Gelatinases and their tissue inhibitors during human ovulation: increased expression of tissue inhibitor of matrix metalloproteinase-1

A.-K.Lind¹, P.Dahm-Kähler, B.Weijdegård, K.Sundfeldt and M.Brännström

Department of Obstetrics and Gynecology, Sahlgrenska Academy, Göteborg University, Göteborg, Sweden
¹To whom correspondence should be addressed at: Division of Gynecology and Reproductive Medicine, Sahlgrenska University Hospital, S-413 45 Göteborg, Sweden. E-mail: annakarin.lind@vgregion.se

Remodelling of the extracellular matrix (ECM) of the follicular wall by matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) has been suggested to be crucial in ovulation. To investigate the expression of the gelatinases, MMP-2 and MMP-9, together with their inhibitors, TIMP-2 and TIMP-1, in the perifollicular ovarian stroma from women just before and during ovulation, we obtained biopsies of the stroma adjacent to the leading follicle. Laparoscopic surgery was performed either before the LH peak or at any of three intervals after ovulation triggering by hCG. Immunoblotting, immunohistochemistry and quantitative RT–PCR were performed. All four proteins were expressed by immunobLOTS, with no detectable changes in the expression of MMP-2, MMP-9 and TIMP-2. Scattered immunostaining for MMP-9 and TIMP-2 was seen, and MMP-2 was demonstrated in a concentric layer. A significant increase in TIMP-1 protein and mRNA was seen during the three ovulatory phases, and a strong and patchy immunostaining for TIMP-1 was shown. This is the first study that has demonstrated an ovulation-associated expression of these ECM-remodelling enzymes around the human follicle at ovulation. The increased expression of TIMP-1 may reflect a specific temporal inhibition of collagenolysis and thereby a time-dependent regulation of ECM breakdown in areas surrounding the apex of the follicle.

Key words: human/ovary/ovulation/follicle/MMP/TIMP

Introduction

Ovulation is initiated by the pre-ovulatory surge of LH, which sets in motion several parallel biochemical pathways that ultimately lead to follicular rupture and release of the oocyte. There is now robust evidence that tissue-remodelling processes (Curry and Osteen, 2003) and vascular changes (Brannstrom et al., 1999) in and around the follicle are important for this biologically essential process to take place.

Several intraovarian mediators are active in the tissue-remodelling process during ovulation with the activation of the progesterone receptor being a central event to induce the expression of these proteins to start the ovulatory cascade (Chen et al., 1995; Lydon et al., 1995; Robker et al., 2000). Animal studies have indicated that the collagenous layers in and around the theca layer and in the overlying tunica albuginea make up the tensile strength of the follicle (Espey, 1967a). Thus, in the rabbit, two layers of interstitial collagen (collagen type I and III) and two basal membranes with collagen type IV have to be broken down to allow the oocyte to be released from the follicle at ovulation (Bjersing and Cajander, 1974a-1). Important proteolytic enzymes in the degradation of these types of collagens are matrix metalloproteinase-2 (MMP-2), which is mainly active on collagen type I and III that have been initially cleaved by collagenases, and MMP-9, which mainly is active on collagen type IV (Woessner and Nagase, 2000). These MMPs are activated by the cleavage of a proenzyme and inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) (Gomez et al., 1997), with TIMP-1 and TIMP-2 mainly inhibiting MMP-9 and MMP-2, respectively.

There are morphological signs of a breakdown of follicular collagen preceding ovulation. A fragmentation and dissociation of collagen and a 3-fold decrease in the density of collagen fibrils before the time of ovulation were demonstrated in the rabbit follicle wall (Espey, 1967b).

Several studies in rodents have pointed towards the importance of MMPs and TIMPs in ovulation. With the inhibitors of varying selectivity for the MMPs, ovulation in the rat ovary could be inhibited both in vivo (Reich et al., 1985) and in vitro (Brannstrom et al., 1988). Taken together, the sign of degradation of the extracellular matrix (ECM) associated with follicular rupture and the blockage of ovulation by synthetic MMP inhibitors provide compelling evidence for the essential role of MMPs in follicular rupture.

