Secretion of matrix metalloproteinases by human endometrial cells *in vitro*

M. Martelli¹, A. Campana² and P. Bischof^{1*}

¹Department of Obstetrics and Gynecology, University of Geneva; and ²Clinic of Infertility and Gynecologic Endocrinology, Geneva, Switzerland

At each menstrual cycle, the uterine endometrium undergoes intense remodelling. Of the many factors implicated in tissue remodelling, the matrix metalloproteinases (MMPs) play a central role owing to their capacity to degrade the extracellular matrix. The aim of this study was to examine the nature and cellular origin of endometrial proteases as evaluated in culture systems with clearly characterized cell types. Endometrial cells from hysterectomy specimens were prepared using collagenase digestion. Bone-marrow-derived cells (a known source of proteases) were removed by immunopurification. Cells were cultured on different substrates (matrigel, agarose, glass or plastic). Purity of cell preparations was examined by immunocytochemistry, and proteases were characterized by zymography on SDS-PAGE containing gelatin. The cell phenotype in culture was largely influenced by the type of substrate. Gelatin-degrading enzymes detected in culture supernatants of stromal and epithelial cells had molecular masses ranging from 42 to 248 kDa, and were identified as metalloproteinases. We conclude that human endometrial stromal and epithelial cells express several matrix metalloproteinases, the expression of which clearly depended on the purity of cell preparation, on cell adhesion and on the nature of the substrate on which the cells grew. These enzymes might be involved in endometrium remodelling, blastocyst implantation and trophoblast invasion.

Introduction

The uterine endometrium, a well-known target for steroid hormones, is regularly shed and rebuilt throughout reproductive life, implying an active and continuous tissue remodelling. Uterine endometrial cells therefore provide an effective model for studying the role of enzymes implicated in extracellular matrix (ECM) degradation. A wide variety of factors participate in tissue remodelling including growth factors, cytokines and matrix metalloproteinases (MMPs or matrixins). MMPs are a group of highly homologous proteolytic enzymes believed to play a major role in the remodelling of connective tissues under various physiological and pathological conditions (Masure and Opotenakke, 1989; Woessner 1991). These enzymes participate in the breakdown of the major protein components of the extracellular matrix such as collagen, proteoglycans, fibronectin and laminin (Emonard and Grimaud, 1990; Van Wart and Mookhtiar, 1990). These enzymes depend on Ca^{2+} and Mg^{2+} and are optimally active at neutral pH (Birkedal-Hansen, 1988).

Seven MMPs have been identified in mammalian cells (Woessner, 1991) and are grouped in three different classes, depending on their structural homology and substrate specificity, interstitial collagenases, gelatinases and stromelysins. The fibroblast-type interstitial collagenase (MMP-1) (Stricklin *et al.*, 1977; Goldberg *et al.*, 1986) and the neutrophil-type homologue (MMP-8) (Hasty *et al.*, 1990; Mallya *et al.*, 1990; Mookhtiar and Van Wart, 1990) can degrade collagen type I, II

and III. They are secreted as proenzymes (52 kDa) and are activated by trypsin, plasmin, kallikrein and chymotrypsin *in vitro*. The 72 kDa gelatinase (MMP-2) produced by proliferating fibroblasts (Sato *et al.*, 1985; Hibbs *et al.*, 1987; Wilhelm *et al.*, 1989) and by tumour cells (Sato *et al.*, 1983; Collier *et al.*, 1988), and the 92 kDa gelatinase (MMP-9) produced by neutrophils (Hibbs and Bainton, 1989), macrophages (Hibbs *et al.*, 1987) and by certain transformed cells (Wilhelm *et al.*, 1989; Bernhard *et al.*, 1990) have an identical substrate specificity degrading native collagen type IV, V and X (Wilhelm *et al.*, 1989; Okada *et al.*, 1990). These gelatinases contain a fibronectin-like domain which allows them to bind to gelatin (denatured type I collagen). The 72 kDa gelatinase is activated by organomercurial salts *in vitro* and not by plasmin or trypsin. Human fibroblasts also produce stromelysins (Okada *et al.*, 1986; Whitham *et al.*, 1986; Wilhelm *et al.*, 1987; Saus *et al.*, 1988), a group of three homologous enzymes (MMP-3, MMP-10 and MMP-7), that are also called proteoglycanases. These degrade type IV and IX collagens, laminin, fibronectin, elastin and proteoglycans (Chin *et al.*, 1985; Okada *et al.*, 1986, 1989). All MMPs are secreted as inactive proenzymes that are activated by limited proteolysis (He *et al.*, 1989; Okada and Nikanishi, 1989) by other proteases, losing a 10 kDa peptide upon activation. In the active form, MMPs are regulated by inhibitors present in plasma and in the extracellular matrix. MMPs can be inhibited by α_2 -macroglobulin (Barret and Starkey, 1973) and α_1 -antitrypsin present in plasma and by tissue inhibitor of metalloproteinases (TIMP) located in the extracellular space (Cawston *et al.*, 1981). The purification and isolation of TIMP has previously been described (Finn, 1986; Goldberg *et al.*, 1989).

*Reprint requests.

Received 6 April 1992.

To our knowledge no MMPs have previously been described in the human endometrium. The present study reports the presence, nature and cellular origin of MMPs in human endometrial cell cultures. Cultures were prepared from hysterectomy specimens. In addition, epithelial and stromal cells were cultured separately on different substrates to investigate the role of cell adhesion on the induction of protease secretion.

