In vivo perimenstrual activation of progelatinase B (proMMP-9) in the human endometrium and its dependence on stromelysin 1 (MMP-3) *ex vivo*

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Most matrix metalloproteinases (MMPs) are secreted as inactive proenzymes. Their expression is well documented in several human tissues, but their activators *in vivo* are still unknown. To address this question, the activation of progelatinase B (proMMP-9) in the human endometrium was selected as a model system. ProMMP-9 was detected by gelatin zymography in homogenates of fresh endometrial tissue sampled during all phases of the menstrual cycle, whereas its active form was observed only during the late secretory and menstrual phases. Furthermore, proMMP-9 was expressed and activated in endometrial explants sampled outside the perimenstrual phase and cultured in the absence of both progesterone and oestradiol, mimicking the menstrual condition *in vivo*. Analysis of such tissue cultures by gelatin zymography and Western blotting showed that activation of proMMP-9 depended on a secreted factor and was selectively inhibited by either a synthetic inhibitor of stromelysin 1 (MMP-3) or a monoclonal antibody that specifically blocks MMP-3, thus providing strong evidence for the activation of proMMP-9 *in vivo* by MMP-3. The activation of proMMP-3 was itself inhibited by a broad-range MMP inhibitor in most cultures, but seemed to involve multiple pathways, implying both serine proteinases and metalloproteinases, which could operate in parallel or sequentially.

Key words: matrix metalloproteinases, menstruation and proenzymes.

INTRODUCTION

Several matrix metalloproteinases (MMPs) are implicated in physiological and pathological processes involving the breakdown of extracellular matrix, such as remodelling of bone and endometrium, wound healing, arthritis and tumour invasion [1]. Because most MMPs are secreted as latent proenzymes (proMMPs), their activation by proteolytic cleavage of the N-terminal propeptide represents a critical step for their action. Potential proteolytic activators of human proMMPs have been identified *in vitro* by using purified enzymes or cell cultures [2], but their effective role in humans *in vivo* can currently only be extrapolated from gene-targeting studies in mice. We believe that investigations on freshly isolated human tissues could contribute to the identification of the relevant activators and that the human endometrium represents a most suitable model for this goal. Indeed, the cyclic, hormonally controlled, extracellular matrix breakdown and shedding of this tissue can be mimicked in short-term cultures of tissue explants and depends on the activity of MMPs [3]. The expression of several proMMPs, including proMMP-1, proMMP-3 and proMMP-9, appears or increases *in vivo* in the human endometrium after the decrease in plasma concentration of the ovarian steroids progesterone and oestradiol, i.e. just before and at the beginning of menses, as it does in cultured endometrial explants upon deprivation of these hormones [4,5]. Moreover, the activation of proMMPs has been observed in this experimental model and has been shown to be inhibited by physiological concentrations of the ovarian steroids [4].

The present study focuses on the identification of the effective activator(s) of proMMP-9 in the human endometrium for the following reasons. Previous studies *in vitro* have reported the activation of human proMMP-9 by MMP-3 [6–8], MMP-2 [9], MMP-13 [2], MMP-7 and MMP-1 [10]. Serine proteinases such as tissue kallikrein [11] and plasmin [12,13] are also able to activate proMMP-9; plasmin potentiates the action of MMP-3 on proMMP-9 [14]. However, in plasminogen knock-out mice, the active form of MMP-9 is not found in the aortic wall, but is still detectable in the culture medium of derived fibroblasts [15]. Alternatively, proMMP-9 is still activated in MMP-3-deficient mice, presumably by a plasmin-dependent mechanism [16]. The present investigation of the activation of proMMP-9 in the human endometrium, *in vivo* and in short-term tissue cultures, provides evidence for a critical role of MMP-3 *in vivo* in this process.

