The expression of interstitial collagenase in human endometrium is controlled by progesterone and by oestradiol and is related to menstruation

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Human endometrial tissue, sampled at different periods of the reproductive cycle, expressed interstitial collagenase mRNA, protein and activity only just before and during the menstrual period. This clear-cut correlation and the inhibition of colla- genase expression by progesterone and oestradiol in tissue culture point to a pivotal role of this proteinase in the mechanism of menstrual tissue breakdown and bleeding.

INTRODUCTION

The cyclic fall in progesterone and oestradiol plasma concen- trations in the absence of pregnancy induces a breakdown of the endometrial extracellular matrix, bleeding and tissue shedding. The biochemical and cellular mechanisms leading to menstru- ation remain poorly understood. The hypothesis of the in- volvement of interstitial collagenase (EC 3.4.24.7, MMP-1) and related matrix metalloproteinases [1] was first supported by our previous study of the regulation of collagenase and the related gelatinases A (EC 3.4.24.24) and B (EC 3.4.24.35) by pro- gesterone in cultured endometrial explants [2] and by the study of endometrial matrilysin (EC 3.4.24.23) during the reproductive cycle [3]. The present investigation focused on collagenase and examined whether the regulation *in vitro* and *in vivo* of this pivotal proteinase is related to menstruation. Therefore we compared both the release of the enzyme and the abundance of its mRNA throughout the menstrual cycle and under various culture conditions, using enzyme assays, immunoblotting and Northern blotting. The results indicate that the expression of interstitial collagenase is turned on just before the onset of menstruation and turned off from the beginning of the proliferative phase until the end of the late secretory phase, implying a role for this proteinase in menstruation.

MATERIALS AND METHODS

Organ culture

Normal endometrial tissue was obtained from biopsies for histological dating during routine investigation of infertility ($n = 38$; patients 22–44 years old), or from uteri removed for leiomyomas or cervical dysplasia ($n = 13$; patients 40–49 years old). Progesterone and oestradiol serum concentrations were measured by r.i.a. at the time of sampling. The study was approved by the Ethical Committee of the University of Louvain.

Endometrial explants were cultured in daily-renewed Dulbecco's modified Eagle medium (Gibco) without serum, as described [2]. Insulin (1.5 μM; Actrapid HM, Novo) was added in the first 19 cultures, but was omitted from the next cultures without any effect on tissue viability detected by histological examination and lactate dehydrogenase release [4]. Water-soluble complexes of 2-hydroxypropyl-β-cyclodextrin and progesterone or 17β-oestradiol (Sigma) were added to the culture medium at

the indicated nominal concentrations, or replaced by identical amounts of 2-hydroxypropyl-β-cyclodextrin alone in controls.

After each day of culture, conditioned medium was collected, supplemented with 0.05 vol. of 1 M Tris/HCl buffer (pH 7.5)/ 1 % (v/v) Triton X-100/0.1 M $CaCl_2$/60 mM NaN_3 and kept frozen at -80 °C until use. At the end of the culture, explants were fixed for morphological evaluation, lysed for total RNA extraction or frozen at -80 °C for biochemical analysis.

Enzyme assays

Collagenase activity was determined at 25 °C with [³H]acetylated collagen in solution [5]. One unit of collagenase is defined as the amount of enzyme which degrades 1 μg of soluble collagen/min. Total collagenase activity was assayed after treating the con- ditioned media for 2 h at 37 °C with 2 mM 4-aminophenyl- mercuric acetate (Aldrich). Spontaneously active collagenase was measured without pretreatment of the media. Inactivation of tissue inhibitors of metalloproteinases (TIMPs) [2] was not systematically performed, because it did not appreciably change collagenase activities.

Immunoblotting

Mouse anti-(human MMP-1) monoclonal antibody (IgG_{2a}, clone 41-1E5) [6] was a gift from Y. Okada, University of Kanazawa, and from K. Iwata, Fuji Chemical Industries, Japan. Mouse anti-(human epithelial membrane antigen) monoclonal antibody (IgG_{2a}, Dakopatts) was used as control. Conditioned media were concentrated 10-fold by filtration through a membrane with a 10000-M_r cut-off (Centricon-10, Amicon). Proteins were sepa- rated by SDS/PAGE [7] and blotted on to a polyvinylidene difluoride membrane (Immobilon; Millipore). After saturation with 5 % (w/v) BSA, the membrane was incubated with 32 ng/ml primary antibody, overnight at 4 °C, followed by 1 h at 37 °C. Detection was carried out with biotinylated anti-(mouse Ig) sheep IgG (1 μg/ml, 2 h at 20 °C; Boehringer Mannheim) and 40 nCi/ml ¹²⁵I-streptavidin (40 nCi/ng, 2 h 30 min at 20 °C; Amersham). Blots were exposed for 1 week at -80 °C on Hyperfilm-MP (Amersham).