Materials and Methods

Preparation of endometrial cells

Endometrial tissue was obtained from hysterectomies performed for reasons other than malignancy. The phase of the endometrial cycle was determined on histological sections of the same endometrium. The nine endometria analysed were of the proliferative phase. Furthermore, in three cases, stromal and epithelial cells were obtained from early secretory endometrium bearing a Gravigard intrauterine device (IUD).

All manipulations were performed under sterile conditions. The cell preparation and separation techniques used were adapted from Tseng and Liu (1981) and were based on the fact that collagenase disrupts the stromal cells, leaving the endometrial glands intact. The tissue was minced and washed in Hank's balanced salt solution (HBSS, Sigma, St Louis), containing 200 U penicillin G ml⁻¹ (10⁶ iu), 200 µg streptomycin ml⁻¹ (Hoechst, Darmstadt) and 2.5 µg Fungizone ml⁻¹ (Gibco, Basel). The washed tissue was then incubated three times for 20 min without shaking in a water-bath at 37°C, in complete medium: RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS; Amimed, Birsfelden), penicillin G (10⁶ iu), 200 µg streptomycin ml⁻¹, 2.5 µg Fungizone ml⁻¹, to which 1 mg collagenase ml⁻¹ (0.287 U ml⁻¹ from *Clostridium histolyticum*; Boehringer, Mannheim GmbH, Germany) was added. After each step, the collagenase digestion was stopped by the addition of complete medium to the supernatant.

The supernatants consisting of stromal cells (the endometrial glands sedimented rapidly) were pooled, centrifuged at 700 g for 5 min, and the cells resuspended in culture medium consisting of complete medium in which the 10% FCS was heat inactivated (56°C for 30 min) and acid treated adjusted to pH 3 with 1 mol HCl l⁻¹, for 2 h at room temperature and neutralized with 1 mol NaOH l⁻¹. Cells were counted in a Neubauer cell in the presence of Trypan blue (0.5% Trypan blue, Serva, Feinbiochemica, Heidelberg, in normal saline 0.9% NaCl): half of the cells were placed in culture immediately and the other half were further immunopurified.

The remaining sedimented glands were incubated with shaking at 37°C for 2 h, in complete medium with 300 mg collagenase ml⁻¹ (330 U ml⁻¹ from *Clostridium histolyticum*, Sigma, St Louis). After addition of complete medium, the tubes were centrifuged and the supernatant discarded. The pellet consisting of endometrial glands was cultured for 5–7 days at 37°C in complete medium. Once a monolayer of epithelial cells was obtained, the culture was trypsinized (trypsin (1:250) 0.05%, EDTA 0.02%, Boehringer Mannheim GmbH, Germany) washed with complete medium and the cells counted in the presence of Trypan blue. Half of the cells were put into culture and the other half was immunopurified.

Immunopurification of endometrial cells

Cell suspensions were incubated with 50 µl of a mouse monoclonal antibody against human leucocyte common antigen (anti-LCA, anti-CD45, Dakopatts, Copenhagen) for 30 min at 4°C and shaken occasionally. After incubation, cells were washed with PBS containing: 137 mmol NaCl l⁻¹, 3 mmol KCl l⁻¹, 8 mmol Na₂HPO₄ l⁻¹, 1.5 mmol KH₂PO₄ l⁻¹ at pH 7.4 supplemented with 0.1% BSA (RIA grade, Sigma, St Louis). Prewashed (PBS-BSA 0.1%), magnetic particles (50 µl) coated with a second antibody, sheep anti-mouse IgG (Dynabeads M 280, Dynal, Milan Analytica, Switzerland), were then added to the cell suspension and incubated for 20 min at 4°C. A magnet was applied along the test tube to retain the particles bound to the cells expressing LCA. The supernatant was poured into a clean tube and the cells were washed with complete medium and counted.

Cell culture

One million epithelial or stromal cells per ml were routinely incubated in culture medium in a 12-well tissue culture plate (Costar, Cambridge, USA) and kept at 37°C under a 5% CO₂ atmosphere in air. Wells were either uncoated or precoated with basement membrane-like material (matrigel 300 µl per well: Collaborative Research, Inotech, Switzerland), agarose (300 µl per well: 0.5% in PBS, Agar Noble, Difco, Detroit) or glass (cover slips, Gribi AG, Bern). Medium was replaced every two days, centrifuged at 700 g and the unattached cells (if any) returned to their original well. Aliquots of the supernatants were immediately frozen at -20°C.

Zymography

Zymography was performed according to Fisher *et al.* (1989) with some modifications. Gelatin (Merck, Darmstadt) at a final concentration of 1 mg ml⁻¹ was incorporated into the running gel containing 10% acrylamide (Fluka AG, Buchs, Switzerland), 25% 1.5 mol Tris(hydroxymethyl)-aminomethane l⁻¹ (Tris buffer, Merck, Darmstadt) pH 8.8, 0.4% SDS (Merck), 0.3% ammonium peroxodisulfate (APS; Merck). A stacking gel was layered containing 2% acrylamide, 0.05% *N,N'*-methylene-bis-acrylamide, 25% Tris buffer (Tris 0.5 mol l⁻¹, pH 6.5, 0.4% SDS), 0.4% APS and 0.1% *N,N,N,N*-tetramethylethylenediamine (TEMED).