EXPERIMENTAL

Materials

Enhanced chemiluminescence (ECL®) detection kit and nitrocellulose membranes were purchased from Amersham (Roosendaal, The Netherlands), gelatin and Tween 20 from Merck (Darmstadt, Germany), acrylamide/bisacrylamide mix and ammonium persulphate from Bio-Rad (Nazareth Eke, Belgium), and 4-aminophenylmercuric acetate (APMA) was from Aldrich (Bornem, Belgium). Aprotinin was from Boehringer (Mannheim, Germany), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), dichloroisocoumarin (DIC) and gelatin-agarose beads were from Sigma (St Louis, MO, U.S.A.), and human plasmin and its chromogenic substrate S-2403 from Chromogenix (Mölndal, Sweden). The broad-range MMP inhibitor SC-44463 [17] was provided by Dr P. Mitchell (Pfizer,

Abbreviations used: APMA, 4-aminophenylmercuric acetate; DIC, dichloroisocoumarin; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; MMP, matrix metalloproteinase; SC, SC-44463; L-758,354, {*N*-(4*S*)-(n-butyl)-(2*R*)-[2-(4′-fluoro-4-biphenyl)ethyl-1-glutaryl]-(2*S*)-t-butyl-glycine, *N*-methylamide}.

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Groton, CT, U.S.A.). *N*-(4*S*)-(n-butyl)-(2*R*)-[2-(4′-fluoro-4-biphenyl)ethyl-1-glutaryl]-(2*S*)-t-butyl-glycine, *N*-methylamide (L-758,354), an inhibitor of MMP-3 [18], was a gift from Dr P. L. Durette (Merck Sharp & Dohm, Rahway, NJ, U.S.A.).

Antibodies

Mouse monoclonal antibodies anti-(human MMP-1) (IgG1/κ, clone 78-12G8) [19], anti-(human MMP-3) (IgG1/κ, clone 55-2A4) [20] and anti-(human MMP-9) (A-9) (IgG1/κ, clone 56-2A4) [21] were gifts from Dr Y. Okada (Keio University, Tokyo, Japan) and Dr K. Iwata (Fuji Chemical Industries, Toyama, Japan). Sheep anti-(human MMP-3) antibodies were a gift from Dr H. Nagase (London, U.K.). Horseradish-peroxidase-conjugated rabbit antibodies directed against sheep or mouse immunoglobulins were obtained from DakoA/S (Glastrup, Denmark) and Amersham (Roosendaal, The Netherlands) respectively.

Tissue explant culture and homogenization

The study was approved by the Ethical Committee of the University of Louvain, in accordance with the Declaration of Helsinki of the World Medical Association. Normal endometrial tissue was obtained from either biopsies performed for histological dating or from hysterectomy specimens, collected in ice-cold PBS, pH 7.4, and divided into two parts. Fresh tissue fragments were suspended in 50 mM Tris/HCl, pH 7.5, containing 10 mM $CaCl_2$, 150 mM NaCl, 1 μM $ZnCl_2$, 60 mM NaN_3 and 0.1 % (w/v) Triton X-100, homogenized with a Dounce homogenizer and processed for biochemical analysis. Alternatively, tissue explants were cultured as described previously [3,22]. In brief, tissue pieces approx. 1 mm on a side were cut with a sterile surgical blade and transferred to Biopore membrane of Millicell-CM inserts (12 mm; Millipore, Bedford, MA, U.S.A.). Medium was placed in the lower chamber and replaced daily; it was supplemented or not by either inhibitors or vehicle [0.1 % (v/v) DMSO], by specific or irrelevant mouse monoclonal antibodies at 10 μg/ml, or by 1 nM oestradiol and 100 nM progesterone (E+P). After collection, all conditioned media were 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 at 4 or −20 °C until use. Media conditioned during the second day of culture are referred to as media of day 2. Explants from a total of 20 endometria (10 proliferative, i.e. from day 6 to day 15 of the menstrual cycle; 6 secretory, day 16 to day 26; 4 perimenstrual, day 27 to day 5) were analysed in this study. In some experiments, homogenates of cultured tissue explants were prepared as described above for fresh tissue fragments.

