Focal Expression and Final Activity of Matrix Metalloproteinases May Explain Irregular Dysfunctional Endometrial Bleeding

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Irregular dysfunctional bleeding of the endometrium (ie, metrorrhagia without organic lesion) is common in women, whether treated or not with ovarian hormones. Several matrix metalloproteinases (MMPs) become normally expressed and/or activated at menstruation and cause extracellular matrix breakdown. We therefore explored whether episodes of irregular dysfunctional bleeding could be associated with untimely MMP activity. By histology, foci of stromal breakdown were exclusively found in the endometrium of metrorrhagic women at bleeding. In these foci, 1) expression of estrogen receptor- α and progesterone receptor was altered; 2) collagenase-1 (MMP-1), stromelysin-1 (MMP-3), and gelatinase B (MMP-9) became detected in stromal cells, together with MMP-9 in neutrophils; and 3) gelatinase A (MMP-2) was more expressed and immunolocalized at the membrane of stromal cells. By biochemistry, endometrial lysates from nonbleeding metrorrhagic patients contained more latent and active MMP-2 and -9 than age-matched controls; at bleeding, collagenase activity, MMP-9, and active MMP-2 were strikingly increased whereas tissue inhibitor of metalloproteinases-1 (TIMP-1) was considerably decreased. As a functional assay, *in situ* gelatin zymography revealed large areas of gelatinolytic activity only in endometrium of bleeding patients. Altogether, these results strongly suggest that inappropriate focal expression and activation of several MMPs, combined with decreased inhibition, trigger irregular dysfunctional endometrial bleeding. (Am J Pathol 2004; 165:83–94)

Disturbances of menstrual bleeding are a major medical and social problem.¹ It is a frequent cause for general practitioner referral to outpatient clinics² and discontinuation of contraceptive treatment.³ Abnormal bleeding in the absence of pregnancy or recognizable pelvic or systemic disease (ie, dysfunctional bleeding) may happen during hormonal contraception or replacement therapy. Irregular dysfunctional bleeding (ie, metrorrhagia) occurs at unpredictable times, without any relation to menstruation. It is generally attributed to hormonal imbalance due either to hyperestrogenism during anovulatory cycles or at menopause, or to abnormally high progesterone-to-estradiol ratio in the case of breakthrough bleeding on progestin-only contraception.^{4,5} However, bleeding can occur at any time, regardless of estradiol plasma concentrations and its relationship with sex steroids remains speculative.^{1,4}

Vaginal bleeding of endometrial origin obviously requires both a vascular injury and the rupture of the surface-lining epithelium, but the molecular mechanisms underlying these events are poorly understood. Widespread beliefs explain menstrual bleeding by ischemic necrosis of the superficial layer of the endometrium secondary to vasoconstriction of the spiral arterioles, and/or by the release of hydrolases from labilized lysosomes.⁴ However, cell necrosis is surprisingly difficult to detect during menstruation,⁶ and viability of menstrual fragments can be demonstrated by their successful culture.⁷ Furthermore, a major role of lysosomal enzymes in the induction of menstrual bleeding is not readily supported by biochemical evidence.^{8,9} Actually, menstruation is characterized by the lysis of collagen-rich argyrophilic fibers of the endometrial stroma, followed by tissue collapse and fragmentation, collectively described as "stromal breakdown."^{4,6} Therefore, matrix metalloproteinases (MMPs) are prime candidates for triggering endometrial bleeding, because they are able together to degrade most proteins of the extracellular matrix at neutral pH.¹⁰

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The effective activity of MMPs is finely tuned by a multilevel regulation integrating gene expression, secretion, activation, inhibition, and clearance. They are expressed as zymogens (proMMPs) that need excision of the propeptide to become active. Apart from the membrane-bound MMPs (MT-MMPs), most proMMPs including procollagenase-2 (proMMP-8) and progelatinase B (proMMP-9) are constitutively secreted once expressed and activated outside the cell. However, proMMP-8 and -9 are also stored in secretory granules of neutrophils, where a large fraction of proMMP-9 is covalently complexed to neutrophil gelatinase-associated lipocalin (NGAL).¹¹ Once active, MMPs can be inhibited by α_2 -macroglobulin and various tissue inhibitors of metalloproteinases (TIMPs).