The dimensions of the gel were 80 × 85 × 0.8 mm. Sample buffer (5 µl) consisting of 17.4% SDS, 7% sucrose (Fluka AG, Buchs) and 10 µl bromophenol in water (Siegfried, Zofingen) was added to 30 µl of the standardized (10⁶ cells ml⁻¹) cell supernatants to be analysed and 25 µl of this mixture applied to the gel. Electrophoresis ran for approximately 4 h at 7 mAmp at 5–7°C in 25 mmol Tris-HCl l⁻¹ containing 0.19 mol glycine l⁻¹ and 0.1% SDS, pH 8.6. After electrophoresis, the gel was washed six times for 5 min at room temperature on a moving platform in 2.5% Triton X-100 in water (Sigma), and three times in PBS. The gel was then incubated overnight at room temperature in PBS, pH 7.4, containing CaCl₂ (0.9 mmol l⁻¹) and MgCl₂ (0.5 mmol l⁻¹), or EDTA (1, 5, 10 or 20 mmol l⁻¹; Fluka AG), 1,10-phenanthroline (0.2 or 2 mmol l⁻¹), 1 mmol iodoacetamide

Table 1. Immunocytochemistry of human endometrial cells in culture

Antibody used	Cell detected	Supernatant		Pellet ^a	
		Before purification ^b (%)	After purification (%)	Before purification (%)	After purification (%)
Keratin	Epithelial	2.6 ± 2.1	2.5 ± 0.7	70 ± 18.3	76.7 ± 11.2
LCA	Bone-marrow-derived cells	20.4 ± 5.2	0.2 ± 0.4	2.5 ± 2.1	0.5 ± 0.7
Factor VIII	Endothelial	1.4 ± 0.9	0.8 ± 0.8	2 ± 0	0.5 ± 0.5
Vimentin	Stromal bone-marrow-derived cells endothelial	93.6 ± 10.1	98 ± 2.64	12.5 ± 8.5	3.5 ± 0.7

Values given are means ± SD, *n* = 9 from proliferative phase and *n* = 3 secretory phase bearing a Gravigard intrauterine device.

^aImmunohistochemistry performed after trypsinization of endometrial monolayers obtained from intact glands grown *in vitro* for 5–6 days.

^bStromal or epithelial cell purification with Dynabeads and LCA monoclonal antibody.

LCA: Leucocyte Common Antigen (CD45).

1^{-1} or $1 \mu\text{mol}$ pepstatine 1^{-1} (all from Sigma). After incubation, the gels were stained with Coomassie brilliant blue G 250 (Fluka AG, 0.1% in 25% methanol and 10% acetic acid in water) for 1 h, and destained in 5% methanol with 7.5% acetic acid in water. Proteolytic activity appeared as clear bands on a blue background. An electrophoresis calibration kit containing protein standards (Pharmacia, Uppsala) for molecular mass determination were run on each gel. The kit provided five protein standards covering the molecular mass range from 53 to 212 kDa. The distance of migration of the digestion bands was compared with that of the standards (log molecular mass versus distance of migration in mm) to determine their size.

Immunocytochemistry

Before culture, aliquots of the different cell suspensions were centrifuged onto microscope slides in a cytocentrifuge. The slides were fixed in acetone at -20°C for 5 min, then washed twice with PBS. The following manipulations were performed at room temperature. PBS-BSA, 5% (5 μl), was added on the cells, and after 10 min the liquid was gently blotted and replaced with 50 μl of the first antibody (monoclonal mouse anti-human: LCA, Factor VIII, Vimentin, all from Dakopatts, Copenhagen, anticytokeratine, clones AE1/AE3 from Boehringer, Mannheim, and anticytokeratin CAM 5.2 from Becton Dickinson, Inotech, Switzerland), these last two antibodies were used as a mixture (1:1). After 30 min, the glass slides were washed twice with PBS and the cells incubated with a second antibody bound to alkaline phosphatase (50 μl IgG rabbit anti-mouse, Dakopatts). After incubation and washing, the second antibody was replaced by a third antibody: swine anti-rabbit IgG coupled to alkaline phosphatase (50 μl , Dakopatts) and incubated as above. Slides were then washed and incubated for 5 min with 5 mg naphthol AS-BI phosphate (Sigma) in 50 μl *N,N* dimethyl-formamide containing 10 mg fast red TR salt (Sigma) and 10 mg of levamisolehydrochloride (Sigma) in 0.1 mol Tris-HCl 1^{-1} pH 8.2. After washing, the slides were counterstained with haematoxylin and evaluated under the microscope. The results were expressed in per cent as the number of positive cells per 300 total cells counted.

Results

Immunocytochemistry

The sedimentation technique used to separate stromal and epithelial cells from hysterectomy specimens was highly efficient (see Table 1). The supernatant contained between 80 and 100% stromal cells, whereas the pellet was rich in epithelial cells (77%). The major contaminant of the epithelial cell preparation was the stromal cells. After immunopurification the majority (more than 95%) of bone-barrow-derived cells were removed.