Gelatin zymography

ProMMP-9, MMP-9, proMMP-2 and MMP-2 were analysed by gelatin-substrate zymography as described [23]. Standard medium was prepared by mixing 10 μl of a solution of molecular-mass markers (Bio-Rad) with 3 μl of a conditioned medium containing MMP-9, proMMP-2 and MMP-2.

Immunoblotting of MMP-3 and MMP-9

Conditioned media were subjected to SDS/PAGE under non-reducing (for MMP-3) or reducing conditions (for MMP-9) and electrotransferred to a nitrocellulose membrane (Hybond-C extra; Amersham, Little Chalfont, Bucks., U.K.) for 1 h at 100 V. Non-specific binding sites were saturated for 2 h at room temperature with PBS containing 5 % (w/v) non-fat dried milk (Gloria®; Nestlé, Vevey, Switzerland) and 0.1 % (v/v) Tween 20. Blots were incubated overnight at 4 °C with the primary antibodies at 0.5 μg/ml. After five washes, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated rabbit antibodies directed against sheep or mouse immunoglobulins, as appropriate. After five washes, blots were revealed by the enhanced chemiluminescence system. For detection of MMP-9, 100 μl of conditioned medium was preincubated for 2 h with 20 μl of packed gelatin-agarose beads that were recovered by centrifugation, to achieve a 3-fold concentration of the gelatinases. Beads were then resuspended in the sample buffer for electrophoretic analysis supplemented with 1 mM dithiothreitol and incubated for 5 min at 100 °C.

Enzyme assays

Total collagenase activity was determined with [^{3}H]acetylated collagen in solution at 25 °C after activation by a 2 h preincubation at 37 °C with 2 mM APMA, as described [24]. Total gelatinase activity was measured with [^{3}H]gelatin in solution at 37 °C after 1 h of activation at 25 °C with 0.4 mM APMA [4,25]. Plasmin activity was quantified with the Chromogenix substrate S 2403 in accordance with the manufacturer's instructions, with purified plasmin as standard.

RESULTS

Continuous presence but restricted activation of proMMP-9 in the cycling human endometrium

To the best of our knowledge, the state of activation of human proMMP-9 has not yet been reported in the endometrium during the menstrual cycle. To address this issue, fresh fragments of endometrium, collected at different phases of the cycle, were homogenized and analysed by gelatin zymography (Figure 1). Whereas monomeric proMMP-9 (92 kDa) was observed throughout the cycle, its active form (82 kDa) was clearly observed only in premenstrual and menstrual samples. As a sole exception, a very faint band corresponding to active MMP-9 was detected in the homogenate of one case out of five analysed secretory samples, and none was found in the proliferative samples. These results indicate that significant activation of proMMP-9 is a characteristic feature of the perimenstrual period. The 130 kDa form, described as proMMP-9 in complex with lipocalin [26] and corresponding to neutrophil gelatinase B, was also detected in the fresh endometrial extracts, but it represented only a minor form. ProMMP-2 (72 kDa) did not vary signifi-

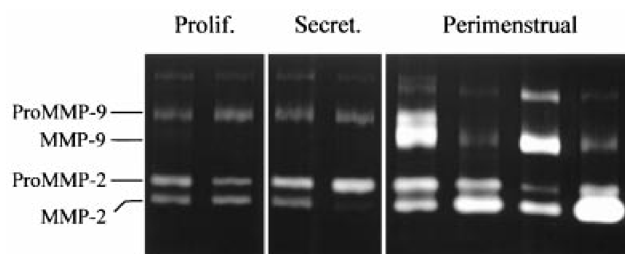

Figure 1 Occurrence and state of activation of endometrial proMMP-9 throughout the menstrual cycle

Tissue samples collected at different phases of the menstrual cycle were homogenized; equal protein loads were analysed by gelatin zymography. Abbreviations: Prolif., proliferative phase (day 6 to day 15), representative of samples from three patients; Secret., secretory phase (day 16 to day 26), representative of samples from five patients; Perimenstrual, premenstrual and menstrual phases (day 27 to day 5), representative of samples from six patients.