TIMP-1 is the TIMP that has been most consistently demonstrated in the ovary. It is secreted as a glycoprotein and binds to the active form of many MMPs, especially to the latent form of MMP-9. In the rat, mRNA for TIMP-1 was localized to the stroma and theca of developing follicles (Curry et al., 2001) and increased after hCG stimulus (Mann et al., 1991). Likewise, there was an increase of TIMP-1 protein in the ovine pre-ovulatory follicle after the LH surge (McIntush et al., 1997).

TIMP-2 forms a complex with the proform of MMP-2 to regulate the activation of pro-MMP-2. In a rat model, it was shown that there was a shift in the expression pattern after the LH surge for TIMP-2 mRNA such that the granulosa cells began to express TIMP-2 (Curry et al., 2001).
Taken together, there is robust evidence that both MMPs and TIMPs are critical in ovulation in animals. However, the knowledge of the intraovarian events of the human ovulatory process is limited. Previous findings are restricted to the observations of increased collagenolytic activity towards ovulation within human follicular fluid (Puistola et al., 1986) and a higher collagenase activity in ovarian tissue of menstruating women compared with that after the menopause (Postawski et al., 1999). Concerning IVF cycles, human granulosa lutein cells contained mRNA for TIMPs (Curry et al., 1990). The expression of MMP-2 and MMP-9 was lower in follicular fluid from IVF patients compared with normally ovulating women with the inverse relationship for TIMP-1 (D’Ascenzo et al., 2004).

The present study differs from these previous investigations, as this is the first study to analyse human ovarian tissues from specific times throughout the periovulatory period. The examination/determination of the protein expression of these mediators in the perifollicular stroma of ovulating women will increase our knowledge of the human ovulatory process.

**Materials and methods**

**Patients and tissue harvesting**

The study was approved by the ethics committee of Sahlgrenska Academy at Göteborg University, and the setting was the Division of Gynecology and Reproductive Medicine at Sahlgrenska University Hospital. Informed written consent was obtained from all patients before the planned laparoscopic sterilization. Twenty-eight women (age 30–38 years, mean 35.4), with previously proven fertility (para >1, mean 2.9), regularly menstruating (cycle length 26–32 days, mean 29 days) and with no chronic systemic diseases were included in the study. The patients had not been on any hormonal contraceptive for a period of at least 3 months before surgery.

All patients were monitored by serial transvaginal ultrasound (TVU; Aloka SSD-900/2000, Aloka Co. Ltd, Tokyo, Japan), for at least one menstrual cycle (mean two cycles) before surgery to decide whether accurate follicular development occurred. During the menstrual cycle of the laparoscopic sterilization with the tissue collection, TVU was performed every 1–2 days to enable the surgery to be planned at the accurate stage of the menstrual cycle. The side of the dominant follicle was identified by TVU, and as the first laparoscopic surgical procedure, the follicle was visualized to confirm that the TVU-determined dominant follicle could be clearly seen and that it was accessible for surgery and not covered by adhesions. Biopsy was then obtained by scissors from the stroma around the dominant follicle. Diathermy was not used, until the tissue sample had been removed. The ovarian biopsy was taken out from the abdomen, immediately placed on ice, taken to the laboratory, washed with ice-cold RNase-free phosphate-buffered saline (PBS) and snap-frozen in liquid nitrogen for western blot and RT-PCR or fixed in 4% formaldehyde overnight for immunohistochemistry (IHC). The frozen biopsies were stored at –70°C until analysis.

**Experimental groups**

The study was designed to obtain tissue material before, during and after ovulation. It was decided to use hCG instead of the endogenous LH surge as the ovulatory trigger to obtain ovarian tissue of more precise stages of ovulation, and for practical reasons to be able to perform surgery during normal working hours. To determine the approximate time of follicular rupture after hCG administration, we first performed a pilot study. Five healthy volunteers (age 25–38 years) participated. When the dominant follicle was of a diameter of 15–17 mm on TVU, an s.c. injection of 250 μg hCG (Ovitrelle®, Serono International S.A., Geneva, Switzerland) was administered. Frequent TVU examinations after hCG administration determined that rupture occurred within 36 h after hCG. Ovulation with luteinization was confirmed by serum progesterone. On the basis of these data and a previous larger study using 6000 IU urinary hCG with follicular rupture detected around 38 h later (Andersen et al., 1995), our study population was divided into four groups (pre-ovulatory phase, early ovulatory phase, late ovulatory phase and post-ovulatory phase) to distinguish between the different stages of the ovulatory process.

