Suppression of matrix metalloproteinase production by hCG in cultures of human luteinized granulosa cells as a model for gonadotrophin-induced luteal rescue

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Granulosa cells were isolated from follicular aspirates collected at ovum recovery for *in vitro* fertilization. Cells were cultured in a defined medium on artificial extracellular matrix (Matrigel) in the presence or absence of hCG as a model for corpus luteum function. Release of cells from this culture system is reduced by hCG and this effect may be mediated through an inhibition of extracellular matrix degradation. Using zymography and western blot analysis, we confirm the identity of matrix metalloproteinases-2 and -9 in culture media. Matrix metalloproteinase-9 was the predominant gelatinase in freshly prepared granulosa cells and in culture media, and also represented a major metalloproteinase component in homogenates of early and mid-luteal phase samples of corpora lutea. Quantitative analysis of matrix metalloproteinases in culture media, obtained throughout the 14 day culture period and expressed per μg of DNA, showed that matrix metalloproteinase-2, undetectable on day 2, rose throughout the culture period and that this rise was significantly inhibited by hCG. In contrast, matrix metalloproteinase-9 was clearly detectable on day 2 and remained relatively constant throughout much of the culture (day 2 to day 12) in the presence of gonadotrophin. Significantly increased production of matrix metalloproteinase-9 (day 6 to day 12) was evident in the absence of hCG. Our results provide further evidence for the hypothesis that the rescue of the corpus luteum in early pregnancy involves the maintenance of cellular function through the stabilization of the extracellular matrix.

Introduction

The formation and regression of the corpus luteum involve intense cellular differentiation and tissue remodelling. Regulated changes in extracellular matrix (ECM) may be important in these events (reviewed by Luck, 1994). During early pregnancy in women, embryo-derived hCG rescues the corpus luteum and prolongs progesterone production, whereas in a nonconception cycle the corpus luteum regresses. It has been proposed that, in the rat corpus luteum, regression may be associated with ECM instability resulting from the action of matrix metalloproteinases (MMPs; Endo *et al.*, 1993). This would be consistent with the idea proposed by Aston *et al.* (1996a) that luteal rescue in women may involve maintenance of ECM stability through the suppression of MMP action by hCG. Matrix metalloproteinases are zinc proteases with a range of specificities directed towards various components of the extracellular matrix (Birkedal-Hansen *et al.*, 1993). They are inhibited by tissue inhibitors of metalloproteinase (TIMP)-1, -2 and -3, which bind to metalloproteinases noncovalently blocking their action (Nagase, 1994). The MMPs present in rat

corpora lutea (Endo *et al.*, 1993) and 4-day-old bovine corpora lutea (Tsang *et al.*, 1995) appear to be MMP-2 and MMP-9, with MMP-2 as the predominant form. Studies on human luteinized granulosa cells (Puistola *et al.*, 1995; Aston *et al.*, 1996a) have also demonstrated the presence of MMP-2 and MMP-9, although there is disagreement as to which MMP predominates. The discrepancy between these studies may derive from the use of different culture conditions. Whether MMP production can be influenced by gonadotrophins remains an open question. Tsang *et al.* (1995) have shown that MMP production was not regulated by LH in cows. However, in cultures of luteinized human granulosa cells, Aston *et al.* (1996a) showed that overall gelatinase activity per cell was suppressed by hCG, an effect that may have been partially mediated by higher concentrations of TIMP in gonadotrophin-stimulated cultures. The possibility that hCG may suppress MMP activity in the human corpus luteum *in vivo* has also been suggested in a preliminary report by Duncan *et al.* (1995).

The present study extends earlier work (Aston *et al.*, 1996a) by positively identifying MMP-2 and MMP-9 in culture media of human granulosa cells by western blotting. The predominance of MMP-9 in fresh and cultured cells is confirmed and shown to be consistent with analysis of MMPs present in

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samples of human corpora lutea. Using a culture system that appears to show aspects of luteal rescue *in vitro*, possibly through stabilization of ECM (Aston *et al.*, 1996a), we show that hCG appears to downregulate the production of MMP-9 and MMP-2. This work provides further evidence that rescue of the corpus luteum of early pregnancy involves hCG-induced suppression of ECM degradation.

Materials and Methods

Patients

Tissue was collected according to protocols approved by our local Ethical Committee and with the informed consent of the patients. Follicular aspirates and washes were obtained at ovum collection for *in vitro* fertilization. The patient protocol (described by Jenkins *et al.*, 1991) involved pituitary down-regulation with gonadotrophin releasing hormone analogue (buserelin; 200 µg intranasally three times a day), followed by ovarian stimulation with human menopausal gonadotrophin (4 ampoules per day) and follicular maturation with hCG (10 000 iu). Follicles aspirated were > 15 mm in diameter and the concentration of circulating oestradiol was consistent with the number and sizes of follicles present.

