Progesterone regulates the activity of collagenase and related gelatinases A and B in human endometrial explants

(matrix metalloproteinases/extracellular matrix)

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ABSTRACT   Explants of human endometrium were cultured to study the release of matrix metalloproteinases (MMPs). Analysis of conditioned media by zymography revealed latent and active forms of collagenase (MMP-1, EC 3.4.24.7), 72-kDa gelatinase A (MMP-2, EC 3.4.24.24), and 92-kDa gelatinase B (MMP-9, EC 3.4.24.35). These proteinases were identified by their M_r, their inhibition by tissue inhibitor of metalloproteinases, and the activation of their zymogens by trypsin or aminophenylmercuric acetate. In the absence of sex hormone, explants released large amounts of enzyme activities, as measured by densitometry of zymograms or in soluble assays. Physiological concentrations of progesterone (10–200 nM) almost totally abolished the release of collagenase, of total gelatinase activity, and of the active form of gelatinase B and largely inhibited the release of the active form of gelatinase A. These effects, which were antagonized by mifepristone (RU 38486), suggest that progesterone restrains endometrial tissue breakdown by blocking the secretion and activation of MMPs.

The stroma of the human endometrium shows striking structural changes, especially during the second half of the menstrual cycle (1). If no pregnancy develops, interstitial shrinkage due to extracellular-matrix breakdown occurs and leads to hemorrhagia and mucosal shedding. The mechanisms of endometrial-tissue breakdown initiated by the fall of plasma progesterone and estradiol are still obscure. A major role of lysosomal enzymes (2) is not supported by biochemical evidence (3), whereas matrix metalloproteinases (MMPs), a family of neutral nonlysosomal enzymes, appear to be plausible agents of the remodeling of the extracellular matrix of the human endometrium (4).

MMPs share similar structural domains and properties (for review, see ref. 5), are secreted as zymogens in the extracellular space, are activated by proteolytic cleavage, and are inhibited by the tissue inhibitors of metalloproteinases (TIMPs). A key feature in the regulation of extracellular-matrix degradation seems to be a fine local balance between MMPs, their activators, and their inhibitors. Interstitial collagenase (MMP-1, EC 3.4.24.7) specifically cleaves the fibrillar collagens into one-fourth and three-fourth fragments, which are susceptible to further degradation by other proteases, among which are two other MMPs, gelatinase A (MMP-2, 72 and 65 kDa, EC 3.4.24.24) and gelatinase B (MMP-9, 92 and 84 kDa, EC 3.4.24.35). Both enzymes are also able to degrade other substrates present in human endometrium (1), such as collagen types V and IV. A third subclass of MMPs has a broad spectrum of substrates and comprises stromelysin 1 (MMP-3, EC 3.4.24.17) and matrilysin (MMP-7, EC 3.4.24.23).

We have studied the possible involvement of MMPs in human endometrial remodeling, using a culture system that maintains the tissue in a similar microenvironment as *in situ* and responds to sex hormones for several days (3). This system allowed us to demonstrate that the human endometrium secretes collagenase and gelatinases A and B. The secretion and activation of these MMPs were inhibited by physiological concentrations of progesterone, and these effects were antagonized by mifepristone.

MATERIALS AND METHODS

Organ Culture. Specimens were obtained from biopsies for histological dating of the endometrium; these biopsies were done with the approval of the Ethical Committee of the University of Louvain. Samples were collected in ice-cold sterile phosphate-buffered saline, pH 7.4, and processed for organ culture as described (3). Because calf serum contains gelatinase, this activity was studied only in medium conditioned without calf serum. In procedure 1, explants were adapted to culture for 1–2 days with 5% (vol/vol) charcoal-treated calf serum (GIBCO) and rinsed; the culture was extended for 1–3 days with serum-free medium renewed daily. In procedure 2, serum was omitted from the start of the culture, and no effect on tissue viability was noticed. At the end of the culture, wet explants were weighed and either frozen at −80°C for biochemical analysis or fixed for morphological evaluation. Thawed explants were solubilized (3), and their protein content was measured with bicinchoninic acid (6). Conditioned medium was supplemented with 0.05 vol of 1 M Tris buffer, pH 7.5/1% (vol/vol) Triton X-100/0.1 M CaCl$_2$/60 mM NaN$_3$ and kept frozen at −80°C until use. Enzyme activities were normalized to the weight or the protein content of the explants.