Pre-ovulatory phase was defined as the stage when the dominant follicle was ≥14 mm and ≤17.5 mm (15.8 ± 0.6 mm; mean ± SEM). Surgery was performed at this pre-ovulatory stage without giving hCG, and serum levels of hormones did not suggest initiated ovulation (n = 7). The majority of women (n = 21) received an s.c. injection of 250 μg hCG (Ovitrelle®) to mimic the natural LH peak when the dominant follicle was of a diameter of ≥14 and ≤20 mm (16.4 ± 0.2 mm). These patients had surgery during any of three different time intervals. Early ovulatory phase was defined as 12 to ≤18 h after hCG (n = 7), late ovulatory phase >18 to ≤24 h after hCG (n = 7) and post-ovulatory phase 44 to 77 h after hCG (n = 7). Samples for the measurement of serum levels of progesterone and estradiol were taken immediately before surgery to confirm their ovulatory phase category. The characteristics of the patient material are summarized in Table 1. The whole dominant follicle with adjacent ovarian stroma was excised from two additional women also undergoing laparoscopic surgery for sterilization. They were monitored and operated on as described above, and care was taken to remove the whole intact follicle during surgery. Instead of freezing, these two follicles were embedded in paraffin for IHC. These two excised follicles were from the pre-ovulatory phase (follicle size = 16 mm, patient age = 37 years) and early ovulatory phase (follicle size = 18 mm, surgery 16 h after hCG, patient age = 39 years).

**Primary antibodies**

The following antibodies were used for both western blot and IHC: MMP-2 (catalogue number IM33L, mouse monoclonal anti-human MMP-2, Calbiochem, EMD Biosciences/Merck KGaA, Darmstadt, Germany); MMP-9 (ab16306, rabbit polyclonal anti-mouse MMP-9, Abcam, Cambridge, UK); TIMP-1 (catalogue number IM41L, mouse monoclonal anti-human TIMP-1, Oncogene Research Products/Calbiochem); TIMP-2 (catalogue number IM11L, mouse monoclonal anti-human TIMP-2, Oncogene Research Products); α-tubulin, Sigma-Aldrich, St Louis, MO, USA) and α-tubulin (product number T 5168, mouse monoclonal anti-α-tubulin, Sigma-Aldrich).

**Table 1.** Characteristics of follicle size, time of surgery in relation to hCG, pre-operative hormone values of peripheral blood (serum), menstrual cycle day of surgery, menstrual cycle length and age of the study population

<table>
<thead>
<tr>
<th></th>
<th>Pre-ovulatory phase</th>
<th>Early ovulatory phase</th>
<th>Late ovulatory phase</th>
<th>Post-ovulatory phase</th>
</tr>
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<tbody>
<tr>
<td>Follicle size (mm)</td>
<td>15.8 ± 0.6 (14–17.5)</td>
<td>16.3 ± 0.3 (15–17)</td>
<td>16.9 ± 0.6 (15–20)</td>
<td>16.7 ± 0.4 (15–18)</td>
</tr>
<tr>
<td>Hours after rhCG</td>
<td>14.0 ± 0.9 (12–18)</td>
<td>21.9 ± 0.8 (18.5–24.0)</td>
<td>49.1 ± 0.6 (44–70)</td>
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<tr>
<td>Estradiol (nmol/l)</td>
<td>0.4 ± 0.5 (0.3–0.6)</td>
<td>1.0 ± 0.13 (0.5–1.4)</td>
<td>2.0 ± 0.4 (1.4–3.0)</td>
<td>3.1 ± 1.2 (0.5–9.0)</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>0.4 ± 0.1 (0.13–1.0)</td>
<td>0.6 ± 0.1 (0.4–0.9)</td>
<td>0.7 ± 0.1 (0.6–0.9)</td>
<td>0.3 ± 0.0 (0.21–0.56)</td>
</tr>
<tr>
<td>Cycle days of surgery</td>
<td>14.0 ± 0.3 (13–15)</td>
<td>12.6 ± 0.5 (11–14)</td>
<td>13 ± 0.6 (11–15)</td>
<td>13.1 ± 0.6 (11–15)</td>
</tr>
<tr>
<td>Cycle length (days)</td>
<td>30.1 ± 0.9 (28–35)</td>
<td>28.1 ± 0.8 (26–32)</td>
<td>29.8 ± 0.8 (28–32)</td>
<td>29.4 ± 0.9 (27–34)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.2 ± 1.1 (30.8–37.7)</td>
<td>34.6 ± 1.0 (30.1–37.8)</td>
<td>36.7 ± 0.4 (34.9–38.2)</td>
<td>35.4 ± 0.7 (33.2–37.0)</td>
</tr>
</tbody>
</table>

