

Menstrual breakdown of human endometrium can be mimicked *in vitro* and is selectively and reversibly blocked by inhibitors of matrix metalloproteinases

(menstruation/extracellular matrix/collagen/proteinases/collagenase)

ETIENNE MARBAIX*†, ISABELLE KOKORINE*‡, PIERRE MOULIN†, JACQUES DONNEZ§, YVES EECKHOUT‡, AND PIERRE J. COURTOT*¶

*Cell Biology Unit and ‡Connective Tissue Group, International Institute of Cellular and Molecular Pathology, University of Louvain Medical School, avenue Hippocrate, 75, B-1200 Brussels, Belgium; and Departments of †Pathology and of §Gynecology, Saint Luc University Clinics, University of Louvain Medical School, avenue Hippocrate, 10, B-1200 Brussels, Belgium

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ABSTRACT The mechanisms underlying the menstrual lysis leading to shedding of the human endometrium and its accompanying bleeding are still largely unknown. In particular, whether breakdown of the endometrial fibrillar extracellular matrix that precedes bleeding depends on aspartic-, cysteine-, serine-, or metalloproteinases remains unclear. In the present study, menstrual regression of the human endometrium was mimicked in organ culture. Whereas sex steroids could preserve tissue integrity only in nonperimenstrual explants, matrix breakdown upon sex steroid deprivation was completely and reversibly inhibited at all stages of the menstrual cycle by specific inhibitors of matrix metalloproteinases, but not by inhibitors of the other classes of proteinases. Matrix metalloproteinases are thus identified as the key class of proteinases involved in the initiation of menstruation.

In his pioneering studies on menstruation using intraocular endometrial transplants in Rhesus monkeys, Markee constantly observed a major shrinking of the tissue within the few days preceding menstrual bleeding (1). Bleeding, which characterizes primates and a limited number of other species, has been linked to hypoxia/reperfusion secondary to spasm or compression of their unique coiled arteries upon tissue collapse (1). Regression of the endometrium also occurs in nonbleeding mammals: in cycling rats, both endometrial wet weight and collagen content decrease during metestrus to 20% of their proestrus value (2), indicating that proteolysis of the extracellular matrix (ECM) takes part in the process (3).

An extensive argyrophilic network of so-called "reticular fibers," containing both type III and type I collagen (4), but whose argyrophilic staining properties are not completely understood (5), is built up in the ECM of human endometrium during the proliferative and early secretory phases of the menstrual cycle. When progesterone concentration declines at the end of the secretory phase, this embryonic-like interstitial fibrillar matrix shows focal breakdown that develops into extensive lysis at menstruation, concomitantly with more restricted sites of basement membrane disruption around vessels and glands. The shrunken tissue remnants then undergo piecemeal shedding (reviewed in ref. 6).

The frequent localization of collagen fibers inside stromal cells just before and during menstruation has been interpreted as lysosomal digestion (7). The lysosomal concept of endometrial bleeding was supported by (i) the high specific activities of several acid hydrolases in this tissue (8); (ii) the increase of

the nonsedimentable fraction of lysosomal enzymes during the late secretory phase (9); and (iii) the cytochemical demonstration of extracellular acid phosphatase in perimenstrual endometria (10). These observations are compatible with a role of lysosomal hydrolases, but do not prove their involvement in the ECM breakdown at menstruation.

Plasmin and plasminogen activators could also be involved. The human endometrium contains both urokinase and tissue plasminogen activator (11), and their concentration increases around menstruation (12). Progesterone inhibits the secretion of both plasminogen activators by cultured endometrial explants (13) and stimulates the expression of plasminogen activator inhibitor-1 by cultured endometrial stromal cells (14), indicating a control of this system by sex steroids during the menstrual cycle and its release before menstruation. Besides its fibrinolytic role, plasmin can degrade some extracellular components by itself (15), and activate other enzymes such as matrix metalloproteinases (MMPs) (16).

MMPs, neutral enzymes that can degrade most proteins of the ECM (17), are a third family of candidates (3). In particular, collagenases are the only mammalian enzymes able to cleave at neutral pH fibrillar collagens including types I and III, major components of the endometrial ECM (18). Most MMPs are secreted as latent proenzymes and are activated by proteolytic processing. In turn, active forms can be inactivated by α_2 -macroglobulin and tissue inhibitors of metalloproteinases. In the human endometrium, expression of interstitial collagenase (MMP-1), stromelysins-1 (MMP-3) and -2 (MMP-10), and gelatinase B (MMP-9) is almost exclusively restricted to the perimenstrual period, as shown by Northern blotting (19, 20) and *in situ* hybridization studies (21, 22). The expression of MMP-1 and MMP-9 is focal and limited to the functional layer, which is subsequently shed (21–23). A role of MMPs in endometrial matrix breakdown, in particular of MMP-1 and MMP-9, is also strongly suggested by the tight control sex steroids exert on their expression, secretion, and activation (23, 24).