Hormonal Treatments. A "micronized" form of progesterone (mikro 20, from Schering) was dissolved in ethanol. Final concentration was adjusted by measuring progesterone adsorption (7) with [^{3}H]progesterone (70 Ci/mmol, 1 Ci = 37 GBq; Amersham). Mifepristone (RU 38486, from Roussel-Uclaf) was dissolved in ethanol and used at 5 or 10 μM nominal concentration. In procedure 1, control culture medium contained no ethanol, whereas in procedure 2 the medium contained the same concentration of ethanol as progesterone-containing medium, ranging between 0.05% and 0.5%. No difference in tissue viability was detected between these conditions by histological examination or assay (8) of the daily release of lactate dehydrogenase.

Assay of Collagenase. Collagenase activity was determined at 25°C with ^{3}H-acetylated collagen in solution (9). One unit of collagenase is defined as the amount of enzyme that degrades 1 μg of soluble collagen per min. Collagenase was

optimally activated either by a 2-hr preincubation at 37°C with 2 mM 4-aminophenylmercuric acetate (APMA; Aldrich) or by a 2-min preincubation at 25°C with 200 nM trypsin (Worthington) followed by a 10-fold excess of soybean trypsin inhibitor (Cooper Biomedical). Inhibition of activated collagenase by endogenous TIMPs was prevented by preincubation of the samples (30 min at 25°C) with 2 mM dithiothreitol (10) followed by a 3-fold dilution with 50 mM Tris·HCl buffer, pH 7.5/150 mM NaCl/5 mM CaCl$_2$/NaN$_3$ at 0.2 mg/ml/0.05% (vol/vol) Triton X-100/10 μM ZnCl$_2$. The partial ($\approx$50%) inhibition of collagenase by 0.7 mM dithiothreitol was reversed by adding 2 mM cystine before activation. This method was shown to completely prevent the total inhibition of endometrial collagenase at 0.6 unit/ml caused by exogenous purified bovine TIMP at 5 units/ml (from Rhône-Poulenc Santé, Vitry-sur-Seine, France).

Assays of Gelatinases. Gelatinase activity was measured in a soluble assay with [^{3}H]gelatin (11) either directly or after activation at 25°C with 0.4 mM APMA for 15 min. Under our assay conditions (16-hr incubation at 37°C), the activity was directly proportional to the amount of enzyme up to 50% substrate degradation. One unit of gelatinase activity is defined as the amount of enzyme that degrades 1 μg of gelatin per min into 12% ice-cold trichloroacetic acid-soluble fragments.

Latent and active forms of gelatinases A and B were also analyzed by gelatin–substrate zymography (12). Unreduced samples were preincubated for 30 min at 25°C in the presence of 2% SDS and submitted to SDS/PAGE on 8 or 10% polyacrylamide (Serva) slab gels copolymerized with gelatin at 0.5 mg/ml (Sigma). SDS was then removed by rinsing with 2.5% Triton X-100, and the gel was incubated for 18 hr at 35°C in 50 mM Tris·HCl buffer, pH 7.5/5 mM CaCl$_2$/NaN$_3$ at 0.2 mg/ml/1% (vol/vol) Triton X-100/1 μM ZnCl$_2$, stained with 0.25% (wt/vol) Serva blue G (Serva), destained with 7% (vol/vol) acetic acid, and analyzed by densitometry. An internal standard was run in each slab gel, and the activities were measured in the linear range of the zymographic assay with at least two dilutions of the conditioned medium.