All data are given as mean ± SEM, and range is given in parentheses; n = 7 in each group.
Western blotting

One hundred milligrams of ovarian stroma from each patient was prepared by homogenization in a lysis buffer consisting of PBS, NaCl (0.15 M), protease inhibitors (Complete Mini™, Roche, Mannheim, Germany) and Tween-20 (0.25%). The homogenate was sonicated (twice, for 15 s) and centrifuged at 10,000 g, 30 min, 4°C. The protein concentration of the supernatant was determined with the Micro BCA protein assay kit according to the manufacturer’s instructions (Pierce, Rockford, IL, USA). Samples were diluted in sodium dodecyl sulphate (SDS) sample buffer (Invitrogen, Paisley, UK) and reducing agent (Invitrogen) and heated at 70°C for 10 min. Fifty micrograms of total protein from each sample was loaded into each lane of an SDS–polyacrylamide gel (NuPAGE 4–12%, Invitrogen) with Bis-Tris-Mops buffer system. A prestained standard (Precision Plus Protein™, All Blue, BioRad, Hercules, CA, USA) was used as weight marker. Control peptides for human MMP-2 and MMP-9 (product numbers M1927 and M4809, respectively; Sigma-Aldrich) and TIMP-1 and TIMP-2 (catalogue number P0191 and PF021, respectively; Calbiochem) were used as positive controls, and human placenta was used on each gel for comparison between blots. Protein separation was obtained at 37°C. Protein bands were detected using a Fluor-S multimager (BioRad) directly after detection in a Fluor-S multimager (BioRad). The optical density of each band was normalized to the control sample (same on each gel), which was set to the numerical value of 1, and the adjusted results were then normalized to the adjusted results of either α-actin or α- Tubulin. This relative number was then used in the statistical analysis.

Immunohistochemistry

Tissues were fixed in 4% formaldehyde overnight and embedded in paraffin. Four-micrometer-thick sections were placed on glass slides, deparaffinized in xylene, rinsed in ethanol and brought to water through a series of decreasing concentrations of ethanol. Antigens were retrieved by boiling with an antigen-retrieving solution (Vector Laboratories, Burlingame, CA, USA) for 20 min at 120°C and then cooled in water for 5 min. Non-specific binding was blocked with normal horse serum for 30 min at room temperature. Primary antibodies against MMP-2 (dilution 1:100), MMP-9 (dilution 1:5000), TIMP-1 (dilution 1:100), TIMP-2 (dilution 1:5000) and α-tubulin (dilution 1:4000), Immunoreactive protein was visualized by chemiluminescence using alkaline phosphatase-conjugated secondary antibodies and CDP-star (Roche) as substrate.

Semi-quantitative measurements of proteins from immunoblots were obtained by densitometry using the Quantity One software package (version 4.2, BioRad) directly after detection in a Fluor-S multimager (BioRad). The optical density (OD) of each band was normalized to the placenta band (same on each gel), which was set to the numerical value of 1, and the adjusted results were then normalized to the adjusted results of either α-actin or α-tubulin. This relative number was then used in the statistical analysis.

Results

TIMP-1 expression and localization

In the immunoblots, several bands were present, and for quantification, a band at ~28 kDa, corresponding to TIMP-1, with positive expression in placenta was chosen. The protein levels of TIMP-1 were low during the pre-ovulatory phase (Figure 1). A clear and significant increase in TIMP-1 protein was seen in all ovulatory phases in comparison with the pre-ovulatory phase. To confirm this increased protein expression of TIMP-1 during ovulation, we performed RT–PCR. A significant increase in TIMP-1 mRNA was also seen during all ovulatory phases as compared with the pre-ovulatory phase. The median levels were 2.4-fold higher during the late ovulatory phase than the pre-ovulatory phase (Figure 2). IHC showed the presence of TIMP-1 in the stroma during all phases. TIMP-1 was localized to most parts of the stromal tissue, but with a clear concentration at specific sites (Figure 3). In samples of the entire pre-ovulatory follicle, TIMP-1 immunoreactivity was seen in the theca cell layer and in the granulosa cell layer (Figure 3). Negative controls showed no immunostaining.