Although striking, the close spatio-temporal correlation between their expression and matrix degradation, as well as their hormonal control, provide only circumstantial evidence for the involvement of MMPs in menstruation. A direct proof could be obtained if the menstrual process was blocked by specific inhibitors of the implicated enzymes (25). The organ culture system of the endometrium (24), which preserves structural relations and paracrine interactions between the different cell types and their ECM, allows the testing not only

Abbreviations: MMP, matrix metalloproteinase; E, estradiol; P, progesterone; DIC, dichloroisocoumarin; BB, BB-2116; Ro, Ro31-4724; RP, RP59794; SC, SC-44463; ECM, extracellular matrix.

¶To whom reprint requests should be addressed.

of the release of enzymes, but also of their effect on their physiological substrates. In this study, menstrual breakdown of human endometrial matrix was mimicked in this system. In addition, the effects of specific proteinase inhibitors demonstrated that at least one MMP initiates the process.

MATERIALS AND METHODS

Organ Culture of Human Endometrium. Endometrial explants from biopsies or from hysterectomy specimens were cultured for 2 or 3 days on Biopore membranes in Millicell-CM inserts (Millipore), at the interface between 5% CO₂/95% air and Dulbecco's modified Eagle's medium devoid of serum, insulin, and phenol red (20). The present study was approved by the Ethical Committee of the University of Louvain. Daily renewed media were either devoid of any addition or supplemented with water-soluble complexes of 1 nM estradiol and 100 nM progesterone (E+P) in 2-hydroxypropyl- β -cyclodextrin (Sigma) or with one of the following proteinase inhibitors, always at the indicated concentration. Specific reversible inhibitors of the family of MMPs were generously provided by Rhône-Poulenc Rorer (Vitry sur Seine, France) [RP59794 (RP), 25 μ M, ref. 26]; Pfizer Diagnostics [SC-44463 (SC), 5 μ M, ref. 27]; British Biotechnology (Oxford, U.K.) [BB-2116 (BB), 5 μ M, ref. 28]; and Roche Products (Welwyn Garden City, U.K.) [Ro31-4724 (Ro), 5 μ M, ref. 29]. In addition, aprotinin (75 μ M), leupeptin (20 μ M), E64 (20 μ M), pepstatin (15 μ M, all from Boehringer Mannheim), aminoethylbenzenesulfonyl fluoride (AEBSF, 100 μ M, ICN) and dichloroisocoumarin (DIC, 100 μ M, Sigma) were used as inhibitors of the other classes of proteinases. Cycloheximide (Sigma), an inhibitor of protein synthesis, was also tested. Final concentrations of solvents were identical within each experiment, maximum concentrations being 0.1% (vol/vol) dimethylformamide (used for RP and DIC) or dimethyl sulfoxide (used for SC, BB and Ro), 1% (vol/vol) ethanol (used for E64 and pepstatin) and 0.3 μ M 2-hydroxypropyl- β -cyclodextrin.

Morphological and Morphometric Analysis. At the end of the culture, explants were fixed in freshly prepared 4% neutral formaldehyde and paraffin sections were silver-impregnated to stain the reticular fibers (30). For immunohistochemical labeling, sections were pretreated for 7 min at 37°C with 0.5 mg/ml pronase E (Merck), and incubated overnight at 4°C with affinity-purified anti-human collagen-I (2 μ g/ml), -III (2 μ g/ml), and -IV (0.7 μ g/ml) rabbit antibodies (all from Monosan, Uden, The Netherlands). Detection was carried out with 1 μ g/ml of biotinylated sheep antibodies directed against rabbit antibodies and 0.5 unit/ml peroxidase-conjugated streptavidin (both from Boehringer Mannheim) (22). Antisense riboprobes transcribed from human MMP-1 cDNA (gift from H. Nagase, Kansas University, Kansas City, KS) and labeled with ³⁵S were used for *in situ* hybridization (22). All histolabeling procedures were performed in parallel for noncultured and the various cultured samples from each biopsy.

Morphometric analysis was performed by an investigator unaware of the culture conditions with a Zeiss microscope connected by a charge-coupled device 72-EX camera (DAGE-MTI, Michigan City, IN) to an IBAS 2000 image analyzer (Kontron, Munich, Germany). The 100 $\times$ or 250 $\times$ video images were resolved into 512² pixels. Projected areas of the fiber network were interactively measured as fractions of the stromal area and expressed as percentages of the corresponding value in noncultured endometrium from the same biopsy. The fractional areas were remarkably constant in noncultured endometria, at 0.38 $\pm$ 0.01 for the argyrophilic fibers (mean $\pm$ SEM, n = 12) and at 0.37 $\pm$ 0.04 for type III collagen (n = 11).