Identification of MMPs and of Their Inhibitors. Endometrial MMPs were compared on gelatin or identically prepared casein (Merck) zymograms with purified human collagenase, gelatinases A and B, and stromelysin (from H. Nagase, University of Kansas Medical Center, Kansas City, KS) in their latent forms and after APMA (1 mM, 60 min at 37°C) or trypsin activation (400 nM, 10 min at 37°C, followed by trypsin inactivation with 2 mM phenylmethanesulfonyl fluoride; Sigma). Inhibition of gelatin degradation was tested with 1 mM 1,10-phenanthroline (Sigma), 2 mM phenylmethanesulfonyl fluoride, 12 μM leupeptin (Protein Research Foundation, Osaka), 1 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64; Sigma) and bovine TIMP at 50 units/ml. Endogenous TIMPs were detected on 13% polyacrylamide-gelatin reverse zymograms, incubated for 24 hr at 37°C in 20 ml of buffer supplemented with 1 ml of APMA-treated (0.4 mM, 2 hr at 25°C) medium conditioned by cultured mouse calvaria.

Statistical Analysis. The nonparametric Wilcoxon two-sample test was used to detect significant differences.

RESULTS

Characterization of Endometrial MMPs and Their Inhibitors. Collagenase was identified in conditioned medium by its activity on native trypsin-resistant collagen in solution at 25°C, by the electrophoretic detection of the characteristic one-fourth and three-fourth breakdown fragments of collagen (data not shown), and by its inhibition by 1,10-phenanthroline and bovine TIMP. Collagenase was entirely latent in most
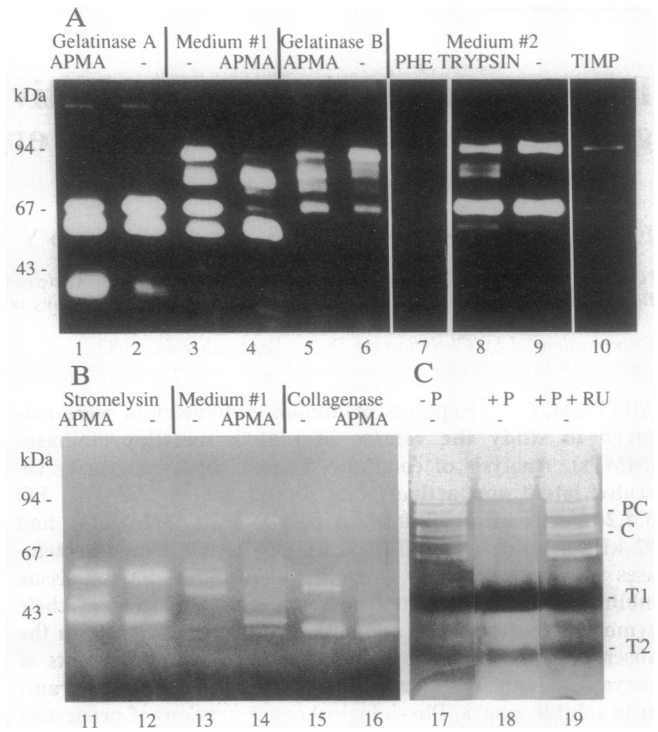

FIG. 1. Characterization of endometrial MMPs and TIMPs. Conditioned media from two cultures and the indicated purified human proMMPs were treated as indicated and analyzed by gelatin (A), casein (B), or reverse gelatin (C) zymography on 8% (A), 10% (B), or 13% (C) polyacrylamide slab gels. Lanes 7 and 10 were incubated separately with either 1,10-phenanthroline (PHE) or TIMP. Media of C were conditioned by explants from the same endometrium, cultured without sex hormone ($-$P), with 200 nM progesterone ($+$P), or with progesterone and 10 μM mifepristone ($+$P$+$RU) and normalized to the protein content of the explants. Procollagenase (PC), collagenase (C), TIMP 1 (T1), and TIMP 2 (T2) are localized in C.

conditioned media and could be activated by APMA or trypsin.