TIMP-2 expression and localization

TIMP-2 protein was expressed at high amounts in all samples. The immunoblots for TIMP-2 showed a single clear band at the expected 21 kDa. There were no changes in the protein expression during the ovulatory phases (Figure 4). IHC showed a scattered immunostaining for TIMP-2 in the perifollicular stroma of all stages (Figure 5). Negative controls did not show any immunostaining.

MMP-2 expression and localization

Immunoblots showed a single band at 77 kDa corresponding to the proform of MMP-2. There were no significant changes in the protein expression of MMP-2 during the ovulatory phases. However, a tendency of a slightly increased expression was seen during the early ovulatory phase as compared with the pre-ovulatory phase (Figure 6). Clear and intense immunostaining for MMP-2 was shown in the perifollicular stroma. The immunoreactivity was concentrated to a concentric layer of the stroma harbouring the primordial follicles (Figure 7). Negative controls showed the absence of immunostaining.
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MMP-9 expression and localization
A clear but weak band at 92 kDa, equivalent to the proform of MMP-9, was identified.

This band was clearly seen in human placenta, and the position on the gel corresponded to the MMP-9 control peptide. There were no detectable changes in protein expression during the ovulatory phases (Figure 8). IHC showed immunostaining for MMP-9 in the perifollicular stroma of all stages (Figure 9), with no immunostaining in the negative controls.

Discussion
The present study focused on enzymes that are primarily involved in the regulation of collagen type IV breakdown. Although the exact composition of the ECM of the human ovary is not clearly established, it is assumed that collagen type IV is an important ECM component of the follicular wall and the surrounding stroma (Irving-Rodgers and Rodgers, 2005). Studies in the rat (Bagavandoss et al., 1983; Palotie et al., 1984) and cow (Zhao and Luck, 1995; Rodgers et al., 1998) have shown that collagen type IV is present in the basal
Figure 3. Immunohistochemical localization of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in ovarian tissues. (A) Perifollicular stroma of early ovulatory phase; scale bar represents 50 μm. (B) Section of whole pre-ovulatory follicle with staining in theca interna (TI) and granulosa cells (GC). Basal lamina (BL) is indicated by arrow. Scale bar represents 100 μm.
membranes separating the granulosa cell compartment and the theca cell compartment as well as separating the surface epithelium from the tunica albuginea.

In the present study, we specially studied the perifollicular stroma situated within 2–3 mm from the theca externa of the pre-ovulatory/ovulatory follicle. The stroma was examined, because previous studies in the rat have shown that both MMP-9 and TIMPs are located primarily in stroma tissue around the follicle and the theca externa (Reich et al., 1991; Hurwitz et al., 1993). Moreover, because the stroma is the most collagen-dense tissue around the pre-ovulatory follicle, we think that it is most relevant to specifically examine this cell compartment. It is likely that more of the collagenolytic activity occurs in the stroma around the pre-ovulatory follicle as compared with the theca and granulosa cell layers because of the dense distribution of collagen in this layer. Additionally, the stromal layer of the human follicle wall is relatively thick. The thickness of this perifollicular stroma in the human is illustrated by a number of fibroblast-rich and collagenous layers of this area in the apex of the human follicle as compared with the same area of the hyperstimulated rodent models. Thus, in the late ovulatory phase of the rat (Brannstrom et al., 1993), which is comparable to a stage just before rupture of the follicle in the human, the stromal part of the apical of the follicular wall was several times thicker in the human (Brannstrom et al., 1994) as compared with that in the rat (Brannstrom et al., 1993) when relating to the thickness of the combined theca and granulosa layers. We therefore speculate that the total collagenolytic activity needs to be proportionally higher in the surrounding of the human follicle as compared with that in the rat, to be able to carry out the complete breakdown of the relatively thicker follicle wall.