Phenotype of cells in culture

Stromal cells. Within 24 h of culture, proliferative endometrial stromal cells adhered to matrigel, glass and plastic, but not to agarose. The cells flattened out on the matrices, and grew into small colonies of aggregated cells. On glass and plastic, cells formed a monolayer (Fig. 1c, d, e and f), whereas on matrigel no monolayer was seen (Fig. 1a, c). On agarose, cells remained viable (negative with Trypan blue); however, they floated in the medium where they grew as aggregates (Fig. 1g, h). With time, these aggregates increased in size with no evidence of attachment. Irrespective of the substrate on which the stromal cells were grown, immunopurified cells grew in much larger colonies. This was particularly evident on matrigel where at day 9 the colonies of immunopurified cells were five times larger than the colonies of normal stromal cells (Fig. 1a, b).

Epithelial cells. After collagenase digestion, the glands remained intact. Glands from proliferative endometrium were shorter, thinner and less tortuous than those obtained during the secretory phase (Gravigard IUD bearing uteri). After 5 to 7 days, the glands were no longer recognizable and the epithelial cells formed a monolayer; these were then trypsinized and grown on different substrates.

Within 24 h of culture, epithelial cells adhered to matrigel (Fig. 2a, b) and formed a pattern consisting of epithelial cells

Fig. 1. Conventional microscopy of endometrial stromal cells obtained from a proliferative endometrium at 9 days of culture. Immunopurified (a, c, e, g) or non-immunopurified (b, d, f, h) cells were grown on matrigel (a, b); glass (c, d); plastic (e, f) and agarose (g, h). Scale bar = 100 μ m.

Fig. 2. Conventional microscopy of endometrial epithelial cells obtained from a proliferative endometrium at 9 days of culture. Immunopurified (a, c) or non-immunopurified (b, d) cells were grown on matrigel (a, b) and agarose (c, d). Scale bar = 100 μ m.

surrounding an empty space, resembling a glandular lumen. Immunopurified epithelial cells (Fig. 2a) grew into larger glandular-like structures than normal epithelial cells. After 9 days, the glandular-like structures formed by immunopurified cells were about twice the size of those formed by normal cells (Fig. 2a, b). On agarose (Fig. 2c, d), cells remained viable and floating in the medium during prolonged culture. Aggregates of cells were seen only if the epithelial cells had been immunopurified (Fig. 2a, c). Minimal aggregation occurred in non-purified epithelial cell cultures on agarose. The phenotypes of the cells obtained from proliferative and secretory phase (Gravigard IUD bearing) endometrium were similar. The viability of endometrial cells grown on different matrices was the same at all incubation times, as analysed with Trypan blue.

Protease release

Conditioned medium of proliferative endometrial cells was analysed by zymography on polyacrylamide–gelatin gel electrophoresis. After 48 h of culture, stromal cell supernatants expressed four to eight digestion bands with molecular masses ranging from 42 to 248 kDa (Fig. 3). Four gelatinase bands were present on zymograms of immunopurified stromal cell supernatants (Fig. 3a, b and c; Table 2), whereas six to eight bands were seen with non-immunopurified cell supernatants (Fig. 3d, e and f). Two of these bands (89 and 64–58 kDa) appeared to be more intense than the others which had molecular masses of 113, 105, 82 and 53 kDa. The number of gelatinases with molecular masses below 53 kDa (49, 46, 43 and 42 kDa) ranged from two to four (Fig. 3). In the supernatants from purified endometrial epithelial cells after 48 h (Fig. 4) only two gelatinases

were expressed (three gelatinases in non-immunopurified epithelial cell preparation), both of which had the same molecular mass as the most intense bands of the stromal cell supernatant (89 and 64 kDa). Culture medium that had not been exposed to cells was always negative during the zymography.

Effect of duration of culture. Gelatinases were analysed at day 2, 5 and 8 of culture. In stromal cell supernatants, the intensity of the gelatinases of higher molecular mass (248, 113, 105 and 89 kDa) reduced as duration of culture increases. The intensity of the 82 kDa gelatinase changed according to the substrate on which the cells were grown, as well as the incubation time, whereas the intensity of the 64–58 kDa gelatinase increased during incubation (Fig. 3a–c and d–f, respectively). For proteases of lower molecular mass (<53 kDa), the duration of incubation did not appear to affect their intensity. Except for a general decrease in intensity of all bands at day 5 of culture, no specific effect could be seen on the intensity of epithelial cell gelatinases (Fig. 4).

Effect of purification. The 248 and 82 kDa gelatinases of stromal cell preparations were clearly of lymphomyeloid origin since after immunopurification these bands could no longer be seen (Fig. 3a–c and d–f, respectively). When LCA-positive cells obtained from stromal cell preparation were cultured alone, they released only two gelatinases of 248 and 82 kDa, whereas cell-free medium incubated on the same substrates released no detectable proteases (results not shown).

Immunopurification reduced the intensity of the 113, 105 and 89 kDa bands indicating that were of stromal and lymphomyeloid origin. The 64–58 kDa gelatinase was always present

Fig. 3. Zymograms of immunopurified (a, b, c) and non-immunopurified (d, e, f) stromal cell supernatants from a proliferative endometrium grown on M: matrigel; A: agarose; G: glass; and P: plastic for 0 to 2 days (a, d); 2 to 5 days (b, e) and 5 to 8 days (c, f).

