing pneumonia cases showed a predominant MMP-2 expression in bronchoalveolar lavage fluid and in tissues. MMP-1 and -9 expressions were elevated during granulation tissue formation and re-epithelialization, whereas in the remodeling phase, MMP-2 and MMP-9 messenger RNAs (mRNAs) were increased in experimental full-thickness wound healing. Collagenases MMP-1, -8 and -13 can cleave collagens into specific fragments for an efficient degradation by gelatinase, and MMP-13 especially can activate latent transforming growth factor-beta 1 (TGF-β1) on cell surfaces. Once TGF-β1 has been activated, it increases collagen synthesis, tissue inhibition of metalloproteinase-1 (TIMP-1), and MMP-2 activity through the inhibition of TIMP-2. Furthermore, TGF-β1 can suppress expression of collagenase. Since these proteolytic activities are regulated by several mechanisms including regulation of gene expression by cytokines or hormones, and extracellular cleavage of the proenzyme to the active form by membrane-type MMP (MT-MMP) inhibition by TIMPs, the qualitative pattern and quantitative levels of MMPs vary among tissues, diseases, tumors, inflammatory conditions, and cell lines.

The objective of the present study was to find a clue on how to break out of the vicious cycle in long-term PD patients with severe PF. In this study, we observed serial and temporal alterations of collagens, MMPs and TIMPs and TGF-β1, associated with tissue fibrosis and remodeling in the process of peritoneal repair after repeated intraperitoneal injection of chlorhexidine gluconate (CG) in mice. We examined the relationship between these alterations and morphological findings in peritoneal repair.

Materials and methods

Animal model

Fifty-one C57BL/6 male mice were purchased at 8 weeks of age from CLEA Japan Inc. (Tokyo, Japan). They were housed in a specific pathogen free (SPF), light- and temperature-controlled room. They had free access to laboratory chow and tap water in standard rodent cages. All animal studies were performed according to the guidelines of the Ethics Review Committee for Animal Experimentation of Juntendo University Faculty of Medicine. Forty-eight mice were given an intraperitoneal injection (i.p.) of 0.35 mL of 0.1% CG and 15% ethanol dissolved in saline three times a week for 3 weeks. The mice were injected with the same dosages of 15% ethanol dissolved in saline without CG as control (n = 3). These mice were sacrificed, and the anterior abdominal walls were collected on days 21, 28, 35, 42, 49, and 56 (n = 8 in each day).

Histological assessment

Serial morphological changes in the peritoneum after repeated CG injection were evaluated. The parietal abdominal walls were fixed in 10% neutral-buffered formaldehyde and embedded in paraffin. Then 4-μm sections
were obtained and stained using Masson’s trichrome. Thickening of the SMC, an area from the surface of the abdominal muscle to the peritoneal cavity, was defined as interstitial fibrosis. We quantified the thickness of SMC from five areas in each of the five figures (a total of 25 points/mouse). Quantification of SMC thickness was performed by image analysis. The images were analyzed using the Release 3.0 KS400 Imaging System, (Carl Zeiss, Oberkocken, Germany).

Quantitative mRNA analyses by real-time PCR

The parietal abdominal walls were dissected and snap-frozen in liquid nitrogen for total RNA extraction. RNA was extracted with Trizol (RNasey Mini Kit; Qiagen, Tokyo, Japan). Complementary DNA was synthesized using random hexamers (Quantum RNA kit; Ambion, Austin, TX, USA) and SuperScript II RNase H Reverse Transcriptase (Life Technologies, Rockville, MD, USA). Primers and fluorogenic probes of collagen types I and III, MMP-13 and −2, TIMP-1 and −2, and TGF-β1 were obtained from a commercial base (Taqman Gene Expression Assays; Applied Biosystems, Foster City, CA, USA). The assay IDs were collagen type I; Mm00801666-g1, collagen type III; Mm00802331-m1, MMP-13; Mm01168713-m1, MMP-2; Mm00439498-m1, TIMP-1; Mm00441818-m1, TIMP-2; Mm00441825-m1 and TGF-β1; Mm03024053-m1. The complementary DNA obtained was further amplified by a real-time polymerase chain reaction (PCR) system (ABI prism 7500 Real-Time PCR System; Perkin-Elmer, Foster City, CA, USA). Initial template concentration was derived from the cycle number at which the fluorescent signals cross the threshold in the exponential phase of the PCR reaction. Relative gene expression was determined based on the threshold cycles (Ct) values. The PCR parameters were 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds and 60°C for 60 seconds.