Assay of Collagen Content and Enzymatic Activities. At the end of the culture, explants were frozen, homogenized in water, and assayed for hydroxyproline (31) after hydrolysis in 6 N HCl for 22 h at 110°C. The values were related to the

protein (32) and DNA (33) tissue content. The protein:DNA ratio did not vary appreciably between the different culture conditions (9 $\pm$ 1 mg protein/mg DNA, mean $\pm$ SEM, n = 17). Collagen content was calculated on the basis of 0.13 mg hydroxyproline per mg collagen.

Collagenase activity was assayed in conditioned media, either without treatment to measure the spontaneously active collagenase, or after treatment with 2 mM aminophenylmercuric acetate for 2 h at 37°C, to measure latent and active enzyme (20). Gelatin zymographies were performed on 8% acrylamide gels copolymerized with 0.5 mg/ml gelatin (24).

Transfer of Matrix Degrading Activity to Noncultured Endometrium. Serial 10 μ m-thick frozen sections from noncultured endometria were fixed for 5 min in acetone or methanol and air-dried. Sections were then incubated for 48 h at 37°C with 40 μ l of media conditioned by explants from unrelated endometria, cultured in various conditions. Media

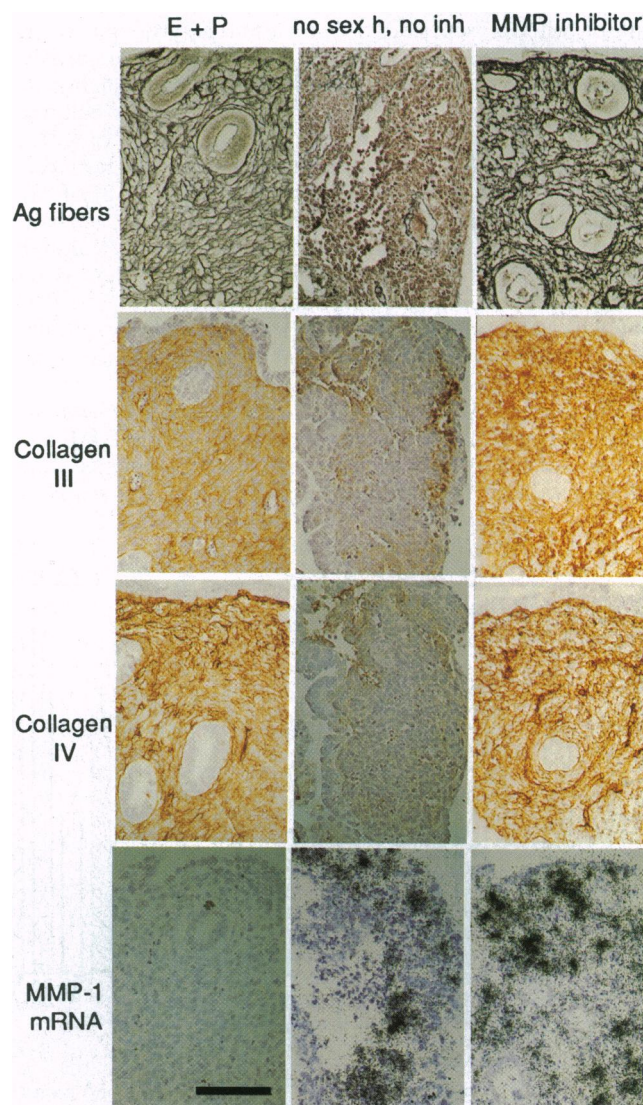


FIG. 1. Endometrial matrix and MMP-1 expression in cultured explants: influence of sex steroids and an MMP inhibitor. In this representative experiment, explants from a late proliferative endometrium were cultured for 2 days either with sex steroids (E+P) or without any addition (no sex h, no inh). Parallel explants were cultured without sex steroid but with RP (MMP inhibitor). At the end of the culture, semi-serial sections of the explants were stained with silver (Ag fibers), immunolabeled for collagen III or collagen IV, or hybridized with a MMP-1 antisense riboprobe (MMP-1 mRNA). (Bar = 100 μ m.)

were supplemented to contain 50 mM Tris·HCl (pH 7.5), 0.05% Triton X-100, 5 mM CaCl₂, 3 mM NaN₃, and, when indicated, 2 mM aminophenylmercuric acetate, with or without proteinase inhibitors. Nonconditioned culture media were used as control. The degradative properties of plasmin were similarly tested by supplementing the nonconditioned medium with 1.5 μ M of plasmin (Boehringer Mannheim). Sections were then fixed overnight in 4% formaldehyde at room temperature and silver-stained (30).