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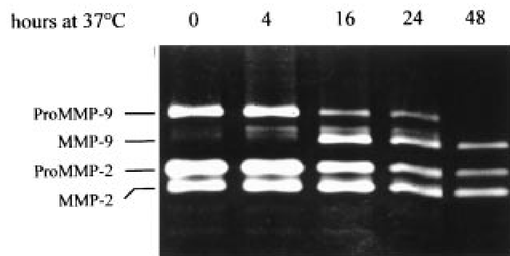

Figure 2 Time course of proMMP-9 activation in conditioned medium

Conditioned medium of day 2 from a secretory endometrium was incubated at 37 °C for the indicated durations and analysed by gelatin zymography. This result is representative of experiments on four distinct endometria.

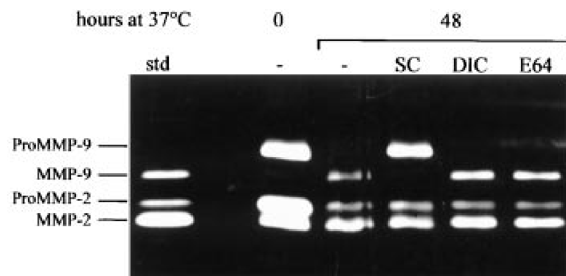

Figure 3 Effect of proteinase inhibitors on proMMP-9 activation in conditioned medium

The same conditioned medium of day 2 as that used in Figure 2 was incubated at 37 °C for 48 h without or with the indicated proteinase inhibitors [5 μM SC-44463 (SC), a broad-spectrum MMP inhibitor; 100 μM DIC, a serine proteinase inhibitor] and analysed by gelatin zymography or with 60 μM E-64 (E64), a cysteine proteinase inhibitor. Abbreviation: std, MMP standards. This result is representative of experiments on two distinct endometria.

cantly during the cycle, whereas MMP-2 (62 kDa) increased during the perimenstrual period.

Spontaneous activation of proMMP-9 *in vitro* occurs in medium conditioned by endometrial tissue

ProMMP-9, proMMP-2 and MMP-2 were also systematically found in conditioned media of day 2, whereas the proMMP-9–lipocalin complex was detected in only one-third of the cultures. A first series of experiments was therefore performed to determine whether proMMP-9 activation required the presence of a tissue-associated factor. Conditioned media of endometrial explants, collected at the end of the second day of culture and selected for the presence of proMMP-9 without detectable MMP-9, were incubated as such at 37 °C for increasing times and analysed by gelatin zymography. As illustrated in Figure 2, a time-dependent activation of proMMP-9 was observed, whereas the active form of MMP-2 did not increase. This demonstrated that cultured endometrial tissue produced a soluble extracellular activator of proMMP-9, but not of proMMP-2.

Similar experiments were performed in the presence of various proteinase inhibitors to determine which class of proteinases might be involved in proMMP-9 activation (Figure 3). In contrast with inhibitors of serine (DIC) or cysteine proteinases (E-64), a broad-range inhibitor of MMPs (SC-44463) blocked the activation of proMMP-9, indicating the involvement of an MMP.

Figure 4 Effect of proteinase inhibitors on proMMP-9 activation during tissue culture

Human endometrial tissue explants (proliferative phase) were cultured in the absence of ovarian steroids with 5 μM SC-44463 (SC), 100 μM DIC, 60 μM E-64 (E64) or 5 μM SC-44463 combined with 100 μM DIC, or in the presence of 1 nM oestradiol combined with 100 nM progesterone (E + P). Conditioned media of day 2 were analysed by gelatin zymography (**A**) or Western immunoblotting with anti-(MMP-9) antibodies (**B**). These results are representative of experiments on eight distinct endometria.