Northern blotting

cDNAs of human MMP-1 (1.7 kbp) [8], stromelysin (gifts from H. Nagase, University of Kansas Medical Center, KS, U.S.A.),

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gelatinase A (gift from G. I. Goldberg, University of Washington Medical Center, U.S.A.), neutrophil collagenase (gift from H. Tschesche, Bielefeld Universität, Germany) and cyclophilin (0.7 kbp; gift from E. Hofer, Sandoz Forschungsinstitut, Austria) [9] were inserted in pBluescript (KS+, Stratagene). MMP-1 and cyclophilin probes were random labelled with [α-^{32}P]dCTP (Megaprime kit, Amersham) to a specific radioactivity of (2–9) × 10^6 c.p.m./ng. The specificity of the MMP-1 probe was assessed by hybridization with sense RNA transcribed from the cDNAs of MMP-1, gelatinase A, stromelysin and neutrophil collagenase. In addition to the estimation of 28 S and 18 S rRNAs after ethidium bromide staining, the house-keeping cyclophilin probe was used as an internal standard of the mRNA loaded in each lane.

Total RNA was isolated from fresh and cultured endometrium by the method of Smale and Sasse [10], with some modifications. The tissue was directly solubilized in lysis buffer and stored at −20 °C. After extraction with phenol/chloroform and then chloroform, RNA was precipitated with 0.1 M sodium acetate/ acetic acid, pH 5.2, and 2.5 vol. of ethanol, and resuspended in water containing 0.1 % (v/v) dimethyldicarbonate and 1 unit/μl RNasin (Promega). Samples (1–10 μg) of total RNA, estimated by spectrophotometry at 260 nm, were electrophoresed in a denaturing agarose gel and transferred to a Hybond–N nylon membrane (Amersham) by capillary blotting. The membrane was incubated for 2 h at 65 °C in a prehybridization solution [1 M NaCl/1 % (w/v) SDS/10 % (w/v) dextran sulphate and 100 μg/ml preboiled herring sperm DNA] and hybridized for 16 h at 65 °C with the appropriate probe (about 10^6 c.p.m./ml). Each blot was washed three times at increasing stringencies, in 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) with 0.1 % SDS at 20 °C, in 2 × SSC with 0.1 % SDS at 50 °C, and in 0.2 × SSC with 0.1 % SDS at 50 °C successively. The membranes were analysed as the immunoblots, after 3 days' exposure to the X-ray film.

Statistical analysis

The Wilcoxon two-samples test was used to detect significant differences within each culture. The Student t test or, when appropriate, paired Student t test was used to compare medians from distinct endometrial cultures. All statistics were studied by using two-tailed tests.

RESULTS AND DISCUSSION

Influence of the menstrual cycle on the initial release of total and active collagenase

Endometria explants sampled at various phases of the menstrual cycle were cultured for 1 day in the absence of added sex steroid. Total collagenase activity (Figure 1) was below the detection limit of the assay in the media conditioned by 18 early- or mid-secretory endometria (mean ± S.D. = 0.07 ± 0.08 unit/ml per 24 h) or by eight proliferative endometria cultured with insulin (0.05 ± 0.05 unit/ml per 24 h), in striking contrast with elevated activities released by nine out of the ten perimenstrual (late secretory or menstrual) endometria (1.32 ± 0.78 units/ml per 24 h, $n = 10$; $P < 0.001$). Moreover, immunoblotting of seven representative conditioned media (Figure 1c) confirmed that immunoreactive (pro)collagenase was released by perimenstrual endometria (lanes 4, 5 and 6), but not by the other ones (lanes 2, 3, 7 and 8).