MMP-13 enzyme activity

MMP-13 (mouse interstitial collagenase) activity was detected on days 21, 28, 35, 42, 49, and 56 using an Enzolyte 520 MMP-13 assay kit (Anaspec, San Jose, CA, USA). Mouse peritoneal tissue samples from each group (n = 4) were homogenized in assay buffer and then centrifuged at 13,500 rpm at room temperature for 15 minutes. Supernatant was collected and stored at −80°C until use. The activity assay was performed according to the manufacturer’s instructions using 5-FAM/QXL520 FRET peptide (Anaspec) as the substrate. Fluorescence on cleavage was monitored at excitation/emission wavelengths of 490/520 nm, and the results were expressed as relative fluorescent units. This phase of the experiment was repeated twice.

Immunohistochemistry

Peritoneal tissues were stained for F4/80 and MMP-13. Using the same 4-μm sections, immunohistochemical analyses were performed. All sections were deparaffinized in xylene, followed by 100% ethanol, and then placed in freshly prepared methanol/0.3% H2O2 solution for 15 minutes to block endogenous peroxidase activity. Microwave antigen retrieval was performed with hot 0.01 mol/l-citrate buffer for 12 minutes. The sections were returned to room temperature before subsequent procedures were performed, and then blocked with blocking solution containing 2% bovine serum albumin, 2% fetal calf serum, and 0.2% fish gelatin in 0.01 mol/L phosphate buffered saline (PBS; pH 7.4) for 30 minutes, followed by overnight incubation with rat anti-mouse F4/80 antibody diluted 1:100 (AbD Serotec, Oxford, UK) and sheep anti-human MMP-13 antibody diluted 1:200 (AbD Serotec) at 4°C. The sections for F4/80 were incubated with goat anti-rat IgG, which was conjugated to peroxidase-labeled polymer (Histofine Simple Stain MAX-PO (Rat); Nichirei Biosciences, Tokyo, Japan) at room temperature for 30 minutes. The sections for MMP-13 were incubated with polyclonal rabbit anti-sheep immunoglobulins, which were conjugated with horseradish peroxidase, diluted 1:100 (Dako, Glostrup, Denmark) at room temperature for 30 minutes. The bound antibodies were visualized with 3,3’-diaminobenzene containing 0.003% H2O2. The negative control was confirmed by incubation without primary or secondary antibody to show no positive cells. The sections were washed with PBS (pH 7.4) three times after each incubation, except before addition of the primary antibodies. All sections were counterstained with Mayer’s hematoxylin at room temperature for 3 minutes before mounting with glycergel mounting medium (MOUNT-QUICK; Daido Sangyo, Saitama, Japan).

Double immunofluorescence

Immunohistochemical analysis was performed using the same 4-μm sections. All sections were deparaffinized in xylene, followed by 100% ethanol. Thereafter, microwave antigen retrieval was performed with hot 0.01 mol/l-citrate buffer for 10 minutes. The sections were then blocked with blocking solution for 30 minutes. They were reacted with anti-mouse F4/80 diluted 1:200 (Serotec, Raleigh, NC, USA), which is a marker of macrophages. After overnight incubation at 4°C, the sections were washed in PBS three times to stop the reaction, followed by reaction with tetramethyl rhodamine isothiocyanate as secondary antibody at room temperature for 40 minutes. The sections were washed with PBS. They were reacted with anti-MMP13 diluted 1:20 (Anaspec), which reacted with MMP-13. After an overnight incubation at 4°C, the sections were washed with PBS and mounted in diluted @@FITC as secondary antibody at room temperature for 40 minutes.

Results

Morphologic changes of the anterior parietal peritoneum

In control mice, peritoneal tissues consisted of a mesothelial monolayer and thin connective tissues under the mesothelial layer (Fig. 1A). On day 21, repeated CG stimulation induced edematous thickening of the peritoneum, marked mononuclear cell infiltration, and
angiogenesis (Fig. 1B). On day 28, marked mononuclear cell infiltration and thickening of the SMC remained (Fig. 1C). On day 35, cell infiltration and thickness of the peritoneum were decreased (Fig. 1D). On day 42, marked regression of SMC thickening and cell infiltration were observed (Fig. 1E). A mesothelial monolayer was found on the peritoneal surface (Fig. 1E). Thickening of SMC induced by CG was gradually decreased after cessation of CG (control, 2.67 ± 0.85 μm; day 21, 199.73 ± 36.21 μm; day 28, 197.08 ± 26.71 μm; day 35, 152.13 ± 24.35 μm; day 42, 133.5 ± 31.88 μm; day 49, 87.86 ± 20.07 μm; day 56, 32.47 ± 6.60 μm).