Statistical Comparisons. The one-tailed Student's *t* test was used for all statistical analyses.

RESULTS

An *in Vitro* Model of Menstrual Matrix Breakdown. Explants from endometria sampled throughout spontaneous menstrual cycles (*n* = 28) or from patients under oral contraceptives (*n* = 6) were cultured for 2–3 days, after which the network of argyrophilic fibers was examined (Fig. 1). Upon culture without sex steroid, interstitial argyrophilic fibers essentially vanished, but basement membranes were partially preserved around vessels and glands, as observed in menstrual endometrium *in situ*. In contrast, parallel explants cultured either with 4 μ M cycloheximide (*n* = 4, not shown) or with 1 nM E and 100 nM P, hereafter referred to as “sex steroids,” showed a well-preserved argyrophilic network (*n* = 21 of 23), except in 2 perimenstrual endometria where it was partially degraded already before culture. Moreover, explants cultured without any addition were more friable and smaller, as reflected by the area of their histological section [0.27 ± 0.02 mm² without sex steroid vs. 0.59 ± 0.05 mm² with sex steroids; mean $\pm$ SEM of the median explant area (4–19 explants per condition) in 12 experiments; *P* < 0.001].

Inhibitors of MMPs Prevent Lysis of the Argyrophilic Fibers Network. To determine the contribution of MMPs in the breakdown of the endometrial matrix, explants were cultured without sex steroid, a condition inducing synthesis

and secretion of these enzymes (20, 24), and in the presence of one of four distinct peptide hydroxamates (RP, SC, BB, and Ro) that specifically and reversibly inhibit the activity of the MMPs. We verified that the IC₅₀ for human endometrial collagenase activity was about 100 nM for RP and about 10 nM for the 3 other inhibitors.

In all experiments, despite the absence of sex steroid, the network of argyrophilic fibers was not altered after 2–3 days of culture with RP (two batches; *n* = 29) or with any of the three other MMP inhibitors (*n* = 8 for each inhibitor), in striking contrast with parallel explants cultured without inhibitor (Fig. 1 and 2a). Only MMP inhibitors prevented lysis of the fibers in perimenstrual endometria.

Immunohistochemical Study of Endometrial Matrix Degradation. To characterize the modifications of the endometrial matrix, histological sections were immunolabeled for collagens I, III, and IV. Type III collagen immunostaining (Figs. 1 and 2b) paralleled the network of argyrophilic fibers. Indeed, it largely decreased in the stroma of explants cultured without any addition, whereas it was well-preserved in explants cultured either with sex steroids or with MMP inhibitors. Morphometric analysis even showed an increase of the relative area of immunolabeled collagen III with supplemented media, in particular in explants cultured with the MMP inhibitors, suggesting the occurrence of collagen synthesis during culture. In cultures without any addition, type IV collagen (Fig. 1) was degraded largely around predecidual cells but inconsistently around vessels and glands. Type I collagen pattern was essentially similar to that of type III collagen, except for a more focal breakdown in the wall of vessels in explants cultured without any addition (not shown).

Quantitative Assessment of Collagen Degradation in Endometrial Explants. Collagen degradation was quantified by assaying the residual hydroxyproline content in explants after culture (Fig. 2c). In all experiments, explants cultured without any addition contained significantly less collagen than noncultured tissue and parallel explants cultured with sex steroids

