Original Article

Identification of complexes of gelatinase A and tissue inhibitor of metalloproteinase-2 in human follicular fluid

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Background and Aims: Ovulation involves considerable tissue remodeling in normal ovarian function. These processes are expected to involve matrix metalloproteinases (MMP). Follicular rupture is caused by the degradation of the basement membrane between the thecal and granulosa layers, as well as disruption of the extracellular matrix (ECM) at the site of rupture. We report on the existence of the complexes of progelatinase A (proMMP-2), MMP-2 and a tissue inhibitor of metalloproteinase-2 (TIMP-2) using zymographic and immunological techniques in human follicular fluid (HFF).

Methods and Results: Partial purification of the complexes was achieved by using gelatin affinity column chromatography. The peak (tubes 68–73) in this chromatography showed gelatinase activities by gelatin-zymography, and also an inhibition by EDTA (metalloproteinase inhibitor). The molecular

weights of the gelatinase activities were approximately 72 and 67 kDa, and were consistent with standard proMMP-2 and MMP-2, as found by using gelatin-zymography. Similarly, the band in this peak was consistent with standard recombinant full-length TIMP-2, as found by the use of western blot analysis, and the molecular weight of the band was approximately 21 kDa.

Conclusion: As proMMP-2, MMP-2 and TIMP-2 exist in the peak from the gelatin affinity column, we expected that these form the complexes. These results indicate that the complexes of proMMP-2/TIMP-2 and MMP-2/TIMP-2 exist in HFF. (Reprod Med Biol 2003; 2: 115–119)

Key words: gelatinase, human follicular fluid, matrix metalloproteinase, tissue inhibitor of metalloproteinase.

INTRODUCTION

MATRIX METALLOPROTEINASES (MMPs) and the tissue inhibitor of metalloproteinases (TIMPs) are involved in the regulation of tissue remodeling of the extracellular matrix (ECM). Regulation of ECM remodeling by MMPs and TIMPs provides an environment that supports growth, migration and differentiation in the body.¹

Ovulation involves considerable tissue remodeling in normal ovarian function. These processes are expected to involve MMPs. Matrix metalloproteinases have been shown to play an essential role in the mechanism of follicular rupture in ovulation. Follicular rupture is

caused by the degradation of the basement membrane between the thecal and granulosa layers, as well as the disruption of the ECM at the site of rupture.²

The participation of MMPs in ovulation has been reported by several groups.^{3–7} In addition, the participation of TIMP has also been reported. The tissue inhibitor of metalloproteinase was released in thecal and granulosa cells.^{8,9} Curry *et al.* reported that TIMP-1 was contained in HFF.¹⁰ The expression of TIMP-2 is reported in sheep pre-ovulatory follicles after an ovulatory stimulus.¹¹

Furthermore, progelatinase A (proMMP-2) and progelatinase B (proMMP-9) are also known as 72 kDa gelatinase/type IV collagenase and 92 kDa gelatinase/type IV collagenase, respectively. Both gelatinases have three repeat domains that are homologous to the type II domain of fibronectin. These domains are responsible for the ability of MMP-2 and MMP-9 to bind to gelatin.¹²

In contrast, it is known that TIMP inhibits MMP by forming a 1:1 molecular complex. The construction of

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human TIMPs indicates that the molecules consist of an N-terminal domain possessing inhibitory activity and a C-terminal domain that appears to influence the interaction with MMPs.¹² Progelatinases, proMMP-9 and proMMP-2, often form complexes with TIMP-1 and TIMP-2, respectively. These specific complexes are formed through the interaction of the C-terminal domains of proMMPs and TIMPs, and the N-terminal domains of TIMPs in the proMMP-9/TIMP-1 and proMMP-2/TIMP-2 complexes are unoccupied. Recently, the crystal structure of proMMP-2/TIMP-2 was reported.¹³

Here, we report on the existence of proMMP-2/TIMP-2 and MMP-2/TIMP-2 complexes in human follicular fluid (HFF).