In the human endometrium, sensitive biochemical methods detect (pro)collagenase-1 (MMP-1), (pro)stromelysin-1 (MMP-3), and MMP-9 only at menstruation. Levels of (pro)gelatinases A (MMP-2) and B (MMP-9) are higher at menstruation than during the other phases of the menstrual cycle.^{12–17} When cultured explants of non-menstrual human endometrium are deprived of progesterone, these various proMMPs become expressed and/or activated and the extracellular matrix undergoes menstrual-like breakdown, which can only be prevented by inhibitors of MMPs.^{7,18} These experiments provide strong *in vitro* support for a key role of MMPs in triggering menstruation.

Metrorrhagia occurring on progestin-only contraception is associated with endometrial foci of menstrual-like stromal breakdown. Moreover, endometrial explants from metrorrhagic women release considerably more MMP-1, -2, -3, and -9, and lower amounts of TIMP-1, when sampled at bleeding episodes.^{5,19} The present study was therefore designed to address the following questions: 1) does a similar pattern of focal tissue breakdown occur in irregular dysfunctional bleeding, irrespectively of hormonal treatment; 2) is breakdown spatially and temporally associated with MMPs activity *in vivo*; and 3) is breakdown accompanied by a systematic alteration in the focal expression of estradiol and progesterone receptors (PRs)?

Materials and Methods

Tissue Sampling

The investigations, approved by the Ethical Committee of the Université Catholique de Louvain, Medical School, combine a retrospective and a prospective study. The retrospective study was performed on biopsies systematically retrieved from the files of the Pathology Department, Saint-Luc University Clinics. The collection included 40 endometrial biopsies from 25- to 62-year-old women (median, 41 years) that had presented with metrorrhagia or postmenopausal bleeding. Nineteen patients were on oral steroid treatment: progestin-only contraception (lynestrenol, $n = 7$; or dydrogesterone, $n = 4$); combined ethinylestradiol and desogestrel ($n = 4$) or gestodene ($n = 2$) contraception; or combined estradiol

and norgestrel replacement therapy ($n = 2$). The other 21 patients were not treated with sex hormones. As positive controls, we tested six endometrial biopsies sampled during spontaneous menstruation in regularly cycling women (median age, 37 years; range 26 to 42 years). As negative controls, we used four biopsies sampled outside menstruation in infertile women without bleeding problems (median age, 41 years; range, 33 to 45 years). Tissues had been routinely fixed for 12 to 24 hours in 10% formalin buffered at pH 7.0 and embedded in paraffin. Serial sections were prepared for histological, immunohistochemical, and *in situ* hybridization analysis (see below). The slides were examined by two experienced pathologists unaware of whether the patient was bleeding or not at the time of the biopsy; this information was collected afterward from the files of the Gynecology Department.

For the prospective analysis, conducted for 4 months, endometrial tissue was collected from 58 consecutive women who underwent hysteroscopy in the outpatient Gynecological Clinic because of abnormal bleeding. Sixteen cases were excluded because of organic lesion (such as myoma, polyp, atypical hyperplasia, adenocarcinoma, or spontaneous abortion), or inadequate tissue retrieval (only mucus and/or blood). The remaining 42 patients, who qualified for the diagnosis of dysfunctional uterine bleeding, were 27 to 51 years old (median, 42 years). Nineteen patients were on oral hormonal contraception, based on progestin alone (lynestrenol, $n = 7$, or dydrogesterone, $n = 1$), or a combination of ethinylestradiol and desogestrel ($n = 5$), gestodene ($n = 3$), levonorgestrel ($n = 2$), or norgestimate ($n = 1$). The other 23 patients were not treated with sex hormones. Fifteen patients were bleeding at the time of hysteroscopy, including two patients with fresh blood in the uterine cavity but without noticeable vaginal discharge. The other 27 patients did not bleed at the time of hysteroscopy. As controls for the prospective study, we analyzed endometrial biopsies performed in 18 additional patients who underwent hysteroscopy for infertility during the same period. These patients were 25 to 42 years old (median, 37 years) and were not treated with ovarian steroids.