Table 2. Comparison of metalloproteinases in different cell types

Cell type	Molecular mass (kDa)								References
	248	197	113	105	89	82	64–58	53	
Embryonic cells	–	–	+	–	+	+	–	+	Adler <i>et al.</i> , 1990
Rabbit corneal fibroblasts	–	–	–	–	+	–	+	+	Fini and Girard, 1990
Alveolar macrophages	–	–	–	–	+	–	+	–	Shapiro <i>et al.</i> , 1990; Welgus <i>et al.</i> , 1990
Sertoli cells	–	–	+	–	–	+	–	–	Sang <i>et al.</i> , 1990
Stromal cells	+	–	+	+	+	+	+	(+)	Bischof <i>et al.</i> , 1990
Immunopurified stromal cells	–	–	+	+	+	–	+	(+)	Bischof <i>et al.</i> , 1990
Epithelial cells	–	–	–	–	+	+	+	–	Bischof <i>et al.</i> , 1990
Immunopurified epithelial cells	–	–	–	–	+	–	+	–	Bischof <i>et al.</i> , 1990
Cytotrophoblast cells	+	+	+	+	+	+	+	–	Bischof <i>et al.</i> , 1990; Fisher <i>et al.</i> , 1989
Immunopurified cytotrophoblast cells	–	+	–	–	+	+	+	–	Bischof <i>et al.</i> , 1990

Fig. 4. Zymograms of immunopurified (lanes 2, 4, 6, 8, 12) and non-immunopurified (lanes 1, 3, 5, 7, 9, 11) epithelial cell supernatants from early proliferative endometrium grown on matrigel (lanes 1, 2, 5, 6, 9, 10) and agarose (lanes 3, 4, 7, 8, 11, 12) for 0 to 2 days (lanes 1–4); 2 to 5 days (lanes 5–8) and 5 to 8 days (lanes 9–12). Std: standard.

with the same intensity irrespective of immunopurification, indicating an exclusively stromal origin of this protease (Fig. 3).

The 82 kDa gelatinase from epithelial cell supernatants was not seen after immunopurification, whereas the intensity of the 89 kDa band was reduced and the 64–58 kDa band remained unchanged (Fig. 4). These results indicate a lymphomyeloid origin for the 82 kDa gelatinase, a dual origin, bone-marrow and epithelial, for the 89 kDa gelatinase and an epithelial origin for the 64–58 kDa gelatinase.

Effect of substrates. The pattern of expression of gelatinases secreted in the supernatant of cells growing on different substrates varied with cell adhesion and the nature of the substrates. Stromal cells grown on agarose did not adhere to this substrate and the gelatinases secreted in the supernatant produced less intense digestion bands than those secreted by cells grown on other substrates (Fig. 3a–c, d–f). After 2–5 days no digestion band could be seen on the zymogram of the supernatant of cells cultured on agarose indicating a strict adhesion dependence for the expression of the proteases (Fig. 3a–c). Immunopurified stromal cells grown for more than two days on matrigel strongly expressed the 82 and 89 kDa gelatinases, whereas the same cells grown on plastic expressed only the 89 kDa gelatinase and on glass none of these two bands was seen (Fig. 3a–c). The pattern of expression of the 68–54 kDa gelatinase did not vary with the nature of the substrate, but its expression increased with duration of culture.

Variations during the endometrial cycle. Numerous gelatinases were expressed by stromal cells obtained from secretory endometrium bearing a Gravigard IUD. After immunopurification, only two to six gelatinases were seen (compare Fig. 5a, b). The same digestion bands could be seen when stromal cell supernatants from early or late proliferative endometrium were compared with the previous phase, thus the intensity of the digestion band seemed to vary with phases of the cycle. During the early proliferative phase, the 64–58 kDa gelatinases were more intense than they were later in the cycle. In contrast, the 89 kDa band was apparently more intensely expressed during

the proliferative phase than in the early proliferative or early secretory bearing a Gravigard IUD phases (Fig. 5).

Effect of inhibitors and pH changes. The effect of different inhibitors and pH changes were tested on stromal cell preparations obtained from a proliferative endometrium. When the gels were incubated overnight with 1, 10 phenanthroline (2 mmol l^{-1}), no digestion bands were visible. When the phenanthroline was replaced by EDTA (20 mmol l^{-1}) the intensity of the digestion bands was drastically reduced. No inhibition was observed with pepstatine A ($1 \mu\text{mol l}^{-1}$), PMSF (20 mmol l^{-1}) or iodoacetamide ($1 \mu\text{mol l}^{-1}$, results not shown). The endometrial gelatinases observed here were clearly neutral. Their highest activity was seen at pH 7.5 and bands were less intense when the gels were incubated at higher or lower pH values.

Discussion

Human endometrial cells isolated by enzymatic digestion and cultured *in vitro* are known to yield a mixed cell population. The endometrium is composed not only of stromal and epithelial cells (surface and glandular cells), but in the stroma, besides the stromal and endothelial cells of the blood vessels, many different bone-marrow-derived cells have also been identified. These include macrophages (Hunt, 1989), mast cells (Finn, 1986) and large granular lymphocytes (King and Loke, 1990). Different methods have been proposed for separating these endometrial cells from each other before plating (Lindenberg *et al.*, 1984; Kirk *et al.*, 1978; Satyaswaroop *et al.*, 1979).