Zymographic analysis disclosed four major bands of neutral gelatinolytic activity (Fig. 1A, lane 3), all inhibited by 1,10-phenanthroline and by bovine TIMP but not inhibited by serine or cysteine proteinase inhibitors. The relative electrophoretic mobilities of these bands were identical to those of human progelatinase B (band 1) or gelatinase B (band 2) and progelatinase A (band 3) or gelatinase A (band 4). Upon activation by APMA (lane 4), the activity of the latent forms (bands 1 and 3) decreased concomitantly with the increase of active forms (bands 2 and 4). Trypsin (lane 8) had a similar effect on progelatinase B but no significant effect on progelatinase A. The activation of both progelatinases frequently generated multiple bands poorly resolved on slab-gel scans. Taken together, these data identify the first band as the latent 92-kDa gelatinase B, and the multiple second bands as its activated forms. Similarly, the third band corresponds to the latent 72-kDa gelatinase A, and the multiple fourth bands correspond to its activated forms.

Concentrated medium revealed additional bands on gelatin and casein (Fig. 1B) zymograms, corresponding respectively to prostromelysin, procollagenase, stromelysin, and collagenase. Reverse zymography (Fig. 1C) disclosed inhibitors of the same relative electrophoretic mobilities as TIMP-1 and TIMP-2.
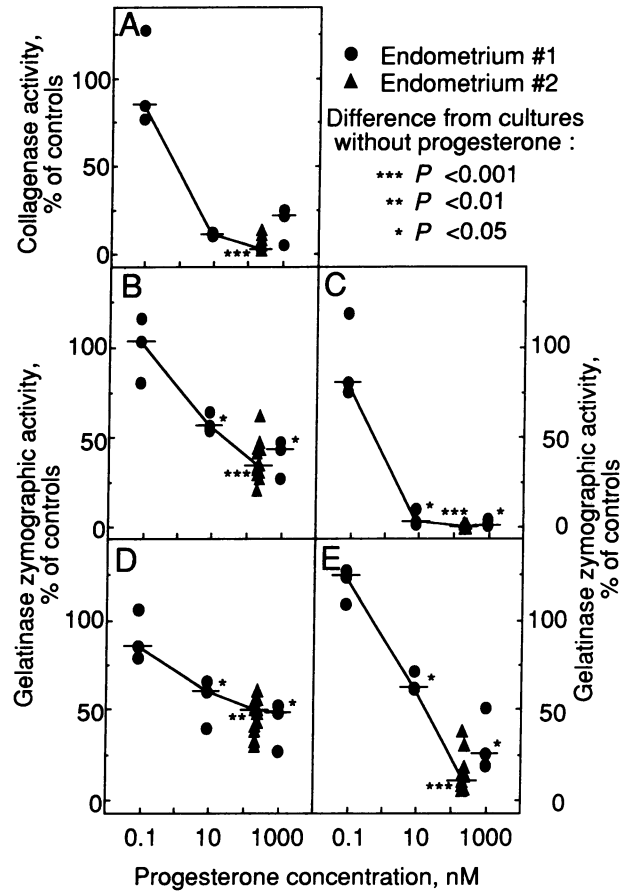
Time Course and Progesterone Regulation of the Release of Endometrial Collagenase and Gelatinase Activities. The rate of release of collagenase and gelatinase activities measured in solution after APMA activation increased >10-fold for collagenase and $\approx$50-fold for gelatinase in cultures without
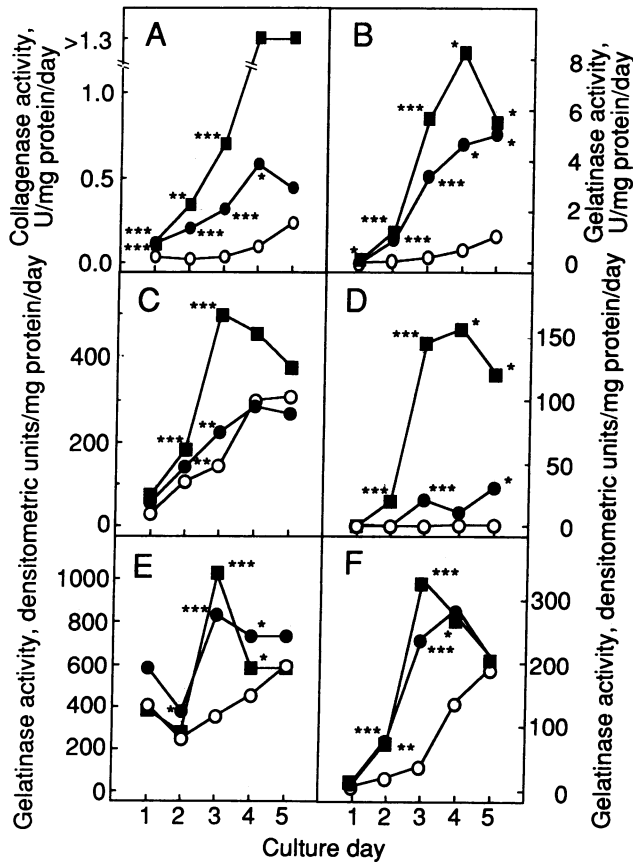
FIG. 2. Time course of the release of collagenase and gelatinases. Collagenase (*A*) and gelatinase (*B*) activities were measured in solution after APMA activation and by gelatin zymography. (*C*) Total gelatinase B. (*D*) Active form of gelatinase B. (*E*) Total gelatinase A. (*F*) Active form of gelatinase A. Endogenous TIMPs were inactivated before assaying collagenase. Each point corresponds to the median of activities released by groups of explants cultured (procedure 2) without progesterone (■, *n* = 5), with 200 nM progesterone (○, *n* = 11 until 54 hr of culture, thereafter *n* = 3) or with 200 nM progesterone and 10 μM mifepristone (●, *n* = 10 until 54 hr of culture, thereafter *n* = 5). Statistical comparisons were made with activities released in the presence of progesterone. ★, *P* < 0.05; ★★, *P* < 0.01; and ★★★, *P* < 0.001.