Fresh endometrial biopsies were collected in ice-cold phosphate-buffered saline, pH 7.0, immediately transferred to the Pathology Department, and frozen at -20°C . A 8- μm -thick frozen section stained with hematoxylin and eosin (H&E) was examined to establish the absence of organic lesion. Five 40- μm -thick frozen sections were then collected and solubilized in 0.5 ml of 50 mmol/L Tris-HCl buffer, pH 7.5, containing 150 mmol/L NaCl, 10 mmol/L CaCl_2 , 3 mmol/L NaN_3 , 0.05% (v/v) Triton X-100, and 1 $\mu\text{mol/L}$ ZnCl_2 , to assay for DNA content, collagenase activity, and to perform gelatin and reverse zymographies on lysates (see below). A further 8- μm -thick frozen section was again stained with H&E to verify the histological appearance of the analyzed samples. In 14 cases, additional 6- μm -thick frozen sections were prepared for *in situ* gelatin zymography (see below). The remaining tissue was fixed overnight in 10% formalin buffered at pH 7.0 and embedded in paraffin for histo-

logical, immunohistochemical, and *in situ* hybridization analyses as in the retrospective study. The area of tissue sections was estimated with a grid of 10×10 squares inserted into an eyepiece of the microscope. The side of each square corresponds to $100 \mu\text{m}$ with the 10-fold magnifying objective.

Histological and Immunohistochemical Staining

Serial histological sections were stained with H&E, impregnated with silver,²⁰ hybridized for MMP-1 and MMP-2 mRNA as described below, and immunolabeled for (pro)MMP-1, -2, -3, and -9; estrogen receptor (ER)- α ; and PR-A and PR-B. Purified mouse monoclonal antibody against (pro)MMP-1 (IgG2a, clone 41-IE5), (pro)MMP-2 (IgG1, clone 42-5D11), (pro)MMP-3 (IgG1, clone 55-2A4), and (pro)MMP-9 (IgG1, clone 56-2A4) were kindly provided by Y. Okada (Keio University, Tokyo, Japan) or K. Iwata (Fuji Chemical Industries Ltd., Toyama, Japan). They were used at 0.2, 0.7, 5.0, and $0.5 \mu\text{g/ml}$, respectively. Mouse monoclonal antibodies were used to detect ER- α (IgG1 kappa, clone 1D5; $0.5 \mu\text{g/ml}$; DakoCytomation, Glostrup, Denmark) and PR-A and PR-B (IgG1, clone 1A6; $1.0 \mu\text{g/ml}$; Novocastra laboratories, Newcastle-on-Tyne, UK). In each case, negative controls were performed by replacement of the primary antibody by a nonrelevant antibody of the same isotype (IgG2a against proinsulin, Novo Biolabs, Bagsvaerd, Denmark; IgG1 against adrenocorticotrophic hormone, DakoCytomation); all these controls consistently produced no signal.