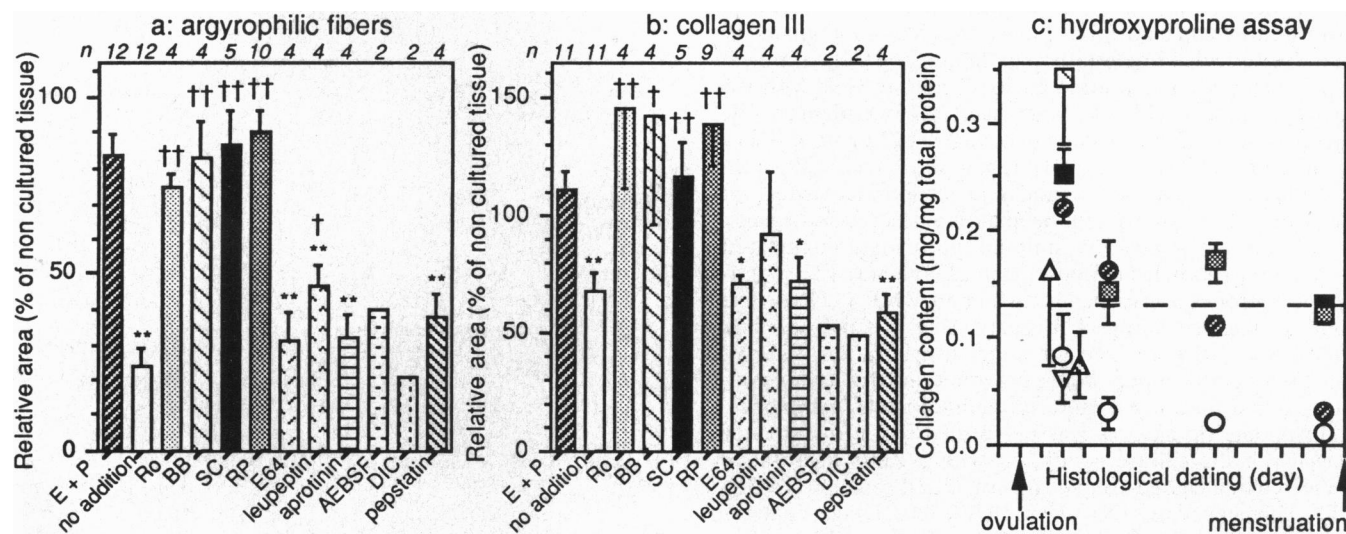


FIG. 2. Measurement of the effects of proteinase inhibitors on the matrix of endometrial explants. (a and b) Morphometric analysis of the argyrophilic fibrillar network (a) and of the collagen III immunolabeling (b). Explants from the indicated number of endometria (*n*) were cultured for 2–3 days in the indicated media, and histological sections were silver-stained (a) or immunolabeled for type III collagen (b). The abundance of silver-stained (a) or collagen III immunolabeled (b) fibers in histological sections of these explants was determined by morphometry and expressed as a percentage of the value for noncultured tissue. In each experiment, the median explant value was determined out of 4–19 (9 on average) explants analyzed per condition, and results are means $\pm$ SEM of these median values. Statistical comparisons were: *, *P* < 0.05 and **, *P* < 0.005 vs. E + P; †, *P* < 0.05 and ††, *P* < 0.005 vs. no addition. (c) Assay of collagen content. Explants from three secretory and one premenstrual endometria were cultured for 3 days with sex steroids (⊙), without any addition (○) or with one of the following proteinase inhibitors: BB (⊞), SC (■), RP (⊠), all inhibitors of MMPs; E64 (Δ), an inhibitor of cysteine-proteinases; DIC (▽), an inhibitor of serine-proteinases; or pepstatin (▲), an inhibitor of aspartic-proteinases. Hydroxyproline was assayed in the explants at the end of the culture. Results are means $\pm$ SEM of 4–5 groups of 10–18 explants per condition in each experiment, distributed according to histological dating of the noncultured tissue. The dashed line corresponds to the collagen content in eight noncultured secretory endometria sampled outside the menstrual phase (0.13 ± 0.02 mg/mg total protein, mean $\pm$ SEM).

or with a MMP inhibitor. In nonperimenstrual explants, the collagen content was fully preserved upon culture with sex steroids or with an MMP inhibitor. In an early secretory sample, the collagen content even increased upon culture in such conditions, indicating that synthesis took place *in vitro*. On the other hand, explants from a perimenstrual endometrium contained much less collagen than the noncultured tissue despite culture with sex steroids, reflecting a loss of inhibitory potential of sex steroids upon menstrual collagen degradation. In that experiment, sex steroids only weakly inhibited the secretion and the activation of collagenase, confirming our previous observations (20). In contrast, the collagen content of the perimenstrual explants was fully preserved by the MMP inhibitors.

Role of Other Proteinases. In a complementary set of experiments, inhibitors of the other classes of proteinases were tested and compared with MMP inhibitors (Fig. 2). When explants were cultured with pepstatin ($n = 4$), a specific inhibitor of aspartic-proteinases, or with aprotinin ($n = 4$), AEBSF ($n = 5$), or DIC ($n = 4$), all specific inhibitors of serine-proteinases, the lysis of argyrophilic fibers (Fig. 2a) and the loss of collagen III immunoreactivity (Fig. 2b) were extensive, reaching similar levels as in explants cultured without any addition. Instead, when explants were cultured with leupeptin ($n = 4$), an inhibitor of both serine- and cysteine-proteinases, or with E64 ($n = 9$), a specific inhibitor of cysteine-proteinases (34), fibers underwent a characteristic fragmentation (not shown) so that the network was no longer identifiable. In one experiment, the collagen content of explants cultured with an inhibitor of these other classes of proteinases decreased as for explants cultured without any inhibitor (Fig. 2c).