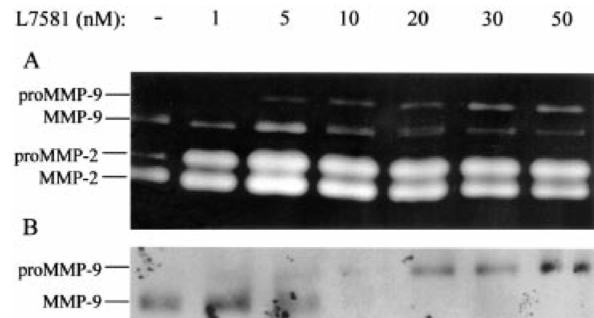

Figure 5 Concentration-dependent inhibition of proMMP-9 activation by an MMP-3 inhibitor during tissue culture

Endometrial explants (mid-secretory phase) were cultured for 2 days in the absence of ovarian steroids, with the indicated concentrations of L-758,354 (L7581), a specific inhibitor of MMP-3. Conditioned media of day 2 were analysed by gelatin zymography (**A**) or Western immunoblotting with anti-(MMP-9) antibodies (**B**). These results are representative of experiments on six distinct endometria.

Activation of proMMP-9 during culture of endometrial tissue explants depends on MMP-3

We have previously reported that culture of endometrial explants in the presence of various class-specific proteinase inhibitors alters neither tissue viability nor the release of several MMPs [3]. Proteinase inhibitors were therefore added from the beginning of explant culture, to check whether an MMP was indeed responsible for the activation of proMMP-9. Analyses by gelatin zymography (Figure 4A) and Western immunoblotting (Figure 4B) provided comparable patterns: activation of proMMP-9 was abrogated by the broad-spectrum MMP inhibitor (SC-44463), but was not affected by inhibitors of serine (DIC) or cysteine (E-64) proteinases. Similar experiments further revealed that a selective inhibitor of MMP-3 (L-758,354) prevented proMMP-9 activation in a concentration-dependent manner between 1 and 50 nM (Figure 5), in agreement with the reported K_i of 10 nM for MMP-3 [18]. We have ascertained that this MMP-3 inhibitor did not affect total collagenolytic activity, total gelatinolytic activity or MMP-3 activation in the tested samples (results not shown). Moreover, the specificity of the observed effect of L-758,354 was confirmed with monoclonal antibodies: activation of proMMP-

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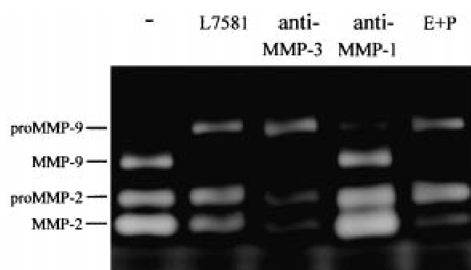

Figure 6 Inhibition of proMMP-9 activation by antibodies neutralizing MMP-3 during tissue culture

Endometrial explants (mid-secretory phase; different patient from that in Figure 5) were cultured for 2 days in the absence of ovarian steroids, with 10 nM L-758,354 (L7581), 10 μg/ml anti-(MMP-3) antibodies or 10 μg/ml anti-(MMP-1) antibodies, or with 1 nM oestradiol combined with 100 nM progesterone (E + P). Conditioned media of day 2 were subjected to gelatin zymography. This result is representative of experiments on three distinct endometria.

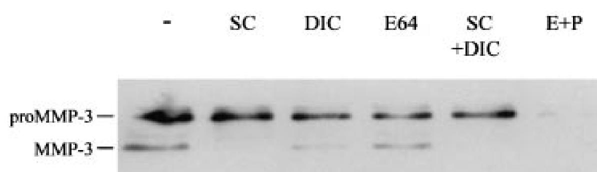

Figure 7 Suppression of proMMP-3 activation during tissue culture by an MMP inhibitor

Endometrial explants (early secretory phase) were cultured for 2 days with 5 μM SC-44463 (SC), 100 μM DIC, 60 μM E-64 (E64) or 5 μM SC-44463 combined with 100 μM DIC, or with 1 nM oestradiol combined with 100 nM progesterone (E + P). Conditioned media of day 2 were analysed by Western immunoblotting with anti-(MMP-3) antibodies. This results is representative of the three cases in which proMMP-3 activation was inhibited by SC-44463 alone (see the Results section).