After removal of paraffin and inactivation of endogenous peroxidases with $0.3\% \text{H}_2\text{O}_2$ for 30 minutes at room temperature, sections for the detection of (pro)MMPs were incubated in 10 mmol/L sodium citrate buffer, pH 5.8, and heated in a water bath at 98°C for 75 minutes to retrieve the antigenic sites. For the same purpose, sections for the detection of ER- α and PR-A and PR-B were boiled by heating with microwaves (one cycle at 750 W for 30 minutes and three cycles at 150 W , for 10 minutes each) in citrate buffer. Nonspecific binding was blocked by a 30-minute incubation with 50 mmol/L Tris-HCl, pH 7.4, containing 10% (v/v) normal goat serum and 1% (w/v) bovine serum albumin. Sections were then incubated overnight at 4°C with the primary antibody. After three washes in 50 mmol/L Tris-HCl, pH 7.4, specifically bound antibodies were labeled for 1 hour at room temperature with Envision (DakoCytomation), and the peroxidase activity was revealed by a 10-minute incubation with 0.5 mg/ml of diaminobenzidine in Tris-HCl buffer. Sections were finally washed in tap water and slightly counterstained with hematoxylin.

In Situ Hybridization

Radiolabeled probes for MMP-1 mRNA *in situ* hybridization were prepared as described.²¹ The anti-sense and sense riboprobes for *in situ* hybridization of MMP-2 mRNA were prepared from a 340-bp fragment cut with *Bgl*II from a polymerase chain reaction-amplified 450-bp fragment (nucleotides 1350 to 1800) of the human MMP-2 cDNA,

cloned into a KS- pBlueScript plasmid (Southern Alberta Cancer Research Centre, Calgary, Canada). The sense probe was synthesized using a T7 polymerase after digestion with *Bam*HI and the anti-sense probe was synthesized using a T3 polymerase after digestion with *Xba*I. The riboprobes were labeled with ^{35}S -UTP (Amersham, Rosendaal, The Netherlands); sense and anti-sense riboprobes of MMP-1 and MMP-2 mRNAs were always tested in parallel.

The procedure for *in situ* hybridization was performed as described,²¹ except that temperature was increased at 54°C for hybridization of MMP-2 mRNA and at 57°C for its stringent washing, because of the higher GC content of the MMP-2 riboprobes. Briefly, after paraffin removal, tissue sections were treated with $1 \mu\text{g/ml}$ of proteinase K (Boehringer Mannheim, Brussels, Belgium) at 37°C for 20 minutes, acetylated, dehydrated, and air-dried. Sections were then hybridized overnight at 50°C or 54°C with anti-sense and sense riboprobes ($30,000$ to $50,000 \text{ cpm}/\mu\text{l}$) that had been denatured for 2 minutes at 80°C just before hybridization. The hybridization mixture contained 100 mmol/L dithiothreitol, 50% (v/v) formamide, 300 mmol/L NaCl, 20 mmol/L sodium acetate, 5 mmol/L ethylenediaminetetraacetate (EDTA), together with 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 10% dextran sulfate (all w/v), and $700 \mu\text{g/ml}$ yeast tRNA. After hybridization, sections were washed three times for 15 minutes and one time for 45 minutes at room temperature in $4\times$ standard saline citrate (SSC) ($1\times$ SSC is 150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7.0), rinsed in $2\times$ SSC, and incubated 25 minutes at 55°C or 57°C in a stringent wash with 50% (v/v) formamide, 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L dithiothreitol, and 20 mmol/L Tris-HCl, pH 8.0. Slides were then treated for 30 minutes at 37°C with $30 \mu\text{g/ml}$ of RNase A and $1 \mu\text{g/ml}$ of RNase T1 (Boehringer Mannheim), washed, dehydrated, air-dried, and dipped into K5 Ilford emulsion (Ilford Imaging Benelux, Wilrijk, Belgium) diluted with the same volume of 300 mmol/L ammonium acetate and 1% (v/v) glycerol. Slides were exposed for 1 week at 4°C , developed with G150 Agfa (Agfa-Gevaert, Mortsel, Belgium) for 75 seconds, and fixed with G350 Agfa (Agfa-Gevaert) for 15 minutes. Sections were slightly counterstained with hematoxylin.