Experiments described so far were performed without serum to avoid α_2 -macroglobulin and other proteinase inhibitors. By the same token, the system was not supplied with plasmin. The addition of 0.1 μ M plasminogen ($n = 3$) or 10% calf serum ($n = 1$) to the medium had no effect on the argyrophilic network in explants cultured either with sex steroids or with a MMP inhibitor (RP). Moreover, breakdown of explants cultured without any addition was not enhanced by plasminogen/plasmin in our system, even though collagenase was totally activated in the media conditioned in the presence of plasminogen, as expected (16) (not shown; see also Fig. 5e).

Effects of Proteinase Inhibitors on Production and Activation of MMPs. Secretion and activation of (pro)MMPs in conditioned media, as examined by assay of total (open bars) and spontaneously activated (solid bars) collagenase, are shown in Fig. 3a. Sex steroids completely inhibited the synthesis and secretion of collagenase by explants from nonperimenstrual endometria, whereas inhibition was only partial in the two perimenstrual endometria, where degradation had started before sampling. In all media of cultures with any of the MMP inhibitors, collagenase activity was essentially undetectable. However, removal of the MMP inhibitor by gel filtration of conditioned media through a Sephadex G-25 NAP-5 column (Pharmacia) allowed the recovery of $74 \pm 13\%$ (mean $\pm$ SEM, $n = 12$) of the collagenase activity secreted by parallel explants cultured without inhibitor, demonstrating that synthesis and secretion of procollagenase were not impaired by the inhibitor. None of the other proteinase inhibitors altered the production of collagenase or its activation during culture.

Gelatin zymograms of the conditioned media (Fig. 3b) showed that RP did not impair the secretion of both (pro)gelatinases A and B (MMPs-2 and -9), as well as of a faint gelatinolytic (pro)enzyme migrating at the same level as (pro)MMP-1 (24). In contrast, the activation of these proenzymes was strongly inhibited during culture with the MMP inhibitor, indicating crossactivation (35, 36) or auto-activation by MMPs during culture. None of the other proteinase inhibitors affected the activation of these MMPs.

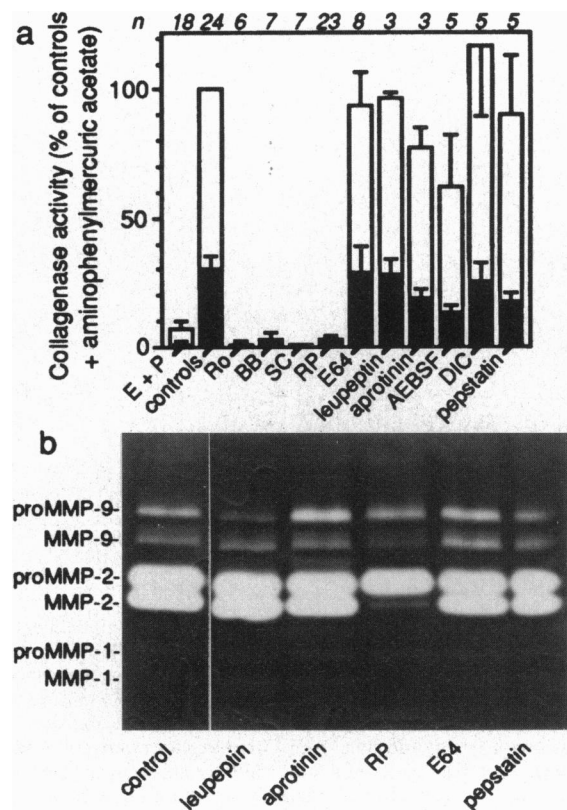


FIG. 3. Effects of proteinase inhibitors on the secretion and activation of MMPs. (a) Collagenase assay. Explants from the indicated number of endometria (n) were cultured either with sex steroids (E+P), without any addition (controls), or with the indicated proteinase inhibitor. The spontaneously active collagenase (solid bars) and the total collagenase (latent and active enzyme, open bars) secreted during the second day of culture were assayed. Results are means $\pm$ SEM expressed as a percentage of the total collagenase activity released by explants cultured without any addition (2.03 ± 0.37 units/ml). (b) Zymography. Representative (out of five experiments) gelatin zymogram of media conditioned during the second day of culture of a mid-secretory endometrium (10μ l per lane). Culture conditions are indicated at the bottom of each lane (control, no addition).

Kinetics of Matrix Degradation and Reversibility of Inhibition. At the concentrations tested, none of the proteinase inhibitors appeared toxic, as assessed by the absence of tissue necrosis (Figs. 1 and 4), by the lack of enhanced release of lactate dehydrogenase (8) (not shown), and by the unaltered production of MMPs (Fig. 3b). Furthermore, *in situ* hybridization studies confirmed that RP did not appreciably affect the abundance of MMP-1 mRNA, in striking contrast to physiological concentrations of sex steroids (Fig. 1).