Samples of corpora lutea were obtained from ovaries previously removed at hysterectomy performed for reasons unconnected with the study. Patients were not on medication. An approximate dating of luteal age was obtained from menstrual history.

Cell preparation and culture

Granulosa cells were prepared from combined follicular aspirates and washes according to the method described by Richardson *et al.* (1992). Each experiment refers to work carried out with cells from an individual patient. The medium used for preparation and culture was a mixture (50:50) of Ham's F12 and Dulbecco's modified Eagle's medium (Imperial Laboratories, Andover) supplemented with glutamine (2 mmol l⁻¹), penicillin (50 mg l⁻¹), streptomycin (60 mg l⁻¹), amphotericin (2.5 mg l⁻¹) and ITS-plus (providing insulin 6.25 mg l⁻¹, transferrin 6.25 mg l⁻¹, selenious acid 6.25 µg l⁻¹, BSA 1.25 g l⁻¹ and linoleic acid 5.35 mg l⁻¹; Universal Biologicals, London). Twenty-four-well culture plates were coated with a thin layer of Matrigel (Strattech, Luton) which provides a model for basement membrane. The Matrigel preparation was diluted 1:1 with medium; 50 µl was dispensed and spread over the surface of each well; the plate was tilted and the excess removed. The cells were plated out at approximately 2–5 × 10⁵ cells ml⁻¹ (incubate volume 1 ml) and cultured at 37°C in 5% CO₂ in air. The amount of hCG used in culture was chosen to be just maximal for progesterone production (Richardson *et al.*, 1992). The medium was changed every 48 h and the cells were cultured for a total of 14 days. At each medium change, adherent cells were assessed by DNA assay. Duplicate wells for each treatment were scraped into assay buffer, sonicated and assayed for DNA content by the method described by Labarca and Paigen (1980) using calf thymus DNA as standard.

Corpus luteum homogenization

Samples of corpora lutea were placed in PBS and stored in liquid nitrogen. The tissue was thawed and homogenized using an Ultra-turrax T8 homogenizer (BDH, Poole) in homogenization buffer (approximately 10 mg tissue ml⁻¹; 0.25 mol l⁻¹ sucrose, 25 mmol l⁻¹ Tris-HCl, pH 7.4). The protein content of the samples was assayed using a protein assay kit (Bio-Rad, Hemel Hempstead) based on the method described by Bradford (1976).

Zymography

Granulosa cell cultured conditioned medium collected every 48 h was assessed by zymography for gelatinase activity. SDS-PAGE was used with the addition of 0.8 mg gelatin ml⁻¹ (Herron *et al.*, 1986) to a 7.5% resolving gel. Samples of culture media (15 µl) were electrophoresed directly, while homogenates of corpora lutea and freshly prepared granulosa cells were diluted in homogenization buffer (0.25 mol sucrose l⁻¹, 25 mmol Tris-HCl l⁻¹, pH 7.4) to a final concentration of 10 µg protein per 15 µl per lane. Positive controls included pure MMP-2 (Biogenesis, Poole) and MMP-9 (kindly donated by G. Murphy, Strangeways Laboratory, Cambridge). The samples were electrophoresed at 40 mV for the first 10 min and then at 80 mV for a further 90 min in buffer (25 mmol Tris l⁻¹, 0.4 mol glycine l⁻¹ and 1% (w/v) SDS). Subsequently, the gels were washed in 2.5% (v/v) Triton-X100 for 30 min and incubated overnight in buffer (50 mmol Tris-HCl l⁻¹, 5 mmol CaCl₂ l⁻¹, pH 8.0). Addition of zinc chelators such as 5 mmol EDTA l⁻¹ or 2 mmol 1,10 phenanthroline l⁻¹ to the incubation buffer abolished gelatinase activity; this served as a negative control. Gelatinase activity was visualized as clear bands against a blue background, after staining in Coomassie blue and destaining in 30% methanol:10% acetic acid in water.

Densitometric analysis

Bands of gelatinase activity were analysed for intensity and surface area by scanning densitometry on a Sharp JX-330P transmissive scanner using the Intelligent Quantifier™ software package (BioImage[®], Crewe). An intensity index was calculated for each band, expressed per µg DNA, converted into a percentage of day 14 control (for MMP-2) and day 2 control (for MMP-9) and plotted against days in culture (Fig. 4).

Western blotting

Before western blot analysis for MMP-2, media samples were purified as follows. Fifty millilitres post-day 2 control culture medium (that is, from cultures not treated with hCG) was dialysed against 20 mmol sodium phosphate l⁻¹ (pH 7.0) in the presence of 10 µl sheep anti-human MMP-2 (kindly donated by G. Murphy). The dialysed material was then applied to a HiTrap Protein-G column (Pharmacia, St Albans) which removed all MMP-2 as assessed by zymography. MMP-2 was subsequently eluted with 1% (w/v) SDS in buffer. The sample was then concentrated a further tenfold using an Ultrafree-MC filter unit (Sigma, Poole). Culture media analysed for MMP-9 were used directly.