Figure 1 Morphological findings in anterior parietal peritoneum. (A) Mesothelial monolayer and thin submesothelial compact zone (SMC) were observed in control mesothelium. (B and C) Edematous thickened mesothelium with many infiltrating mesothelial cells and angiogenesis was found on days 21 and 28. (D–F) Thickness of SMC and number of infiltrating mononuclear cells were decreased on days 35, 42, and 49. (E and F) Mesothelial monolayer on the peritoneal surface and many spindle-shaped cells in SMC were found on days 42 and 49. (G) Thin SMC was observed on day 56. ×100. b–d. ×400. Masson’s trichrome staining.
Assessment of mRNA expressions

Messenger RNAs isolated from parietal peritoneal tissues at days 21, 28, 35, 42, 49, and 56 were analyzed by real-time PCR. CG enhanced expressions of collagen type I and type III, MMP-13 and -2, TIMP-1 and -2, and TGF-β1 mRNAs in the parietal peritoneum (Fig. 2A–G). Levels of TGF-β1, collagen type I, and type III mRNA were elevated until day 28 (Fig. 2A, B and G). These levels then gradually decreased in the following weeks (Fig. 2A, B and G). However, the highest level of MMP-13 mRNA was at day 21, and then rapidly decreased in the following weeks (Fig. 2C). Levels of MMP-2 and TIMP-1 and -2 mRNA were elevated until day 28, and then gradually decreased following the observation period (Fig. 2D, E and F).

MMP-13 enzyme activity

Levels of MMP-13 activity increased compared with those in the control on day 21, and then rapidly decreased following the observation period (Fig. 3).

Figure 2  Serial expressions of mRNA in anterior parietal peritoneum. Blank boxes, control; dotted boxes, at cessation of CG; filled boxes, after cessation of CG. (A) Collagen type I, a: p < 0.05 vs. control. b: p < 0.0001 vs. control. c: p < 0.002 vs. on day 21, d: p < 0.01 vs. on day 21, e: p < 0.001 vs. on day 28. (B) Collagen type III, a: p < 0.05 vs. control. b: p < 0.001 vs. control. c: p < 0.02 vs. on day 21, d: p < 0.05 vs. on day 28, e: p < 0.0001 vs. on day 28. (C) MMP-13, a: p < 0.05 vs. control. b: p < 0.001 vs. on day 21, c: p < 0.05 vs. on day 28. (D) MMP-2, a: p < 0.05 vs. control. b: p < 0.02 vs. on day 21, c: p < 0.02 vs. on day 28. (E) TIMP-1, a: p < 0.01 vs. control. b: p < 0.01 vs. on day 21, c: p < 0.01 vs. on day 28. (F) TIMP-2, a: p < 0.05 vs. control. b: p < 0.01 vs. on day 21, c: p < 0.01 vs. on day 28. (G) TGF-β1, a: p < 0.01 vs. control. b: p < 0.01 vs. on day 21, c: p < 0.01 vs. on day 28.
Immunohistochemical analysis of the peritoneum

Many macrophages (i.e., F4/80-expressing cells) were found in the whole thickness of the SMC on day 21 (Fig. 4A). Infiltrating macrophages in SMC were decreased on days 28 and 35 (Fig. 4B and C). Many round-shaped MMP-13 expressing cells were observed in the whole SMC on day 21 (Fig. 4D). On days 28 and 35, some MMP-13 expressing cells were found in SMC (Fig. 4E and F). Most MMP-13 expressing cells appeared round-shaped on day 21 (Fig. 4G). Spindle- and round-shaped MMP-13 cells were found simultaneously on days 28 and 35 (Fig. 4H and I). On day 21, some macrophages in SMC expressed MMP-13 (Fig. 5).

Discussion

Characteristics of morphological alterations in long-term PD patients include the marked thickening of the peritoneum.
and vasculopathy in the SMC. It is still unclear whether severely injured peritoneum is associated with long-term PD repair. Recovery from PF was involved in the destruction of fibrosis in the peritoneum. In such events, MMPs and TIMPs are thought to play important roles in biological processes that involve angiogenesis, tissue remodeling, and tissue degeneration under physiological and pathological conditions. We showed differential expression levels over time of the various gene products that are involved in tissue repair in the PF mouse. The spatial and temporal induction of different key players is linked to the role of these gene products in the healing process. Since alterations of the peritoneal membrane are similar in CG-induced PF animals and in long-term PD patients, we utilized CG-induced PF mice in this study. The common histological findings include increased accumulation of interstitial collagens such as collagen types I and III, infiltration of monocytes/macrophages, and increase in α-SMA-positive myofibroblasts and vascular density in the peritoneum.

In this study, synthesis of collagen types I and III increased after cessation of CG and peaked at day 7. In response to collagen synthesis, the SMC on day 28 was thicker than that on day 21. Therefore, cessation of stimulant does not result in a switch from PF to repair immediately. Ulrich et al reported that fibrosis-specific type of cross-linking of collagens, MMPs and TGF-β1, seen in human scars, plays a harmonic role in the healing process.