9 was strongly inhibited by an anti-(MMP-3) antibody, but not by a control anti-(MMP-1) antibody (Figure 6). In addition, homogenates of cultured tissues showed a response to inhibitors and antibodies comparable to that observed in conditioned medium (results not shown). Taken together, these results establish that MMP-3 is responsible for the activation of proMMP-9 in cultured human endometrial tissue. As reported previously [4], activation of proMMP-9 was also strongly inhibited by the addition of ovarian steroids (1 nM oestradiol combined with 100 nM progesterone) to the cultures.

Mechanism of proMMP-3 activation

To further identify the activator(s) of proMMP-3, endometrial cultures were again performed in the presence of various proteinase inhibitors (Figure 7). The cysteine proteinase inhibitor E-64 did not affect proMMP-3 activation (six experiments). Total inhibition of proMMP-3 activation was observed in six of the nine cultures with the metalloproteinase inhibitor (SC-44463). The same score was obtained with serine proteinase inhibitors (DIC or aprotinin). It should also be noted that, in three of these experiments, there was a complete inhibition of proMMP-3 activation by SC-44463, but no inhibition by a serine proteinase inhibitor, indicating for the first time a metalloproteinase-dependent activation of proMMP-3. Interestingly, in three out of six cultures, both SC-44463 and serine proteinase inhibitors when used alone were sufficient to inhibit proMMP-3 activation totally, suggesting a sequential intervention of a serine proteinase and a metalloproteinase in these tissue samples, without defining their order.

DISCUSSION

The biosynthesis of most MMPs is tightly controlled at the transcriptional level, resulting in restricted expression whenever and wherever tissues are undergoing physiological or pathological breakdown. In addition, because most MMPs are secreted as latent proenzymes, subsequent activation represents a crucial step for their lytic action on extracellular matrix proteins. However, although the expression of MMPs in human tissues has been extensively studied, their actual mechanisms of activation remain largely unexplored and interesting clues provided by studies *in vitro* remain to be checked *in vivo*. In view of its periodical alternation of build-up and breakdown, the cycling human endometrium is a remarkable model tissue in which to study the physiological regulation of both the expression and activation of proMMPs.

The first part of this study demonstrates that, whereas proMMP-9 is detected in homogenates of fresh human endometrial tissue throughout the menstrual cycle, significant spontaneous activity of MMP-9 occurs only when tissues are sampled 1 or 2 days before or during menstruation. A recent report [27] also showed the occurrence of proMMP-9 without detectable MMP-9 in endometria that were all sampled outside the perimenstrual phase. In the cycling human endometrium, four MMP patterns can therefore be distinguished, which are exemplified as follows: (1) MMP-2 is expressed and partly activated throughout the entire menstrual cycle ([5], and this study); (2) MMP-1 and MMP-3 are expressed only during the perimenstrual phase [5]; (3) MMP-7 expression is strong during the perimenstrual phase, but moderate during the proliferative phase [5]; and (4) MMP-9 occurs as a zymogen throughout the menstrual cycle, but is significantly activated only in the perimenstrual phase (this study). Accordingly, the present work focused on the activation of human endometrial proMMP-9.