MATERIALS AND METHODS

Materials

GELATIN-CELLULOSE FOR chromatography columns was purchased from Seikagaku Kogyo Co., Tokyo, Japan. Standard proMMP-9, proMMP-2 and MMP-2 mixture solutions were obtained from Yagai Research Center Co., Yamagata, Japan. Dimethyl sulfoxide (DMSO) and ethylenediaminetetraacetic acid (EDTA) were from Nakalai Tesque Co. Ltd, Kyoto, Japan. Rabbit antihuman MMP-9, MMP-2, TIMP-1 and TIMP-2 peptide antibodies and mouse antihuman fibronectin polyclonal antibody were from Sigma Chemical Co., St Louis, MI, USA. Standard recombinant full-length TIMP-2 was from Fuji Chemical Co., Toyama, Japan. Polyvinylidene difluoride (PVDF) membrane and Precision protein standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories, Hercules, CA, USA. All reagents used, including antibodies and proteins, were of analytical grade or were medically used products.

Human follicular fluid

Mature HFF samples were collected from volunteers (five with normal ovulation and without the polycystic ovary syndrome) who consulted the *in vitro* fertilization and embryo transfer (IVF-ET) program¹⁴ at Tachikawa Kyosai Hospital, Tokyo, Japan. Informed consent was obtained from all volunteers, and ethical approval was obtained from a committee of ethics that was associated with the Tachikawa Kyosai Hospital. Ovarian stimulation for multiple follicular growth was induced with human menopausal gonadotropin (hMG; 150 IU/day × 8 days) under a long downregulation protocol with

gonadotropin releasing hormone (GnRH) agonist use.¹⁵ Follicular growth was monitored by using ultrasonography (USG). Human chorionic gonadotropin (hCG; 10 000 IU) was administered when follicular diameter exceeded 23 mm (three or more pieces). Oocyte recovery was performed by using USG-guided needle aspiration 34 h later. A mature HFF sample after first puncture of the vesicular ovarian follicle was collected from each volunteer and used in the present investigation. The average HFF sample volume was approximately 5 mL, with a distribution ranging from 2 to 8 mL. Uncontaminated mature HFF was centrifuged at 14 000 g for 30 min at 4°C. The supernatant was then filtered through a 0.45-µm Millipore filter and stored at –40°C until use.

Partial purification of MMPs and TIMPs

Purification steps were performed at 4°C. The supernatant (36 mL) of HFF was loaded onto a gelatin-cellulose column chromatography (2.5 × 8 cm), and equilibrated with 50 mmol/L Tris-HCl buffer, pH 7.5, 0.15 mol/L NaCl, 10 mmol/L CaCl₂, and 0.02% NaN₃ (TNC buffer). The column was washed with the TNC buffer (100 mL), and then washed with TNC buffer containing 1 mol/L NaCl. The proteins were eluted with TNC buffer containing 1 mol/L NaCl and 5% DMSO. Finally, the column was washed with TNC buffer containing 1 mol/L NaCl and 6 mol/L urea. The fractions were collected at 2 mL/tube. The homogeneity of the MMP-2 and TIMP-2 following the chromatography was examined by using SDS-PAGE, gelatin-zymography and western blot analysis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The SDS-PAGE was performed by using 12% total acrylamide under reducing conditions, as previously described.¹⁶ The proteins were stained with Coomassie Brilliant Blue R-250 or silver nitrate.

Gelatin-zymography

The chromatography extracts were mixed with a non-reducing SDS gel sample buffer and applied without boiling to a 12% polyacrylamide gel containing 0.1% SDS and 1 mg/mL gelatin.¹⁷ After electrophoresis, the gels were washed in 50 mmol/L Tris-HCl (pH 7.5) containing 0.15 mol/L NaCl, 5 mmol/L CaCl₂, 5 µmol/L ZnCl₂, 0.02% NaN₃, and 0.25% Triton X-100 (three changes) at room temperature, and then incubated in the same buffer without Triton X-100 (two changes) at

37°C for 20 h. The proteins were stained with Coomassie Brilliant Blue R-250 solution.

To examine the inhibition of enzyme activity, samples at a final concentration of 10 mmol/L EDTA were incubated at room temperature for 1 h, mixed with non-reducing SDS gel sample buffer (1:1 (v/v)), then applied without boiling to a 12% polyacrylamide gel containing 0.1% SDS and 1 mg/mL gelatin. After electrophoresis, the gel was washed, incubated in zymography buffer with inhibitor (at a final concentration of 10 mmol/L) at 37°C for 20 h, and stained with Coomassie Brilliant Blue R-250.