Addition of 1.5 μM insulin to the culture medium did not influence the release of total collagenase activity from early-, mid-secretory or perimenstrual endometria. In contrast, pro-
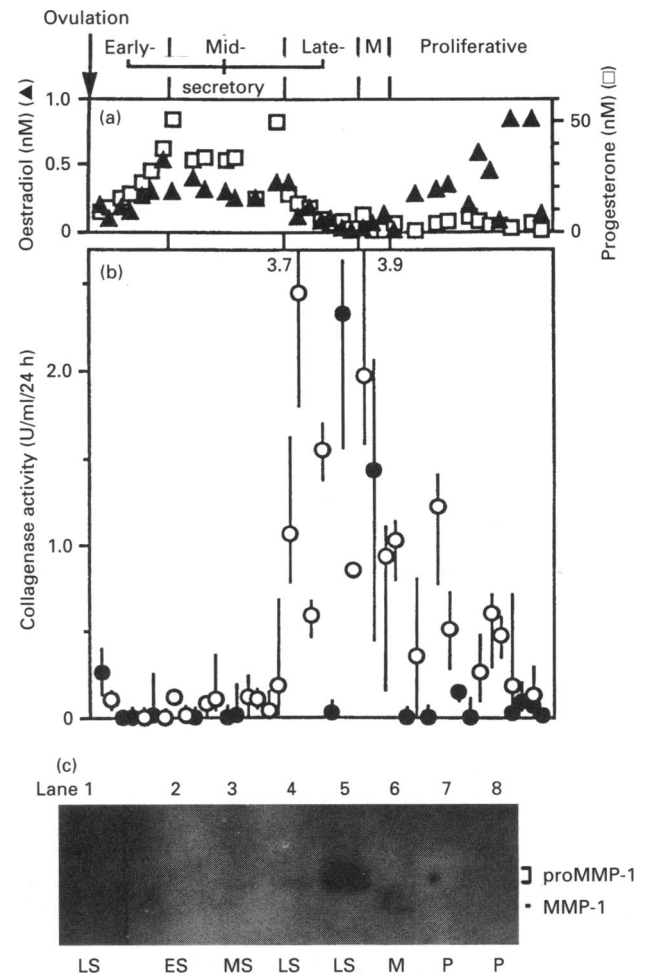

Figure 1   Interstitial collagenase release is related to the menstrual cycle

(a) Oestradiol (▲) and progesterone (□) concentrations in the serum of 41 women at the time of tissue sampling, distributed according to the histological appearance of the endometrium. The menstrual cycle starts with ovulation, at left. M, menstruation. (b) Explants of corresponding endometrial tissues have been cultured during 1 day (15–26 h) without sex steroid added to the culture medium. Culture medium was either devoid of insulin (○) or supplemented with insulin (●). Total collagenase activity released in the conditioned media was normalized for 24 h of culture. For each culture, measurements were made in 3–12 individual conditioned media or in 1–8 pools of 2–24 conditioned media. Circles represent medians, or in four cultures single measurements, with bars between extremes. (c) Explants from seven endometria sampled throughout the menstrual cycle (early secretory, ES, lane 2; mid-secretory, MS, lane 3; late-secretory, LS, lanes 1, 4 and 5; menstrual, M, lane 6; and proliferative phase, P, lanes 7 and 8) were cultured during 1 day with insulin and without sex steroid. Concentrated pools of conditioned media were immunoblotted with the anti-MMP-1 monoclonal antibody (lanes 2–8) or with the control monoclonal antibody (lane 1, same medium as in lane 5) as described in the Materials and methods section. The faint signal shown in lane 4 was from the late-secretory endometrium which released almost no detectable collagenase activity even after inactivation of TIMPs.

liferative endometria released significantly more collagenase when cultured without than with insulin (0.53 ± 0.37 unit/ml per 24 h, $n = 9$; $P < 0.01$), albeit still much less than perimenstrual endometria ($P < 0.02$). At this supraphysiological concentration, insulin could act by increasing the abundance of sex-steroid receptors, as observed in breast-cancer cells [11] and as suggested by our preliminary immunohistochemical observations.