Fig. 1. Zymography of gelatinases present in culture medium from cultures of luteinized granulosa cells compared with reference preparations of MMP-2 and MMP-9 (central panel); MMP-2/MMP-9, reference preparations; d6C, medium from day 6 control cultures maintained without gonadotrophin. Western analysis for MMP-2 after purification from culture media (left-hand panel) and western analysis for MMP-9 (right-hand panel). MWM, positions of molecular markers (142, 97, 50 and 35 kDa).

The samples were electrophoresed on 7.5% SDS-polyacrylamide gels as described above but with the omission of gelatin. The proteins were then transferred on to nitrocellulose membranes at 100 mV for 1 h. The membranes were incubated in BSA (1 mg ml^{-1}) for 1 h to prevent nonspecific binding before overnight incubation at 4°C with the primary antibody of interest (sheep anti-human MMP-2, kindly donated by G. Murphy, and mouse anti-human MMP-9, Oncogene Science, Cambridge). Subsequent incubations with alkaline phosphatase conjugated secondary antibodies and nitroblue tetrazolium (both from Sigma) revealed red/blue bands. All incubations and dilutions were performed in Tris-buffered saline ($50 \text{ mmol Tris l}^{-1}$, $150 \text{ mmol NaCl l}^{-1}$, pH 7.5) supplemented with 0.1% (v/v) Tween 20.

Statistical analysis

The data were subjected to nonparametric analysis of variance of repeated measures using day of culture and $\pm \text{hCG}$ as variables. ANOVA demonstrated significant effects of hCG throughout the culture period. Significance was calculated using the Wilcoxon test for paired nonparametric data.

Results

Two gelatinases were apparent in granulosa cell-conditioned medium (day 6 control) with molecular masses consistent with MMP-9 (92 kDa) and MMP-2 (72 kDa) (Fig. 1). These two MMP species co-electrophoresed with their respective standards. After purification and concentration of the minority MMP-2 component, its identity was confirmed by western analysis using a specific anti-MMP-2 antibody. Similar methodology confirmed the identity of the predominant MMP-9 band without prior concentration.

Fig. 2. Zymography of homogenates of (a) freshly prepared granulosa cells and (b) samples of corpora lutea designated as early (E) or mid-(M) luteal phase. All lanes were loaded with $10 \mu\text{g}$ cellular protein. Positions of molecular markers are noted (left) as well as presumed identities of MMP-2 and MMP-9 (right).

The pattern of gelatinases present in homogenates of freshly prepared granulosa cells from five separate patients is shown (Fig. 2a). All preparations showed the predominant MMP-9 band (with some processing to the lower molecular mass 82 kDa active species) and an absence of MMP-2. A higher molecular mass gelatinase (approximately 130–150 kDa) remained unidentified. Zymography of homogenates from samples of five separate corpora lutea (three early and two mid-luteal phase), loaded and run under identical conditions to those used for granulosa cells, again showed strong MMP-9 bands (particularly in early luteal samples), with narrower MMP-2 bands showing evidence of processing to the lower molecular mass (67 kDa) activated form (Fig. 2b). A higher molecular mass form of gelatinase similar to that described for the granulosa cell homogenates above was also recognized but remained unidentified.

The relationships between MMP concentration and intensity index obtained after densitometric analysis of zymograms obtained after electrophoresis of standard, reference preparations of MMP-2 and MMP-9 are shown (Fig. 3). The two relationships (intensity index against $-\log[\text{MMP-2}]$ and $-\log[\text{MMP-9}]$) were sufficiently linear to allow the intensity index to be used as a quantitative measure of enzyme activity in samples of culture media. The greater activity of the reference MMP-2 preparation (in terms of intensity index

Fig. 3. Relationship of intensity index (as measured by image analysis of bands obtained on zymography) to concentration of (a) MMP-2 and (b) MMP-9, expressed as $\mu\text{g ml}^{-1}$ and converted to $-\log[\text{MMP}]$.

μg^{-1} protein) is only partially explained by its lower molecular mass compared with MMP-9.