Western blot analysis

For western blot analysis, the samples electrophoresed by 12% SDS-PAGE were electroblotted onto polyvinylidene difluoride (PVDF) membranes, as previously described.¹⁸ Non-specific binding of immunoglobulin (Ig) G was blocked by using a 3% skim milk solution. The membranes were incubated with the primary antibodies at a 1:5000 dilution for 1 h. The primary peptide antibodies of rabbit antihuman MMP-9 (N-terminal), MMP-2 (N-terminal), TIMP-1 (third loop), TIMP-2 (first loop) and mouse antihuman fibronectin were used in the experiments. After extensive washing of the membranes, they were incubated with peroxidase-conjugated goat antirabbit or antimouse IgG (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) at a 1:50 000 dilution for 1 h at room temperature. The protein bands were detected by using the ECL+ plus a western blotting detection system (Amersham Pharmacia Biotech. Ltd, Buckinghamshire, UK) with subsequent exposure to X-ray film.

RESULTS

THE SUPERNATANT OF HFF was loaded on a gelatin-cellulofine column (Fig. 1), and the fractions in tubes 68–73 were found to contain gelatinase activities as assessed by gelatin-zymography (Fig. 2). The fractions in tubes 110–120 did not have activities when assessed by gelatin-zymography (data not shown). The peaks (tubes 68–73) were collected and concentrated using a Vivaspin concentrator (Sartorius Co., Tokyo, Japan). The concentrated samples were examined by using SDS-PAGE, gelatin-zymography, and western blot analysis against antihuman MMP-9, MMP-2, TIMP-1 and TIMP-2. As shown in Fig. 2, the purity of the preparation was examined by using gel electrophoresis under reducing conditions (Fig. 2a). The partially purified protein produced several bands; the major bands were 52 and 27 kDa,

Figure 1 Gelatin-cellulofine column chromatography. The supernatant of human follicular fluid (HFF) was loaded to a gelatin-cellulofine column chromatograph. The NaCl concentration is indicated by a dotted line, and the arrow bar indicates 50 mmol/L Tris-HCL buffer, pH 7.5, 0.15 mol/L NaCl, 10 mmol/L CaCl_2 , and 0.02% NaN_3 (TNC) buffer containing 1 mol/L NaCl and 5% dimethyl sulfoxide (DMSO) or 6 mol/L urea. The solid bar over the peaks (tubes 68–73) indicates the pooled fractions.

Figure 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gelatin-zymography and western blot analysis of pooled the fractions from gelatin-cellulofine column chromatography. The concentrated sample (tubes 68–73) from gelatin-cellulofine column chromatography was electrophoresed in 12% SDS-PAGE (a), 12% gelatin-zymography (b–d) and western blot analysis (e–g). (a) Reducing concentrated sample (silver stain), (b) non-reducing mixture of standard human progelatinase B (proMMP-9), progelatinase A (proMMP-2) and gelatinase A (MMP-2), (c) non-reducing concentrated sample, (d) non-reducing concentrated sample with EDTA, (e) reducing concentrated sample against antihuman MMP-2 antibody, (f) reducing standard human tissue inhibitor of metalloproteinase (TIMP-2) against antihuman TIMP-2 antibody, and (g) reducing concentrated sample against antihuman TIMP-2 antibody.

and the minor bands were 72 and 21 kDa on SDS-PAGE. Proteinase activities of a mixture of standard gelatinases (proMMP-9, proMMP-2 and MMP-2) and the concentrated sample were detected by using gelatin-zymography (Fig. 2b,c). The three bands of standard MMP mixture marker were observed on the gelatin-zymography gel (Fig. 2b). The upper band is proMMP-9 (92 kDa), the middle band is proMMP-2 (72 kDa), and the lower band is MMP-2 (67 kDa). In the concentrated sample, one major band of 72 kDa was observed, along with other minor bands of 92 and 67 kDa, by gelatin-zymography (Fig. 2c). The activity of all these proteinases was inhibited by EDTA (metalloproteinase inhibitor), as assessed by gelatin-zymography (Fig. 2d).

On western blot analysis, using the antibodies of antihuman MMP-9, MMP-2, TIMP-1 and TIMP-2, one band was shown against antihuman MMP-2 and TIMP-2 antibodies in each lane (Fig. 2e–g), but the bands were not shown against antihuman MMP-9 and TIMP-1 antibodies (data not shown). The antibodies of MMP-2 and TIMP-2 recognized proteins of 72 and 21 kDa, respectively (Fig. 2e–g). The band of standard recombinant full-length TIMP-2 was detected by the use of western blot analysis (Fig. 2f).