DNA Measurements

The DNA content of tissue samples was assayed in triplicates as described.²² Briefly, lysates were diluted 50-fold in a 10 mmol/L Tris-HCl buffer, pH 7.0, containing 100 mmol/L NaCl, 10 mmol/L EDTA, and 0.1% (v/v) Triton X-100. One hundred μl thereof were added to the same volume of 4',6-diamidino-2-phenylindole dihydrochloride hydrate (Sigma, St. Louis, MO), diluted at 1 mg/ml in the Tris-HCl buffer without Triton X-100, in each well of an Optiplate (Packard, Downers Grove, IL). The resulting fluorescence was quantified with a Fluorocount (Packard) and referred to a standard curve performed with a sodium salt of salmon sperm DNA (Calbiochem, San Diego, CA). The DNA content varied from 3.0 to $22.6 \mu\text{g/ml}$, without significant difference between the groups of patients analyzed.

Collagenase Assay

Total collagenase activity was assayed on ^3H -acetylated collagen, as described.¹⁸ The procedure included preincubation of tissue lysates with 2 mmol/L 4-aminophenylmercuric acetate (Sigma-Aldrich, Bornem, Belgium) for 2 hours at 37°C to activate latent proMMP-1. In this assay, TIMPs and α_2 -macroglobulin present in the lysates are still able to inhibit MMPs. One unit of collagenase is defined as the enzyme activity that degrades 1 μg of soluble collagen per minute.

Gelatin and Reverse Gelatin Zymography

Both latent (ie, pro-) and active gelatinases A (MMP-2) and B (MMP-9) were analyzed in tissue lysates by gelatin zymography, as described.¹⁸ Briefly, nonreduced samples were preincubated for 30 minutes at 25°C in the presence of 2% sodium dodecyl sulfate (SDS) and 10 μl of each sample were resolved by SDS/polyacrylamide gel electrophoresis on a 10% polyacrylamide gel copolymerized with 0.5 mg/ml gelatin (Sigma). After SDS was removed by rinsing with 2.5% Triton X-100, the gel was incubated for 18 hours at 35°C in 50 mmol/L Tris-HCl buffer, pH 7.5, containing 5 mmol/L CaCl_2 , 0.2 mg/ml NaN_3 , 1% (v/v) Triton X-100, and 1 $\mu\text{mol/L}$ ZnCl_2 to allow for digestion by (pro)gelatinases bands. The gel was finally stained with a solution of 0.25% Brilliant Blue R-250 (Sigma), 9% acetic acid, and 45% methanol, destained with 7% acetic acid, 20% methanol, and 20% (all v/v) Norvanol D (Vel, Leuven, Belgium), and analyzed by densitometry using the 1.62 NIH Image software.

In parallel, the amount of TIMPs was determined by reverse gelatin zymography.¹⁸ Twenty μl of each sample were resolved in 13% polyacrylamide-gelatin zymograms. These were further incubated for 24 hours at 37°C in 20 ml of buffer supplemented with 1 ml of medium conditioned by cultured mouse calvaria, that was treated with 0.4 mmol/L 4-aminophenylmercuric acetate for 2 hours at 25°C before incubation, then stained and analyzed as above. Activities were normalized between gels according to an internal standard run in each zymogram, then referred to the individual DNA content of samples.

In Situ Gelatin Zymography

Six- μm -thick frozen sections, prepared from 14 endometria of metrorrhagic patients, 9 of whom were bleeding and 5 nonbleeding at the time of the biopsy, were spread on gelatin-coated slides (Fuji Film; a kind gift from Y. Okada). After incubation for 4 to 8 hours in a moist

chamber at 20°C, slides were stained for 2 minutes with 1% amido black 10B in a 70% methanol, 10% sodium acetate solution (all v/v), briefly rinsed in distilled water, destained in the methanol-acetate solution for 20 minutes, air-dried, and analyzed by light microscopy. The gelatin layer was completely degraded when incubated for 4 hours with a frozen section of an endometrium from a bleeding woman, but no degradation was observed when an adjacent frozen section of the same endometrium was incubated in parallel at 4°C (not shown). The nature of the gelatinolytic activity was defined by pre-treating slides overnight with 1 mmol/L *o*-phenanthroline (Sigma), 25 $\mu\text{mol/L}$ RP59794, a specific inhibitor of MMPs (Rhône-Poulenc Rorer, Vitry-sur-Seine, France), 20 $\mu\text{mol/L}$ leupeptin (Boehringer), or vehicle, before overlay with serial frozen sections of an endometrium from a bleeding metrorrhagic woman. Slides were finally incubated at 37°C for 8 hours with vehicle alone or in the continued presence of the inhibitor.