Several studies *in vitro* have shown that proMMP-9 can be activated by a variety of tissue proteinases, including the serine proteinases cathepsin G, tissue kallikrein, plasmin and mast-cell α-chymase, as well as MMP-1, MMP-2, MMP-3, MMP-7, MMP-10 and MMP-13 [28]. According to an extensive study [6], the activation of human proMMP-9 requires the intervention of a distinct activator. The observation of a conversion of human endometrial proMMP-9 *in vivo* into its active form led us to explore its mechanism in short-term tissue cultures. In this system, which closely mimics the *in vivo* situation, the expression and/or activation of several MMPs are down-regulated, if not totally suppressed, by physiological concentrations of the ovarian steroids progesterone and oestradiol [4,29]. In a first step, proMMP-9 in medium conditioned by endometrial tissue explants was found to be converted into active MMP-9 on incubation at 37 °C in the absence of tissue, in contrast with the lack of increase in MMP-2, indicating the presence of a soluble activator of proMMP-9. The use of various proteinase inhibitors revealed that, both in isolated conditioned medium and in short-term endometrial cultures, proMMP-9 activation was prevented only by inhibitors of MMPs, including a synthetic inhibitor of MMP-3. The specificity of the latter inhibition was confirmed by using a monoclonal antibody that selectively blocked MMP-3 activity. In these experiments, activation of proMMP-9 was analysed in parallel by gelatin zymography and Western blotting. Because homogenates of cultured explants showed a response to inhibitors and antibodies comparable with that observed in con-

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ditioned medium, it can be concluded that, in our *ex vivo* experimental system, MMP-3 is responsible for the activation of proMMP-9 in the tissue itself.

When using cultured endometrial explants, previous studies have indicated that the absence of blood circulation impairs neither menstrual tissue breakdown nor its control by the ovarian steroids [30]. Of course, blood leucocytes and especially plasma proteinases could also contribute to the activation of proMMP-9 *in vivo*, but our results show clearly that they are not required. There are conflicting reports on the cellular origin of endometrial proMMP-9 and on its expression pattern during the menstrual cycle [31–33]. Fresh tissue samples contain variable quantities of blood and leucocytes. Although our gelatin-zymographic analyses showed the occurrence of the 130 kDa proMMP-9–lipocalin complex, characteristic of neutrophils [34], it was much less abundant than the 92 kDa form of proMMP-9 and its release into the conditioned medium was inconstant. In addition, purified endometrial stromal cells could by themselves produce and activate proMMP-9 (results not shown). The apparent loss of MMP-9 upon prolonged incubation has been reported by others and might be due to its processing by MMP-3 or another proteinase into an inactive form [8].

Because proMMP-9 seemed to be activated by MMP-3 in endometrial explants, we finally attempted to identify which type of proteinase was responsible for the activation of proMMP-3. Surprisingly, in three out of nine cultures, proMMP-3 activation was blocked by the broad-range MMP inhibitor SC-44463, but not by an inhibitor of serine proteinases, indicating for the first time the involvement of a metalloproteinase in proMMP-3 activation. No purified MMP has yet been found capable of activating proMMP-3 *in vitro* [28], except MMP-3 itself [35]. However, the SC-44463-sensitive activation of proMMP-3 was insensitive to L-758,354 (results not shown). This observation might indicate that either a still unidentified MMP or a related SC-44463-sensitive metalloproteinase can activate proMMP-3 directly, or that a metalloproteinase is able to activate a serine proproteinase that can then activate proMMP-3. In three other cultures, proMMP-3 activation was blocked by both SC-44463 and serine proteinase inhibitors, whereas in the three other experiments it was blocked only by serine proteinase inhibitors. These results, which require further investigation, suggest alternative parallel and/or sequential pathways for proMMP-3 activation. The inhibition of proMMP-3 activation by an inhibitor of serine proteinase, observed in six of the nine cultures, does not contradict the absence of effect of such an inhibitor on the activation of proMMP-9, because the presence of active MMP-3 and of serine proteinases might vary considerably from one experiment to the other, reflecting differences between patients.

In summary, we have demonstrated that, whereas human endometrial proMMP-9 is present throughout the menstrual cycle, its activation is characteristic of the perimenstrual phase. By using a tissue culture system that mimics the situation *in vivo*, MMP-3 was identified as the physiological activator of proMMP-9. The activator(s) of proMMP-3 remain(s) to be identified but our results indicate both serine proteinases and metalloproteinases, acting in parallel or sequentially.

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