The clinical material we used was well characterized by several methods. Importantly, all women had demonstrated their fertility and they were not on any hormonal medication either during the menstrual cycle of biopsy or during the time when they were closely monitored. The exogenous hCG to mimic the LH surge and the hormonal values at biopsy also ensured that the biopsies were of excellent quality concerning timing of the ovulatory phase. By clinical observations with repeated TVU, before surgery in the specific cycle, we are convinced that we truly examined the preovulatory/ovulatory follicle, because we could follow its daily growth. In the human ovary, the pre-ovulatory/ovulatory follicle is also easily identified at laparoscopy because of its large size and typical appearance.

In the present study, we demonstrated a marked increase of TIMP-1 mRNA in all ovulatory phases, as compared with that before hCG. The mRNA levels, which may not always fully correlate with the protein levels, showed a similar increase of TIMP-1 protein 12–18 h after hCG. By IHC, TIMP-1 protein was undoubtedly present in all ovulatory phases. This is the first study of TIMP-1 expression in the tissue of the ovulatory human follicle, and the demonstrated TIMP-1 increase and its distribution clearly indicate its crucial role in ECM remodelling during ovulation. The first study on the possible regulation of TIMP activity during ovulation showed an increase of unspecified MMP inhibitory activity throughout the periovulatory period in the rat (Curry et al., 1986). Studies in several animal models have also shown an increase of TIMP-1 mRNA. Thus, TIMP-1 mRNA was maximally increased at late pro-estrus in the natural estrous cycle of the mouse (Inderdeo et al., 1996). Moreover, an increase of TIMP-1 mRNA was seen after hCG in the rat (Chun et al., 1992; Curry et al., 2000), mouse (Hagglund et al., 1999) and rhesus monkey (Chaffin and Stouffer, 1999) as well as after the mid-cycle gonadotropin surge in the sheep (Smith et al., 1994). Previous studies on TIMP-1 in the human ovary have been exclusively on cells and follicular fluids obtained from IVF cycles with the apparent disadvantages of using these cells and fluids of hyperstimulated luteinized follicles to study natural ovulation (Shalev et al., 2001; D’Ascenzo et al., 2004).

In the present study, TIMP-1 was apparently localized to the human ovarian stroma of all ovulatory phases, but in a patchy pattern.
Figure 5. Immunohistochemical localization of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in ovarian tissues. (A) Perifollicular stroma of early ovulatory phase with staining concentrated at specific sites. Scale bar represents 100 μm. Inset shows immunostained area at higher magnification (scale bar represents 50 μm). (B) Section of whole pre-ovulatory follicle with no staining in theca interna (TI), theca externa (TE) or in granulosa cells (GC). Basal lamina (BL) is indicated by arrow. Scale bar represents 100 μm.
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Table 3. Expression of MMP-2 protein in different ovulatory phases.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Median (25-75%)</th>
<th>IQR (10-90%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO</td>
<td>1.20</td>
<td>1.00-1.40</td>
</tr>
<tr>
<td>EO</td>
<td>1.30</td>
<td>1.10-1.50</td>
</tr>
<tr>
<td>LO</td>
<td>1.40</td>
<td>1.20-1.60</td>
</tr>
<tr>
<td>PSO</td>
<td>1.50</td>
<td>1.30-1.70</td>
</tr>
</tbody>
</table>

Although there were no significant variations of TIMP-2 protein expression in our study, the median level of TIMP-2 increased during the early ovulatory phase as compared with the pre-ovulatory and late ovulatory phases. The only other study in a primate species examining TIMP-2 mRNA in ovarian tissue found increased levels after hCG in the rhesus monkey (Chaffin and Stouffer, 1999), and we cannot rule out that there may be a similar modest up-regulation in the human.

TIMP-1 and TIMP-2 are capable of inhibiting the activities of most MMPs and as such play key roles in maintaining the balance between (ECM) deposition and degradation (Gomez et al., 1997). Changes in the balance between MMPs and TIMPs can have profound effects on the composition of the ECM as seen in cervical ripening (Stygard et al., 2002), ovarian cancer (Davidson et al., 2001) and endometriosis (Osteen et al., 2003). One can speculate that ECM homeostasis during ovulation also depends on this type of co-ordinated balance between the deposition and removal of ECM components.