Statistical Analyses

For morphological comparison, significance was tested using 2×2 contingency tables. Biochemical data were analyzed using the nonparametric two-tailed Wilcoxon rank-sum test. Comparison between morphological and biochemical data were performed by the Spearman correlation test.

Results

Retrospective Study

Histological Analysis

The overall fibrillar organization of the endometrial extracellular matrix is best demonstrated by silver impregnation. In the four nonbleeding control women, this method revealed the classical reticular network of collagen-rich argyrophilic fibers supporting the entire tissue, including in blood vessels walls and under the glandular and surface-lining epithelia. The six menstrual endometria, used as positive controls, showed no evidence of necrosis but extensive stromal breakdown. Stromal breakdown was defined, on staining by H&E, by stromal shrinking and tissue fragmentation leading to the formation of small clusters of stromal cells; it is associated with disappearance of the argyrophilic network. Among metrorrhagic patients, stromal breakdown was observed in foci of variable extension in 22 of the 40 examined endometrial biopsies, irrespectively of steroid hormone treat-

Figure 1. Focal alterations in a dysfunctional bleeding endometrium. This metrorrhagic patient, not treated with ovarian steroids, was bleeding at the time of biopsy. The histological appearance of her proliferative endometrium suggests dysfunctional bleeding during an anovulatory cycle (**A**: H&E). Serial sections show, in the focus of stromal breakdown at **right** of this field, the disappearance of collagen-rich argyrophilic fibers (**B**: silver impregnation), of ER- α (**C**) and of PR-A and PR-B (**D**: antigen retrieval by microwaves). PRs were heterogeneously immunolabeled in adjacent areas (**inset**). In contrast, proMMP-1 mRNA (**E**) and protein (**F**) were selectively detected in the focus of stromal breakdown. Fields **F** to **K** are higher magnifications of the oblique **square boxed** in **E**, and **G** is a further enlargement of the **box rectangle** at the **bottom** of **F**. These higher magnifications also show that proMMP-2 mRNA is strongly expressed in the breakdown area by essentially all stromal cells (**H**). Notice that immunolabeling for (pro)MMP-2 (**I**), -3 (**J**), and -9 (**K**) is strongest at the margin of clusters, ie, at the border of the clefts because of extracellular matrix rupture (**arrows**). (Pro)MMP-9 is also detected in inflammatory cells (**arrowheads**). Notice also at **I** the membranous pattern of (pro)MMP-2 immunostaining, contrasting with the granular pattern of the other (pro)MMPs. **Insets** at the top of **E**, **F**, **H**, and **I** are negative controls using sense probes for MMP-1 (**E**) and MMP-2 mRNAs (**H**) or nonrelevant primary antibody for (pro)MMP-1 (**F**) and the other (pro)MMPs (**I**). Scale bars, 100 μm .