No spontaneously active collagenase was found in media conditioned by three proliferative, two early-, one mid- and four
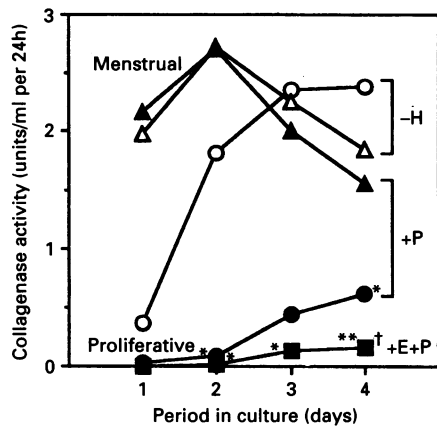
Figure 2 Effect of progesterone alone or combined with oestradiol on collagenase secretion

Explants from a menstrual endometrium (△ and ▲) and from a proliferative endometrium (○, ● and ▲) were cultured for 4 days in the absence of added insulin. Culture medium was devoid of sex steroid (−H, △ and ○; 6 and 7 groups of explants respectively) or supplemented either with 100 nM progesterone (+P, ▲ and ●; 6 and 4 groups of explants respectively) or with both 1 nM oestradiol and 100 nM progesterone (+E+P, ■; 5 groups of explants). Total collagenase activity was measured in all conditioned media. Medians are represented and statistical comparisons were made within each culture either with explants cultured without added sex steroid (*$P < 0.05$; ** $P < 0.01$) or with explants cultured with progesterone alone (†$P < 0.05$).

late-secretory endometria, but a significant activity was detected in culture media of the three menstrual endometria tested, at respectively 100, 31 and 8 % of total collagenase activity. The activation of MMP-1 during the first day of culture of menstrual endometria was confirmed by immunoblotting (Figure 1c, lane 6).

In conclusion, these data show a striking correlation between the initiation of menstruation and the release of collagenase from endometrial tissue, as well as its activation.

Collagenase activity results from synthesis and secretion of interstitial collagenase

Neutrophil polymorphonuclear leucocytes infiltrate the endometrium at the end of the late-secretory phase and during menstruation [12]. Thus the high collagenase activities released from late-secretory and menstrual endometria during the first day of culture could be due to exocytosis of neutrophil collagenase stored in the specific granules. To test for this possibility, explants from a menstrual endometrium were cultured with or without 1 μg/ml cycloheximide, or at 4 °C. In control conditions, high levels of collagenase (0.94 unit/ml per 24 h) were released during day 1 of culture, increasing by 180 % during day 2 of culture ($P < 0.01$). Addition of cycloheximide or culture at 4 °C during day 1 decreased the release of collagenase by 80 and 95 % ($P < 0.05$ and $P < 0.01$) respectively. All these inhibitions were reversible. This demonstrates that collagenase release involves continuous synthesis and secretion, and rules out degranulation of neutrophils and desorption from the extracellular matrix as its major source.

Treatment with 5 mM N-ethylmaleimide (30 min at 25 °C), which activates neutrophil but not interstitial procollagenase [13], resulted in a negligible increase in collagenase activity in media conditioned during the 3 days of culture of four distinct endometrial tissues (two early- and two late-secretory endometria; mean increase ± S.D. = 8.8 ± 5.8 % of collagenase ac-

tivity after 4-aminophenylmercuric acetate treatment). This observation confirms that neutrophil collagenase does not account for the collagenase activity secreted by these endometria in culture.

Effect of progesterone and oestradiol on collagenase secretion

After day 1 of culture without added steroid, collagenase secretion appeared and/or increased in all non-perimenstrual endometria examined (Figure 2). This secretion was constantly inhibited by physiological concentrations (50–150 nM) of progesterone throughout the 2–4 days of culture of three proliferative, two early-, six mid- and four late-secretory endometria (mean inhibition ± S.D. = 69 ± 32 %; $P < 0.001$). Progesterone also decreased the amount of enzyme protein detected by immunoblotting (results not shown). In contrast, collagenase activity secreted by the two menstrual endometria tested was elevated from day 1 of culture, did not increase during the following days, and was not down-regulated by progesterone (Figure 2).

Although 1 nM oestradiol moderately inhibited the release of collagenase by one proliferative, one early- and three mid-secretory endometria during days 2–4 of culture in the presence of insulin (mean inhibition ± S.D. = 34 ± 41 %; $P < 0.02$), combination of 50 or 100 nM progesterone with 1 nM oestradiol further inhibited the secretion of collagenase throughout the 2–4 days of culture of 14 distinct non-menstrual endometria sampled throughout the cycle (mean inhibition ± S.D. = 86 ± 17 % $P < 0.001$). Indeed, oestradiol potentiated the inhibitory effect of progesterone in cultures of one proliferative (Fig. 2), one early- and four mid-secretory endometria ($P < 0.05$ when comparing the combination of sex steroids with progesterone alone). In contrast, the release of a representative lysosomal hydrolase, N-acetyl-β-hexosaminidase [14], did not increase during culture without hormone and was only marginally influenced by the combined sex steroids (mean inhibition ± S.D. = 14 ± 25 %, $n = 11$; $P < 0.02$; results not shown).