In the absence of any addition, the argyrophilic network of explants from nonperimenstrual endometria was preserved after 1 day of culture but largely disappeared after 2 days (Fig. 4), matching the time course of the secretion of several MMPs (20, 24). Fibers were protected after 2 days of culture with an MMP inhibitor (RP), but were lost when culture was extended for a third day without the inhibitor (compare *h* with *g*). The rapid onset of lysis of the argyrophilic fibers during the third day of culture could result from the increased expression of MMPs during the 2 previous days of culture without sex steroid, as observed by *in situ* hybridization (Fig. 1) and by enzyme assays (24). As expected, some protection by sex steroids remained 1 day after their withdrawal (compare *f* with *e*).

Transfer of Matrix Lytic Activity to Noncultured Endometria. To test whether proteinases secreted during culture were indeed able to degrade the ECM of noncultured endometrium,

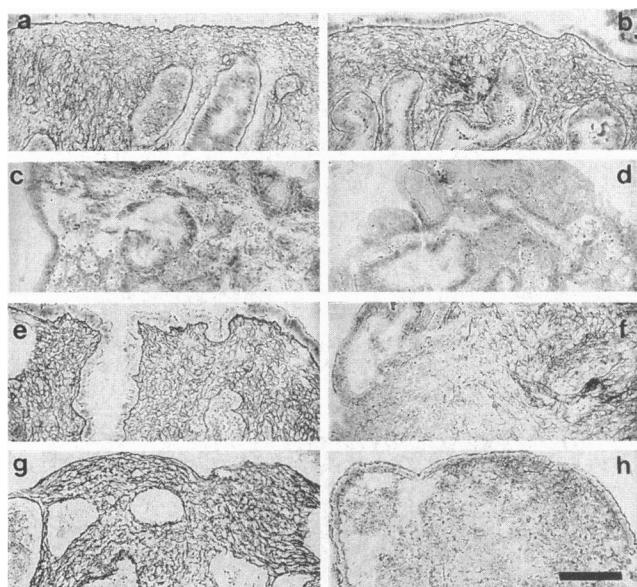


FIG. 4. Kinetics of degradation of the argyrophilic fibers and reversion of the effects of sex steroids and of an MMP inhibitor. In this representative experiment (out of three), histological sections from an early secretory endometrium were silver-stained before culture (a) or after 1 (b), 2 (c), or 3 days of culture (d–h). Some explants were cultured without any addition (b–d). Other explants were cultured with sex steroids for 3 days (e), or for 2 days followed by 1 day without added sex steroid (f). Still other explants were cultured with an MMP inhibitor (RP) for 3 days (g) or for 2 days followed by 1 day without added inhibitor (h). (Bar = 100 μ m.)

acetone- or methanol-fixed histological sections of endometrial biopsies were incubated for 2 days with media conditioned by explants from other endometria, cultured under various conditions (Fig. 5). Media containing high collagenase activities effectively degraded the argyrophilic network, confirming that disappearance of fibers during culture was primarily due to proteolysis, not to impaired synthesis, whereas media containing no collagenase activity did not alter the fibrillar network. When conditioned media contained mostly latent collagenase, digestion of the argyrophilic fibers was enhanced

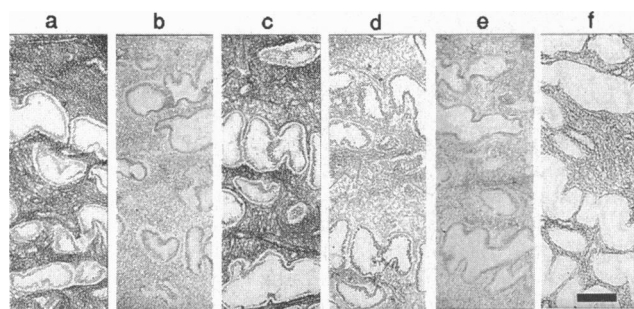


FIG. 5. Matrix degrading activity, transferred to noncultured endometrium, is selectively abolished by an MMP inhibitor. In this representative experiment (out of five), acetone-fixed serial histological sections from an early secretory endometrium were incubated for 48 h at 37°C in the following media: (a) nonconditioned culture medium as control, (b–e) medium conditioned during the second day of culture of explants from an unrelated, late proliferative endometrium, cultured without any addition (6.4 units/ml of total collagenase activity). Conditioned medium was incubated as such (b), or was supplemented with an MMP inhibitor (RP, c), a serine-proteinase inhibitor (DIC, d), or a cysteine-proteinase inhibitor (E64, e). Aminophenylmercuric acetate was added to media (a–e). Another section was incubated in parallel with plasmin added to nonconditioned medium (f). Argyrophilic fibers were stained at the end of the incubation. (Bar = 200 μ m.)