Changes in the activity of MMP-2 and MMP-9, as measured by the intensity index of electrophoretic bands, were examined in culture media obtained throughout 14-day experiments in which cells were cultured with and without a maximal concentration of hCG (100 ng ml^{-1}). Combined data from six separate preparations are illustrated (Fig. 4). MMP-2 was not detectable at the beginning of the culture period (Fig. 4a). Becoming detectable by day 4, MMP-2 production then rose progressively throughout the culture period. This rise was suppressed by gonadotrophin so that by days 12–14, MMP-2 output was clearly and significantly lower in hCG-treated cells. It should be noted that MMP-2 remained a minority component of overall gelatinase activity throughout. Thus, by the end

Fig. 4. Comparative amounts of (a) MMP-2 and (b) MMP-9 in culture media obtained from cultures of luteinized granulosa cells maintained in the presence (●) or absence (○) of hCG (100 ng ml^{-1}). Values were calculated from the intensity index of electrophoretic bands corrected for μg cellular DNA estimated at each time point in the presence and absence of hCG. Results were expressed as percentage of control values on day 14 (for MMP-2) or day 2 (for MMP-9) and means were subsequently plotted from data derived from six separate preparations, with SEM. Absolute values in intensity index units per μg DNA were day 14 control MMP-2, 1015 ± 698 , and day 2 control MMP-9, 1449 ± 638 . * $P < 0.05$.

of the culture period, MMP-2 still represented only 20–30% of total gelatinase activity (MMP-2 plus MMP-9). Changes in MMP-9 production showed a different pattern (Fig. 4b). Substantial MMP-9 output was evident from the beginning of the culture, and this was maintained in the presence of hCG for much of the culture period (particularly days 4–12). In the absence of gonadotrophin, MMP-9 output was more variable and significantly increased (days 6–12).

Discussion

The finding that the predominant form of MMP in luteinized granulosa-cell conditioned medium is MMP-9 confirms an earlier study (Aston *et al.*, 1996a). Western blotting now provides positive identification of both the majority MMP-9 component and the minority MMP-2 enzyme, which needed purification and concentration before analysis. The observation that freshly prepared granulosa cells, when homogenized and subjected to zymography, showed no evidence of MMP-2 is consistent with the lack of this enzyme at the beginning of the culture period and confirms that the predominance of MMP-9 is not simply due to the specific culture conditions used. Our observations are further substantiated by strong bands of MMP-9 activity apparent on zymography of homogenates of corpus luteum, particularly those obtained in the early luteal phase.

The potential physiological importance of MMP-9 in corpus luteum function has not been previously recognized to our knowledge. Thus, although both MMP-2 and MMP-9 have been shown to be present in rat and bovine corpora lutea (Endo *et al.*, 1993; Tsang *et al.*, 1995), it is MMP-2 that predominates in both these species. Furthermore, a previous study on luteinized granulosa cells from women (Puistola *et al.*, 1995), emphasized the presence of MMP-2 which appeared to accumulate preferentially after culture in serum-containing media. It is possible that the greater contribution of MMP-2 evident on zymography of corpus luteum as compared with granulosa cells, may be due to the presence of thecal cells.

Having established the identities of the gelatinases produced by luteinized granulosa cells in culture and how these related to those found in the corpus luteum, the main purpose of this study was to investigate regulation of MMPs by gonadotrophin. We have previously suggested that the culture system used, whereby human granulosa cells are maintained on ECM in serum-free medium, shows features that suggest that it may provide an *in vitro* model for corpus luteum function (Richardson *et al.*, 1992). In the presence of hCG, progesterone production is well maintained for long periods in culture (up to 14 days; Aston *et al.*, 1996b), and in the absence of gonadotrophin, cells eventually become detached from the culture surface, possibly through degradation of ECM (Aston *et al.*, 1996a). We have speculated that this rescue of cellular function *in vitro* by hCG acts as a model for luteal rescue by hCG *in vivo*. The pattern of MMP production in this system does appear to mimic the changes occurring in human corpus luteum during the luteal phase (preliminary report by Duncan *et al.*, 1995), particularly with regard to an increase in MMP-2 content towards the late luteal phase which can be suppressed by the administration of hCG *in vivo*. These *in vivo* studies also noted a predominance of MMP-9 in the early luteal phase and suppression of MMP-9 by hCG (information presented but not detailed in abstract of Duncan *et al.*, 1995).

The general principle that luteolysis may involve increased activity of gelatinases (particularly MMP-2 and MMP-9) is supported not only by work presented here, but also by the preliminary work of Duncan *et al.* (1995), and studies by Endo *et al.* (1993) on rat corpora lutea. The present study has concentrated on the production of MMPs irrespective of the presence of TIMP, which is removed during zymography.

Upregulation of TIMP by hCG in our model system (noted by Aston *et al.*, 1996a) represents another level of complexity. Increased gelatinase activity in luteal regression may result from a combination of effects, including both differential production of MMPs (described in the present report) and falling TIMP expression. Rescue of luteal function by hCG in early pregnancy would involve both a suppression of MMP production and increased expression of TIMP. We suggest that enhanced stability of the ECM, induced by hCG, prevents luteal regression and enables an extension of cell survival into early pregnancy.

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