DISCUSSION

CURRY *ET AL.* reported that two kinds of metalloproteinase inhibitors were contained in human ovarian follicular fluid.¹⁰ These inhibitors were α_2 -macroglobulin and TIMP-1. Particularly, the purification of TIMP-1, a specific matrix metalloproteinase inhibitor, was achieved by using three steps consisting of chromatography on a gel-filtration column and then on two kinds of affinity columns. They suggested that the presence of collagenase inhibitor activity in the pre-ovulatory follicular fluid may be regulated by inhibitors.

The existence of proMMP-2, MMP-2 and TIMP-2 complexes by using gelatin-zymography and western blot analysis in HFF was reported on in the present study.

Partial purification of the complexes was achieved by using gelatin-affinity column chromatography. The concentrated sample (tubes 68–73) produced several bands; the major bands were 52 and 27 kDa, and the minor bands were 72 and 21 kDa on SDS-PAGE (Fig. 2a). These major bands were identified as human heavy and light chain immunoglobulin by N-terminal amino acid sequence analysis (model 477 A/120 A protein sequencer; Applied Biosystems, Rockville, MD, USA). We considered this to be non-specific binding to the gelatin-affinity column because HFF contained a

large amount of immunoglobulin. Gelatinase activities were detected in the concentrated sample by gelatin-zymography (Fig. 2c). The molecular weights of the gelatinase activities were approximately 92, 72 and 67 kDa, and were consistent with standard proMMP-9, proMMP-2 and MMP-2 detected by using gelatin-zymography (Fig. 2b), and they were also inhibited by EDTA (metalloproteinase inhibitor; Fig. 2d). However, proMMP-2 was only detected by using western blot analysis against antihuman MMP-2 antibody (Fig. 2e). The reason why proMMP-9 and MMP-2 were not detected by using western blot analysis was that there were only small quantities of proMMP-9 and MMP-2 compared with proMMP-2 in HFF. The fractions in tubes 110–120 were identified as human fibronectin by western blot analysis using antihuman fibronectin antibody (data not shown).

Similarly, the concentrated sample was consistent with standard recombinant full-length TIMP-2, which was determined by western blot analysis, and the molecular weight was approximately 21 kDa (Fig. 2f–g). Therefore, it was confirmed that TIMP-2 existed in the concentrated sample. These results showed that proMMP-2, MMP-2 and TIMP-2 exist in the concentrated sample, and that they formed complexes of proMMP-2/TIMP-2 and MMP-2/TIMP-2 in HFF.

The follicular plasminogen activator-plasmin system has been suggested to be involved in the mechanism of ovulation. It has been reported that activated collagenases from procollagenases by plasmin degrade the basement membrane, the theca and the tunica albuginea, thereby degrading the ovarian wall to the point of stigma formation, and then rupture.^{19,20}

It has been reported that proMMPs are activated in a step-wise manner by a proteinase, plasmin, plasma kallikrein and neutrophil elastase.¹² The activation of proMMPs is initiated by these proteinases attacking the middle of the prodomain of proMMPs. For example, the sequence of the region (Lys³⁴–Arg–Arg³⁶) in the prodomain of MMP-1 is recognized by its arginine or lysine residues. However, proMMP-2 does not have this region in the prodomain.²¹ Recently, it was reported that proMMP-2 can be activated in a step-wise manner by membrane type-matrix metalloproteinase-1 (MT1-MMP), of which the cleavage site of the region (Asn³⁷–Leu³⁸) is in the prodomain of MMP-2.^{22,23} Another group reported on the expression of MT1-MMP in bovine pre-ovulatory follicles and luteal tissue after an ovulatory stimulus.²⁴ Therefore, the degradation of the ovarian wall may be caused by MMP-2 activated by MT1-MMP, leading to the rupture, and not to the follicular

plasminogen activator-plasmin system. We will examine the existence of MT1-MMP or other proMMP-2 activators in HFF in later studies.

Recently, Lahav-Baratz *et al.* reported that the lower expression of TIMP-1 was found in the follicular fluid of patients with polycystic ovary syndrome (PCOS) compared with normal ovulating patients.²⁵ The complexes of PCOS in women will be examined in the future.

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