FIG. 3. Effect of progesterone concentration on collagenase and gelatinases. Explants from two endometria were cultured (procedure 2) in the presence of the indicated progesterone concentrations. (*A*) Collagenase activities released between 26 and 51 hr (●) and 42 and 65 hr (▲) of culture were measured after inactivation of TIMPs and treatment with APMA. Gelatinase activities released between 51 and 80 hr (●) and 42 and 65 hr (▲) were measured by zymography. (*B*) Total gelatinase B. (*C*) Active form of gelatinase B. (*D*) Total gelatinase A. (*E*) Active form of gelatinase A. Activities in media conditioned with progesterone are expressed in percentage of medians of activities released without progesterone (controls). For each progesterone concentration, 3 (●) or 11 (▲) conditioned media were analyzed, and the median is indicated by a bar. ★, *P* < 0.05; ★★, *P* < 0.01; ★★★, *P* < 0.001.

hormone from day 1 to day 4 (Fig. 2 *A* and *B*). Progesterone almost totally inhibited this release and was antagonized by mifepristone. No significant activities were detected when the conditioned media were not activated.

The individual gelatinases were further analyzed by densitometry of zymograms (Fig. 2 *C–F*). This method allowed the discrimination of their latent and active forms and the dissociation of preexisting gelatinase–TIMP complexes (5). Zymographic activities correlated well with those assayed in solution after activation by APMA (culture 1, *n* = 8, *r* = 0.97; culture 2, *n* = 26, *r* = 0.88) but proved less reproducible. Moreover, zymographic analysis revealed the presence of spontaneously active gelatinases A and B (Fig. 2 *F* and *D*). In the absence of progesterone, the rate of release of total (i.e., latent and active forms) gelatinase B (Fig. 2*C*) increased ≈10-fold from day 1 to day 4, whereas that of total gelatinase A (Fig. 2*E*) increased, at most, 3-fold. Progesterone had more inhibitory effect on the release of total gelatinase B than of total gelatinase A and had much more effect on both active forms than their zymogens. The complete inhibition of active gelatinase B release from day 1 to day 5 was particularly striking. The antagonistic effect of mifepristone was more pronounced on gelatinase A than on gelatinase B and decreased or disappeared at the end of the culture. Reverse