Before characterizing the proteases secreted by endometrial cells, we chose to develop a technique for eliminating the bone-marrow-derived cells from the endometrial cell suspension because these cells are known to produce proteases in other tissues (Shapiro *et al.*, 1990). Since leucocyte common antigen (LCA or CD45) is expressed on all bone-marrow-derived cells including those of the human decidua (Starkey *et al.*, 1988) and endometrium (King *et al.*, 1989), we used an antibody to this

Fig. 5. Zymograms of stromal cell supernatants from endometria obtained at different phases of the menstrual cycle, cultured on matrigel, agarose and glass (days 0–2). (a) Non-immunopurified cells; (b) immunopurified cells; early proliferative endometrium (lane 1); early secretory endometrium bearing a Gravigard IUD (lane 2) and late proliferative endometrium (lane 3).

surface antigen to immunopurify the endometrial cell suspension before plating. This immunopurification procedure was very efficient since very few LCA-positive cells could be seen after immunopurification. This procedure yielded a stromal cell culture of over 95% purity. The epithelial cell culture however was still contaminated by 3–4% stromal cells.

As reported previously (Schatz *et al.*, 1990; White *et al.*, 1990), the phenotype of endometrial cells changes with the nature of the substrate on which they grow. Epithelial cells grew into glandular structures when cultured on matrigel (White *et al.*, 1990; the present report) and stromal cells organized themselves into clusters (Schatz *et al.*, 1990; this study). To our knowledge, the study reported here is the first attempt to grow human endometrial cells on agarose on which neither stromal nor epithelial cells attached. The inability of cells to attach to agarose serves as a good control to appreciate the effect of cell attachment on cell growth and protease production. Irrespective of the nature of the substrate on which the cells were cultured, immunopurified stromal or epithelial cells grew into much larger colonies than when bone-marrow-derived cells were present in the culture together with the other endometrial cells. This observation indicates that LCA-positive cells produce a factor or factors that inhibit endometrial cell–cell association. If this is also true *in vivo*, it would attribute to the

bone-marrow-derived cell an important paracrine role in the remodelling of the endometrium.

Epithelial and stromal cells in culture expressed gelatinases as shown by zymography. Since serum (including FCS) contains well-known protease inhibitors such as α_2 -macroglobulin and α_1 -antitrypsin, the FCS added to our culture media were heat inactivated and acid treated to denature those inhibitors. This cell-free medium did not have intrinsic gelatinase activity even when incubated for 2 days over matrigel. The observed gelatinases were thus cell specific. These were active at neutral pH and were inhibited by EDTA or phenanthroline but not by other protease inhibitors, indicating that the observed gelatinases were metalloproteinases. Zymography, as used here, has its limitations which should be kept in mind before discussing the metalloproteinases observed. First, the molecular mass of the digestion bands is an approximation of the true molecular mass of the enzyme because the substrate included in the gel (gelatin) influences the electrophoretic behaviour of the enzymes. Second, SDS has to be used for the electrophoresis to be performed, but since this compound artificially activates the proenzymes into active enzymes, it is not possible to determine whether the metalloproteinases released in the culture medium are activated or not. Finally, the use of gelatin instead of collagen or casein or other substrates limits our observations to

the gelatinases only. With these limitations in mind, it was very interesting to note that the molecular mass of the endometrial metalloproteinases corresponded to those of the MMPs described in other cell types (Fisher *et al.*, 1989; Adler *et al.*, 1990; Fini and Girard, 1990; Sang *et al.*, 1990; Sato and Mori, 1990; Shapiro *et al.*, 1990; Welgus *et al.*, 1990; Bischof *et al.*, 1991).

Comparison between immunopurified and non-immunopurified endometrial cell cultures showed that the stromal cells released four gelatinases, two of which were also produced by bone-marrow-derived cells (113, 105 kDa): one (89 kDa) was produced by stromal, lymphomyeloid and epithelial cells, whereas the 64–58 kDa band was seen only in purified stromal and epithelial cell supernatants. This last gelatinase corresponds to the 72 kDa collagenase (MMP-2) which is known to migrate with a molecular mass of 66 kDa on gelatin-containing gels when activated (Fini and Girard, 1990). This band was also clearly expressed by cytotrophoblast cells (Bischof *et al.*, 1991). The intensely expressed 89 kDa gelatinase probably represents the 92 kDa collagenase (MMP-2) that degrades collagen type IV and V and which is also expressed in leucocytes, monocytes, macrophages (Welgus *et al.*, 1990) and cytotrophoblast cells (Bischof *et al.*, 1991). The characterization of these gelatinases will require the use of antibodies before their identity can be assessed.

Before discussing the quantitative changes of MMPs, it must be said that zymography is only a semi-quantitative technique and that the apparent increases or decreases in MMPs on the zymogram might be due to changes in cell number rather than changes in production rates since samples have been standardized according to the number of plated cells.

The expression of the endometrial MMPs seemed to be regulated by cell attachment and by the nature of the matrix on which the cells were grown. In the absence of cell–substrate adhesion (cultures on agarose) the expression of all gelatinases was very weak or absent in comparison to cells grown on glass, plastic or matrigel. Although zymography is only a semi-quantitative technique, it seems that the 64–58 kDa gelatinase secreted by stromal but not epithelial cells depends strictly on adhesion, as it was never expressed by cells grown on agarose. This observation also suggests that there is a cell-specific regulation of the MMPs. The fact that expression of MMPs also depends on the nature of the matrix is illustrated by the fact that stromal cells grown on plastic or glass did not express the 82 kDa gelatinase, whereas this enzyme was strongly expressed when the same cells were grown on matrigel. Besides these *in vitro* effects on protease activity, ovarian hormones appear to influence metalloproteinase expression. The 64–58 kDa gelatinase appeared to be expressed more during the follicular phase, whereas the 89 kDa gelatinase was more active during the luteal phase of endometrium bearing IUDs. It cannot be ruled out that this effect is due to the IUD rather than to the presence of progesterone at this stage of the cycle. In the cases where an IUD was present, the number and activity of the gelatinases was increased but this was essentially attributable to proteases secreted by bone-marrow-derived cells, as this was no longer after immunopurification.