The results of the present study demonstrated an abundance of MMP-2 protein and presence of MMP-9 protein in human ovarian tissue. IHC showed that MMP-2 was mainly localized in a concentric layer of the ovarian stroma.

Previously, collagenolytic activity against collagen type IV was demonstrated in human follicular fluid from the natural cycle (Puistola et al., 1986) and in human luteinized granulosa cells of IVF cycles (Puistola et al., 1995). This is now recognized as being due to the activity of MMP-2 and MMP-9. A recent study found that the concentration of MMP-2 and MMP-9 protein in human follicular fluid of natural and IVF cycles was similar during the pre-ovulatory stage of natural cycle and IVF cycles (D’Ascenzo et al., 2004). On the contrary, studies in the rat have demonstrated that the highest expressions of these tissue-remodelling enzymes were in the theca and the surrounding stroma (Reich et al., 1991; Curry et al., 2001). Thus, the perifollicular stroma would be the relevant cell layer to examine for MMP expression.
Figure 7. Immunohistochemical localization of matrix metalloproteinase-2 (MMP-2) in ovarian tissues. (A) Perifollicular stroma of pre-ovulatory phase with clear staining in layers of the stroma. Ovarian surface is located upwards in the photograph. Scale bar represents 100 μm. (B) Magnification of indicated area in A. Scale bar represents 10 μm.
Although we are not able to detect any significant changes in MMP-2 and MMP-9 protein during the ovulatory process, we observed that MMP-2 was expressed at high quantities. The median levels of MMP-2 and MMP-9 were higher, although not significant, during early ovulatory phase, indicating a small up-regulation during ovulation. This is in line with results from a study in the eCG–hCG-primed rat showing a 4-fold increase in unspecified gelatinase activity (activity against collagen type IV) during the periovulatory period (Curry et al., 1992). Moreover, in situ hybridization studies of rat ovaries showed MMP-2 mRNA in the granulosa cells after hCG stimulus; MMP-9 mRNA, on the contrary, was mainly detected in the stroma (Curry et al., 2001). However, a study in the mouse, also by in situ hybridization, indicated that MMP-2 mRNA seemed to be up-regulated in the theca–interstitial cells adjoining the large pre-ovulatory follicles just before ovulation (Liu et al., 1998). In the macaque ovary, MMP-2 mRNA increased 5-fold in granulosa cells within 12 h of hCG, and a tendency to an increase throughout the periovulatory interval was shown (Chaffin and Stouffer, 1999). In the latter study, MMP-9 mRNA did not increase until 36 h after the ovulatory bolus, and a tendency to a decline between 0 and 24 h after hCG was seen.

In the present study, which is exclusively on well-defined human material of the ovulatory cycle, some differences concerning the expression of MMP-2 and MMP-9 compared with previous findings in animals are found as described above. There are several possible explanations for these differences. In human material, often with a restricted number of patients compared with inbred laboratory animals, a greater interindividual difference is expected, despite the very well-defined material we used. Thus, smaller changes in this human material may not reach the significance level. In most animal studies, mRNA levels were examined, and it is possible that the levels measured do not adequately reflect the level of the active MMP protein because there is a balance between pro-MMPs and active forms and that post-translational changes may occur. Furthermore, the size of the ovaries used in the animal studies only allows comparisons between levels in the granulosa cells and in the residual ovaries, containing a mixture of theca cells, interstitial cells, vascular and granulosa cells of pre-antral and early antral follicles.

The present study is the first and only study with timed human ovulatory specimens. We have, in more detail, investigated the protein expression of MMPs and TIMPs in the stroma around the human follicle of ovulating women. Altogether, our data show the presence of MMP-2, MMP-9 and TIMP-2 in the human perifollicular stroma, and an increase of TIMP-1 expression after hCG which may be related to a restricted time-specific inhibition of collagenolysis and thereby of collagen breakdown in areas surrounding the apex of the follicle to allow for site-directed collagen breakdown.

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TIMP-1 in human ovulation

References


Figure 9. Immunohistochemical localization of matrix metalloproteinase-9 (MMP-9) in the perifollicular stroma. Section of stroma, at the apex of a pre-ovulatory follicle, with diffuse immunostaining in tunica albuginea (TA) and underlying stroma (S). Ovarian surface epithelium (OSE) is indicated by arrow. Scale bar represents 50 μm.