Figure 4  (A) Many macrophages, expressing F4/80 cells, spread over the whole thickened SMC on day 21. (B, D, and F) MMP-13 expressing cells also spread over the whole thickened SMC on days 21, 28, and 35. (C) Some macrophages were found in the whole thickened SMC. (D) The number of infiltrating macrophages was decreased on day 28. (E) Some macrophages were found over the whole thickened SMC on day 35. ×200. (G) Many round-shaped infiltrating cells in the thickened SMC expressed MMP-13 on 21 day. (H and I) Spindle-shaped MMP-13 expressing cells also spread over the whole thickened SMC on 28 and 35 days. Arrowheads show MAP-13 positive cells. ×1000.
Evaluated TGF-β1 synthesis reached a peak not at cessation of CG, but after 7 days. Synthesis of MMP-2, MT1-MMP, and TIMP-1 and -2 increased until 7 days after cessation of CG, and then rapidly decreased.

MMP-13 synthesis peaked at cessation before these increments. MMP-13 mainly cleaves fibrillar collagen types

Figure 4  (continued).

Figure 5  Fluorescent immunostaining of infiltrating cells in SMC on 21 day. (A) Many MMP-13 expressing cells were found in SMC. ×1000. (B) Many macrophages and F4/80 positive cells were also observed. ×1000. (C) Some macrophages coexpressed MMP-13 (arrowheads). ×1000.
I, II, and III into characteristic 3/4 and 1/4 fragments. However, the degradation activity of MMP-13 is weaker than that of gelatinase A. Biochemical characterization of recombinant human MMP-13 has shown that this enzyme cleaves type II collagen about 6-fold more effectively than types I and III collagens, and displays a 40-fold stronger gelatinase activity than those of the collagenases. MMP-13 is expressed when either rapid or effective remodeling or excessive degradation of collagenous extracellular matrices takes place. In human wounds, MMP-13 expression is reported to occur only in connective tissues of chronic wounds. Wu et al suggested that MMP-13 expression was biphasic, with peak activities at days 15 and 37 after injury in free-electron laser and scalpel incisions. They noted that early expression of MMP-13 by fibroblasts contributed to re-epithelialization, and late expression was involved in long-term collagen remodeling and skeletal muscle repair. Hattori et al also reported that MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. In this study, peak levels of MMP-13 synthesis and activity were observed at cessation of CG and then decreased immediately. In spatial assessment, the expressing cells were observed in the whole thickened parietal peritoneum in this study. The level of MMP-13 expression at day 21 significantly increased compared with that during CG stimulation (data not shown). Therefore, MMP-13 may contribute not only to re-epithelialization, and late expression was involved in long-term collagen remodeling in the CG-induced PF mouse.

The degradation activity of collagen types I and III for MMP-13 was higher than that of the gelatinases. However, once collagen fragments are cleaved by collagenases, the fragments are effectively digested by MMP-2 and -9. Improvement of the thickened SMC was found to contribute to amplification of gelatinase synthesis in this study. Gelatinases, such as MMP-2 and MMP-9, also degraded components of collagen type IV and fibronectin, which comprise the basement membrane, and play important roles in angiogenesis and cell migration. Once TGF-β1 is activated, it stimulates the synthesis of collagens and increases MMP-2 activity through suppression of TIMP-2. Additionally, TGF-β1 suppresses MMP-13 activity via inhibition of synthesis and activation of TIMP-1. Therefore, synthesis of MMP-13 might be rapidly decreased since TGF-β1 synthesis was increased in this study. MMP13 also has a central position in the MMP activation cascade, both activating and being activated by several MMPs. Mesothelial monolayer was observed at day 14 after cessation of CG in this study, and then peritoneal repair was accelerated (data not shown). Therefore, it appears that clearance of fragmented collagen by increased MMP-2 facilitates reconstruction of tissue destroyed during peritoneal repair.

Fallowfield et al suggested scar-associated macrophages are a major source of hepatic MMP-13 and facilitate the resolution of murine hepatic fibrosis. Macrophages are recognized as a major source of MMPs. Macrophages can influence matrix turnover both directly by producing MMPs and indirectly by secretion of cytokines including IL-1 and TNF-α that modulate MMP production by other resident cell types. We propose that macrophages represent the primary source of MMP13 in relevant models of PF.

In this study, some infiltrating macrophages in the thickened peritoneum induced and used MMP-13 to mediate the resorption of interstitial matrix through interaction with other MMPs and cytokines in peritoneal remodeling. It appears that MMP-13 might play a prominent role in PF and may be a key player in peritoneal remodeling after cessation of stimuli.

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