upon activation of the proMMPs by aminophenylmercuric acetate, an agent that had no additional effect if collagenase had been spontaneously activated during culture. Addition of an MMP inhibitor (1 μ M RP or 0.1 μ M BB) to a medium with high collagenase activity fully preserved the argyrophilic fibers (by morphometry, 93 and 90% of controls, respectively, vs. 20% without inhibitor), whereas inhibitors of cysteine- or serine-proteinases were ineffective. Plasmin at physiological concentrations did not degrade the argyrophilic fibers by itself.

DISCUSSION

This study reports that menstrual breakdown of human endometrial ECM can be mimicked in an organ culture system, reproducing the menstrual shrinking of *in situ* endometrium (6). The collagen-rich argyrophilic fibers are well-preserved in nonperimenstrual explants cultured with physiological concentrations of sex steroids, as observed *in situ* during phases of the menstrual cycle when sex steroids are abundant. In contrast, these explants shrink and their ECM is degraded when sex steroids are omitted during culture, reproducing the drop of sex steroid plasma concentrations before menstruation. Cycloheximide suppressed the degradation of the fibers in such a condition, indicating that it requires protein synthesis. Moreover, the kinetics of fiber degradation is in keeping with the time course of menstrual regression *in situ*, as well as in intraocular endometrial transplants in Rhesus monkeys (1). Admittedly, some components are absent from the experimental system, in particular the blood circulation that brings plasminogen/plasmin and neutrophil leukocytes. However, the latter are not essential since menstruation induced *in vivo* by mifepristone occurs without leukocytic infiltration of the endometrium (37). Moreover, addition of plasminogen/plasmin to the culture medium did not alter the pattern of degradation of argyrophilic fibers, nor could plasmin by itself degrade these fibers.

More importantly, this study provides the awaited direct evidence (3, 25) that MMPs are responsible for initiating the breakdown of the collagen-rich fibers network of the human endometrium, at least in culture conditions that mimic menstrual regression. Both morphological and biochemical data indeed demonstrate that inhibitors of MMPs completely prevent the menstrual-like degradation of argyrophilic fibers and of collagen, in particular of collagens I, III, and IV. This protective effect on the matrix, obtained with four distinct specific inhibitors of MMPs, but not with inhibitors of the three other classes of proteinases, is not due to cell toxicity because it was fully reversible in living explants and was also observed in a purely *in vitro* test, where the degrading activity was transferred with conditioned media to previously fixed tissue. Of course, we cannot rule out the possibility that other metalloproteinases not belonging to the MMPs participate in the endometrial matrix breakdown. This possibility is however unlikely in view of the substrate specificities, the hormonal control, and the exclusive perimenstrual expression of several MMPs (19–24).

It is likely that several MMPs are directly involved in endometrial matrix breakdown. The identification of the key enzyme in the initiation of the degradation and the respective contribution of the various MMPs remain unknown at this stage, and more selective inhibitors are needed to clarify this issue. Although MMP(s) clearly initiates the degradation of collagen fibers, involvement of other enzymes downstream can be expected. In particular, whether one or several cysteine-proteinases also participate at some further step of matrix degradation needs additional study. Proteinases of the plasminogen/plasmin system are not required for tissue degradation in our culture system, but their involvement *in vivo* cannot be excluded.

The MMP zymogens need to be converted into their active forms to exert their proteolytic effects. The activation of both proMMP-2 and proMMP-9 clearly depends on one or several MMPs, such as a membrane type-MMP (35, 36) and MMP-3 (38). The physiological activators of procollagenase are still unknown, but plasmin, kallikrein, and cathepsin B are good candidates (16). In our culture system, serine- and cysteine-proteinase inhibitors did not prevent the activation of procollagenase, indicating that these enzymes are not required. Alternative pathways of activation involving MMPs (39) could compensate for the lack of serine- or cysteine-proteinase activities.

Our demonstration that MMPs play a crucial role in menstrual breakdown clarifies this physiological process. In turn, this knowledge may lead to unravelling the mechanisms of abnormal endometrial bleeding and pave the way to new diagnostic and therapeutic prospects. In particular, it may help to better manage menorrhagias and dysfunctional uterine bleeding, which too often lead to surgical removal in perimenopausal women (40). Moreover, if inhibitors of MMPs prevent endometrial bleeding, their pharmacological use could greatly improve the tolerance of long acting steroids that are being proposed for world-wide use to control human populations (41).

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