zymograms indicated that progesterone inhibited the release of both latent and active forms of collagenase (Fig. 1*C*).

Effects of Progesterone Concentration on Release of Collagenase and Gelatinase Activities. When progesterone concentration was increased from 0.1 nM to 10 nM (Fig. 3), the release of collagenase was inhibited by ≈90% and that of active gelatinase B was completely inhibited, whereas that of active gelatinase A was only reduced by ≈50%. On the other hand, total release of gelatinases B and A was inhibited by ≈50% when progesterone concentration was increased from 0.1 nM to 100 nM.

Gelatinase activities released in the absence of progesterone varied largely between distinct endometria (Fig. 4), and no correlation could be found with the time of sampling during the menstrual cycle. Despite this variability, addition of progesterone constantly decreased the release of all forms of gelatinase activities.

DISCUSSION

This study demonstrates that explants of human endometrial tissue in culture secrete collagenase (MMP-1) and the related
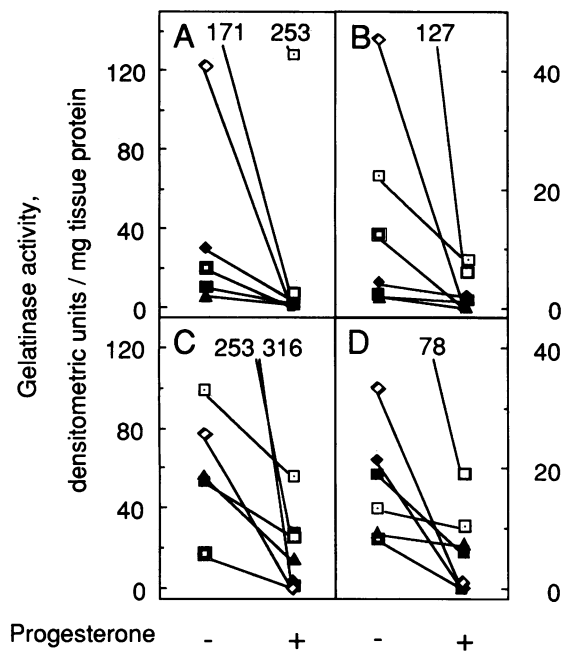
FIG. 4. Effect of progesterone on release of gelatinases. Gelatinases released during day 3 (without calf serum) by two to four groups of explants of seven endometria (procedure 1) were measured on zymograms. Medians of the activities released in the absence or the presence of 60–600 nM progesterone are linked and represented by a distinct symbol for each endometrium or by their absolute values when lying outside the scale. (*A*) Total gelatinase B. (*B*) Active form of gelatinase B. (*C*) Total gelatinase A. (*D*) Active form of gelatinase A.

gelatinases A (MMP-2) and B (MMP-9). These MMPs were identified by their characteristic M_r, their activation by APMA and/or trypsin, their activities on collagen or gelatin, and their inhibition by 1,10-phenanthroline and TIMP. Stromelysin, TIMP-1, and TIMP-2 were also detected.

When cultured without sex steroid, endometrial explants produce 10–100 milliunits of procollagenase per day and per mg of tissue. Such elevated activities are in the same range as those produced by explants of human embryonic, but 20-fold higher than human adult, skin (13). The rate of release of collagenase and gelatinase activities strikingly increases during the first days of culture. The affinity of collagenase for collagen and the presence of TIMPs could explain why no active collagenase and low gelatinase activities were found in the conditioned medium, when assayed with biochemical methods that do not detect TIMP–proteinase complexes. These complexes are dissociated in zymography (5), but activity comparisons between different bands of the same proteinase and between different proteinases must be interpreted with caution because the influences of the electrophoretic treatments on the zymographic activities are ignored. For example, zymographic activities of the zymogens could be underestimated, owing to partial refolding of the propeptide after removal of SDS.