In conclusion, this study shows for the first time that human endometrial cells release MMPs, the expression of which seems to be regulated by cell attachment and by the nature of substrates as well as other factors. We postulate that endometrial

MMPs are implicated in uterine tissue remodelling after menses or parturition, and in the regulation of blastocyst hatching and implantation as well as trophoblast invasion.

The authors thank L. Haenggeli for her skilful assistance. This work was supported by Swiss National Fund for Scientific Research, grant number 32-29806.90.

References

- Adler RR, Brenner CA and Werb Z (1990) Expression of extracellular matrix-degrading metalloproteinases and metalloproteinase inhibitors is developmentally regulated during endoderm differentiation of embryonal carcinoma cells *Development* **110** 211–220
- Barret AJ and Starkey PM (1973) The interaction of alpha-2-macroglobulin with proteinases. *Biochemical Journal* **133** 709–713
- Bernhard EJ, Muschel RJ and Hughes EN (1990) Mr. 92,000 gelatinase release contents with the metastatic phenotype in transformed rat embryo cells *Cancer Research* **50** 3872–3877
- Birkedal-Hansen H (1988) From tadpole collagenase to a family of matrix metalloproteinases *Journal of Oral Pathology* **17** 445–451
- Bischof P, Friedli E, Martelli M and Campana A (1991) Expression of extracellular matrix-degrading metalloproteinases by cultured human cytotrophoblast cells: effects of cells adhesion and immunopurification *American Journal of Obstetrics and Gynecology* **165** 1791–1801
- Cawston TE, Galloway WA, Mercer E, Murphy A and Reynolds JJ (1981) Purification of rabbit bone inhibitor of collagenase *Biochemical Journal* **195** 159–165
- Chin JR, Murphy G and Werb Z (1985) Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase: biosynthesis, isolation, characterization, and substrates *Journal of Biological Chemistry* **260** 5213–5216
- Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He C, Bauer EA and Goldberg GI (1988) H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen *Journal of Biological Chemistry* **263** 6579–6587
- Emonard H and Grimaud JA (1990) Matrix metalloproteinases. A review *Cellular and Molecular Biology* **36** 131–153
- Fini ME and Girard MT (1990) The pattern of metalloproteinase expression by corneal fibroblasts is altered by passage in cell culture *Journal of Cell Science* **97** 373–383
- Finn CA (1986) Implantation, menstruation and inflammation *Biology Reviews* **61** 312–328
- Fisher SJ, Cui T, Zhang L, Hartman L, Grahl K, Guo-Yang Z, Tarpey J and Damsky CH (1989) Adhesive and degradative properties of human placental cytotrophoblast cells in vitro *Journal of Cellular Biology* **109** 891–902
- Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA and Eisen AZ (1986) Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein *Journal of Biological Chemistry* **261** 6600–6605
- Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S and He C (1989) Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteinase designated TIMP-2 *Proceedings of the National Academy of Sciences, USA* **86** 8207–8211
- Hasty KA, Pourmotabbed TF, Goldberg GI, Thompson JP, Spinella DG, Stevens RM and Mainardi CL (1990) Human neutrophil collagenase: a distinct gene product with homology to other matrix metalloproteinases *Journal of Biological Chemistry* **265** 5647–5659
- He C, Wilhelm SM, Pentland AP, Marmer BL, Grant GA, Eisen AZ and Goldberg GI (1989) Tissue cooperation in a proteolytic cascade activating human interstitial collagenase *Proceedings of the National Academy of Sciences, USA* **86** 2632–2636
- Hibbs MS and Bainton DF (1989) Human neutrophil gelatinase is a component of specific granules *Journal of Clinical Investigation* **84** 1395–1402
- Hibbs MS, Hoidal JR and Kang AH (1987) Expression of a metalloproteinase that degrades native type V collagen and denatured collagens by cultured human alveolar macrophages *Journal of Clinical Investigation* **80** 1644–1650
- Hunt JS (1989) Macrophages in human uteroplacental tissues: a review *American Journal of Reproduction and Immunology* **21** 119–122