All these observations are in keeping with the known up-regulation of progesterone receptors by oestrogen [15] and with the considerable decrease in endometrial progesterone receptors at menstruation [16]. Moreover, the effects of progesterone and oestradiol alone or combined confirm in well-defined culture conditions the hormonal regulation of the release of endometrial collagenase, in agreement with our hypothesis of a pivotal role of collagenase in the mechanism of menstruation.

Influence of the menstrual cycle and of sex steroids *in vitro* on interstitial collagenase mRNA

In fresh tissue, the 2.2 kb mRNA of MMP-1 was detected by Northern blotting only in extracts (5 or 10 μg of total RNA) of the two menstrual endometria analysed (Figure 3, lane 1). In striking contrast, no signal at all was observed in five proliferative, four early- or mid-secretory (Figure 3, lane 2) and four late-secretory endometria, whereas the control cyclophilin mRNA was at least as abundant as in the menstrual endometria. These observations confirm those recently made by Hampton and Salamonsen [17], and point to the conclusion that the transcription of pro-MMP-1 is turned on just before the onset of menstruation and turned off from the beginning of the proliferative phase until the late-secretory phase.

Moreover, our Northern-blot analysis of cultured endometrial explants revealed MMP-1 mRNA in explants from all cultures performed without sex steroid (two proliferative, two early-, five mid- and five late-secretory endometria), after 1–3 days of culture. Culture with progesterone decreased the steady-state
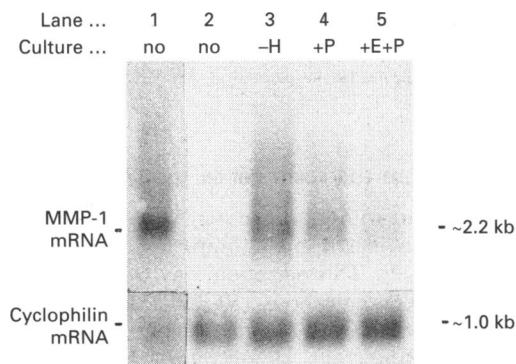
Figure 3   Northern blot of endometrial interstitial collagenase

Northern blot of total RNA extracted from a non-cultured menstrual endometrium (day 2 of the menstrual cycle; lane 1) and from a non-cultured mid-secretory endometrium (day 22; lane 2). Explants from this last endometrium were cultured for 2 days without insulin and without sex steroid (lane 3, −H), with 100 nM progesterone alone (lane 4, +P) or with both 1 nM oestradiol and 100 nM progesterone (lane 5, +E+P). Total RNA was extracted from the explants at the end of the culture. A 5 µg sample of total RNA was run in each lane and successively hybridized with the interstitial collagenase and the cyclophilin probes.

level of MMP-1 mRNA in explants from all endometria except from one late-secretory endometrium, an effect that was potentiated in some experiments by combination with oestradiol (e.g., compare lanes 4 and 5 in Figure 3).

Summary and general discussion

These observations on the expression of interstitial collagenase in human endometrium, on its inhibition by progesterone (and oestradiol), and on its relation to the menstrual cycle strongly suggest that MMP-1 is involved in the molecular events leading to menstruation. Significant activities of neutrophil collagenase were not detected in our cultures, but we cannot exclude a contribution of this or another neutrophil proteinase to the tissue degradation. Although other MMPs are likely participants in this process, for instance gelatinases A and B [2], matrilysin [3] and stromelysin [17], 'true' collagenases appear to be crucial, as

being the only proteinases capable of cleaving the native triple-helical domain of fibrillar collagen. Immunolocalization and mRNA-hybridization studies *in situ* will help to determine in which cells and where in the tissue procollagenase in transcribed, synthesized, secreted and activated. The extension of such studies to endometrial diseases could also pave the way to new diagnostic and therapeutic perspectives.

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