In spite of these limitations and of the variable activities produced by endometrial explants in the absence of hormone (Fig. 4), our results demonstrate that physiological concentrations of progesterone inhibit the production of the three MMPs from the first to the third day of culture. These effects were antagonized by the progesterone-antagonist mifepristone (RU 38486). The inactivation of endogenous free TIMPs by dithiothreitol did not significantly increase the collagenase activities, thus indicating that the inhibitory effects of progesterone were not primarily due to an increased release of TIMPs. However, preliminary data (Fig. 1*C*) suggest a stimulatory effect of progesterone on the release of TIMP-1,

as already reported on rabbit uterine cervical fibroblasts (14, 15).

The influence of progesterone concentration on collagenase and on the zymographic activity of proenzyme and active forms of gelatinases A and B (Fig. 3) is of great interest because it confirms a trend appearing in Figs. 2 and 4. In both types of experiments, total collagenase and the active form of gelatinase B are clearly the most inhibited by progesterone, suggesting a prominent role of these two MMPs in endometrial remodeling. Gelatinases A and B have identical *in vitro* substrate specificities, but their transcriptional control and their activation mechanisms differ. Macrophages and neutrophils constitutively secrete gelatinase B but do not secrete gelatinase A, whereas skin fibroblasts constitutively produce gelatinase A but produce very low amounts of gelatinase B (16). The regulatory elements found in the 5'-end flanking region of the human gene for collagenase (5) and gelatinase B (17) differ from those found in the gelatinase A promoter (18). The transcriptional regulation of collagenase and gelatinase B actually differs from that of gelatinase A in rabbit corneal fibroblasts (19).

The physiological activators of the zymogens of collagenase and gelatinases A and B are still unknown. Plasmin, kallikrein, and lysosomal cysteine proteinases are potential activators of procollagenase (20). The marked inhibitory effect of progesterone on the release of the two plasminogen activators (t-PA and u-PA) from endometrial tissue in culture (21) points to the plasminogen activator/plasmin cascade as the most likely activator of endometrial procollagenase. Stromelysin has been implicated in the full activation of procollagenase (22), in the activation of progelatinase B (23), and in the degradation of a variety of extracellular matrix proteins (24). Stromelysin is produced by a mixture of ovine endometrial epithelial and stromal cells in primary culture (25) and was detected in our organ culture system of human endometrium, where its role remains to be examined. Progelatinase A is not activated by any known proteinase (26), but it can be activated by a fibroblast cell surface activator (27).

Inhibition by progesterone of the production and/or activation of procollagenase has been observed in other tissues or cells: postpartum rat uterine explants (28) and smooth muscle cells in culture (29), rabbit (14) and guinea pig (30) uterine cervical fibroblasts, and peritoneal macrophages (31). On the other hand, progesterone had no effect on the production of collagenase by human myometrial smooth-muscle cells (32). In contrast, synthesis by stromal cells of human endometrium of a membrane metalloproteinase (EC 3.4.24.-11), acting on enkephalins and bioactive peptides, is stimulated by progesterone (33).

In summary, our data indicate that collagenase and related MMPs participate in the breakdown of the extracellular matrix of the human endometrium. The rapid and striking inhibitory effect of progesterone on the release and activation of several MMPs from endometrial explants in culture supports the hypothesis that progesterone controls the maintenance of the endometrial connective tissue by downregulating MMPs. Further investigation of the cellular origin and the transcriptional control of collagenase, related MMPs, and TIMPs in human endometrium should help clarify the molecular and cellular mechanisms of endometrial remodeling.

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