- King A and Loke YW (1990) Uterine large granular lymphocytes: a possible role in embryonic implantation? *American Journal of Obstetrics and Gynecology* **162**, 308–310
- King A, Wellings V, Garner L and Loke YW (1989) Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle *Human Immunology* **24**, 195–205
- Kirk D, King RJB and Heyes J (1978) Normal human endometrium in cell culture. I. Separation and characterization of epithelial and stromal component in vitro *In Vitro* **14**, 651–662
- Lindenberg S, Lauritsen JG, Nielsen MH and Larsen JF (1984) Isolation and culture of human endometrial cells *Fertility and Sterility* **41**, 650–652
- Mallya SK, Mookhtiar KA, Gai Y, Brewk, Dioszegi M, Birkedal-Hansen H and VanWart HE (1990) Characterization of 58-Kilodalton human neutrophil collagenase: comparison with human fibroblast collagenase *Biochemistry* **29**, 10 628–10 634
- Masure S and Opotenakke G (1989) Cytokine-mediated proteolysis in tissue remodelling *Experientia* **45**, 542–549
- Mookhtiar KA and Van Wart HE (1990) Purification to homogeneity of latent and active 58-Kilodalton forms of human neutrophil collagenase *Biochemistry* **29**, 10 620–10 627
- Okada Y and Nakanishi I (1989) Activation of matrix metalloproteinase 3 (stromelysin) and matrix metalloproteinase 2 ("gelatinase") by human neutrophil elastase and cathepsin *FEBS Letters* **249**, 353–356
- Okada Y, Nagase H and Harris ED, Jr (1986) A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components: purification and characterization *Journal of Biological Chemistry* **261**, 14 245–14 255
- Okada Y, Konomi H, Yada T, Klimata K and Nagase H (1989) Degradation of type IX collagen by matrix metalloproteinase 3 (stromelysin) from human rheumatoid synovial cells *FEBS Letters* **244**, 473–476
- Okada Y, Morodomi T, Enghild JJ, Suzuki K, Yasui A, Nakanishi I, Salvesen G and Nagase H (1990) Matrix metalloproteinase 2 from human rheumatoid synovial fibroblast *European Journal of Biochemistry* **194**, 721–730
- Sang QX, Stetler-Stevenson WG, Liotta LA and Byers SW (1990) Identification of type IV collagenase in rat testicular cell culture: influence of peritubular-Sertoli cell interactions *Biology of Reproduction* **43**, 956–964
- Sato T, Liotta LA and Tryggvason K (1983) Purification and characterization of a murine basement membrane collagen-degrading enzyme secreted by metastatic tumor cells *Journal of Biological Chemistry* **258**, 3058–3063
- Sato T, Turpeenniemi-Hujanen T and Tryggvason K (1985) Tumor-promoting phorbol esters and cell proliferation stimulate secretion of basement membrane (type IV) collagen-degrading metalloproteinase by human fibroblasts *Journal of Biological Chemistry* **260**, 8526–8531
- Sato T, Ito A and Mori Y (1990) Interleukin 6 enhances the production of tissue inhibitor of metalloproteases (TIMP) but not that of matrix metalloproteases by human fibroblasts *Biochemical and Biophysical Research Communication* **170**, 824–829
- Satyaswaroop PG, Bressler RS, De la Pena MM and Gursipide E (1979) Isolation and culture of human endometrial glands *The Journal of Clinical Endocrinology and Metabolism* **48**, 639–641
- Saus J, Quinones S, Otani Y, Nagase H, Harris ED, Jr and Kurkinen M (1988) The complete primary structure of human matrix metalloproteinase-3. Identity with stromelysin *Journal of Biological Chemistry* **263**, 6742–6745
- Schatz F, Gordon RE, Laufer N and Gursipide E (1990) Culture of human endometrial cells under polarizing conditions *Differentiation* **42**, 184–190
- Shapiro DS, Campbell EJ, Kobayashi DL and Welgus HG (1990) Immune modulation of metalloproteinase production in human macrophage: selective pre-translational suppression of interstitial collagenase and stromelysin biosynthesis by gamma interferon *Journal of Clinical Investigation* **18**, 1204–1210
- Starkey PM, Sarget IL and Redman CWG (1988) Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry *Immunology* **65**, 129–134
- Stricklin GP, Bauer EA, Jeffrey JJ and Eisen AZ (1977) Human skin collagenase: isolation of precursor and active forms from both fibroblast and organ cultures *Biochemistry* **16**, 1607–1615
- Tseng L and Liu HC (1981) Stimulation of arylsulfotransferase activity by prostaglandins in human endometrium in vitro *The Journal of Clinical Endocrinology and Metabolism* **53**, 418–421
- Van Wart HE and Mookhtiar KA (1990) Biological response modifiers for tissue repair, pp. 23–50 Eds G Grotendarst, LM Hjelmeland and JP Gills. Portfolio, The Woodlands, TX
- Welgus HG, Campbell EJ, Cura JD, Eisen AZ, Senior RM, Wilhelm SM and Goldberg GI (1990) Neutral metalloproteases produced by human mononuclear phagocytes *Journal of Clinical Investigation* **86**, 1496–1502
- White TEK, Di Sant'Agnese PA and Miller RK (1990) Human endometrial cells grown on an extracellular matrix form simple columnar epithelial and glands *In Vitro Cellular and Developmental Biology* **26**, 636–642
- Whitham SE, Murphy G, Angel P, Rahmsdorf H-J, Smith BJ, Lyons A, Harris TJR, Reynolds JJ, Herrlich P and Docherty AJP (1986) Comparison of human stromelysin and collagenase by cloning and sequence analysis *Biochemical Journal* **240**, 913–916
- Wilhelm SM, Collier IE, Kronberger A, Eisen AZ, Marmer BL, Grant GA, Bauer EA and Goldberg GI (1987) Human skin fibroblast stromelysin: structure, glycosylation, substrate specificity and differential expression in normal and tumorigenic cells *Proceedings of the National Academy of Sciences, USA* **84**, 6725–6729
- Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA and Goldberg GI (1989) SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages *Journal of Biological Chemistry* **264**, 17 213–17 221
- Woessner JF, Jr (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling *FASEB Journal* **5**, 2145–2154