Table 1. Morphological Results of the Breaking Down Areas in the Retrospective Study

Patients Stromal breakdown	Nonmenstrual	Menstrual	Metrorrhagic (n = 40)	
	– (n = 4)	+ (n = 6)	– (n = 18)	+ (n = 22)
Lysis of argyrophilic fibers	0/4	6/6	0/18	22/22
Estrogen receptor- α	4/4	0/6	18/18	0/22
Progesterone receptors-A and -B	4/4	0/6	12/18	0/22
proMMP-1 mRNA	0/4	6/6	0/18	20/22
proMMP-2 mRNA	NT	1/1	0/5	8/9
(pro)MMP-1	0/4	4/6	0/18	16/20
(pro)MMP-2*	0/4	4/6	0/18	20/22
(pro)MMP-3	0/4	4/6	0/18	18/22
(pro)MMP-9†	0/4	4/6	0/18	13/22

This table compiles analyzed features in biopsies from nonmenstrual, menstrual, and metrorrhagic patients, retrieved retrospectively from the Pathology Department files. Biopsies from the metrorrhagic patients were further divided in two groups, according to the presence (n = 22) or absence (n = 18) of stromal breakdown. In the 6 menstrual women and 22 metrorrhagic women with stromal breakdown, features exclusively refer to the areas of stromal breakdown (for description outside breaking down areas, see text).

*Only strong (pro)MMP-2 immunolabeling as illustrated in Figure 1I.

†Only immunolabeling of stromal cells; NT, not tested; proMMP-2 mRNA and (pro)MMP-1 immunolabeling could not be analyzed in all patients. Expression of the analyzed MMPs in the stromal cells was significantly associated with the presence of stromal breakdown ($P < 0.002$ for proMMP-1 mRNA, (pro)MMP-1, (pro)MMP-3, and (pro)MMP-9 detection and strong (pro)MMP-2 immunolabeling; $P = 0.01$ for proMMP-2 mRNA).

ment (Figure 1A). In these foci, the argyrophilic network had completely vanished (Figure 1B). In a few cases, argyrophilic fiber lysis slightly extended beyond the limits of stromal breakdown areas, but the argyrophilic network was preserved elsewhere.

Immunolocalization of Ovarian Steroid Receptors

The endometrium of the four nonbleeding control women showed a diffuse immunolabeling of ER- α and PR-A and PR-B. In the few preserved areas remaining in the endometrium of menstrual controls, ER- α was also labeled as in nonbleeding control women, but not in the zones of stromal breakdown. In the metrorrhagic patients, ER- α vanished in the foci of stromal breakdown and was heterogeneously detected in the preserved areas (Figure 1C, Table 1). PR immunostaining also disappeared in the foci of stromal breakdown, and was strongly decreased and heterogeneous or even absent in the preserved areas of the normal menstrual samples and the metrorrhagic patients (Figure 1D, Table 1). The marked heterogeneity in the expression of ER- α and PR in both epithelial and stromal cells of the preserved tissues from metrorrhagic patients contrasted with their homogeneous labeling in nonmetrorrhagic patients. In particular, PR could not be detected in the endometrium from 16 metrorrhagic patients, including 6 cases without stromal breakdown, when retrieving antigens by the usual microwave procedure. This heterogeneity was no longer evidenced when using the more sensitive water bath procedure for antigen retrieval: ER- α and PR were strongly and uniformly labeled by this method, except in areas of stromal breakdown (not shown).

In Situ Hybridization and Immunolocalization of (pro)MMPs mRNA and Protein

ProMMP-1 and -2 mRNAs were both detected together in the stromal cells of the majority of stromal breakdown

foci from most metrorrhagic patients, and in all menstrual controls, but never elsewhere (Figure 1, E and H; Table 1). The signal for proMMP-2 mRNA was less intense than for proMMP-1 mRNA but more cells were labeled. In the large majority of these foci, immunolocalization confirmed the presence of (pro)MMP-1 and (pro)MMP-3 as discrete spots in the cytoplasm of stromal cells (Figure 1; F, G, and J; Table 1); and of (pro)MMP-2 as a strong membranous signal (Figure 1I; this level of intensity was selected for Table 1), contrasting with a diffuse but faint immunostaining outside the stromal breakdown areas.

In all endometrial samples examined, inflammatory cells were present and found to be more numerous in the foci of stromal breakdown. These cells, most of which were identified as neutrophils in adjacent sections stained with H&E, showed a strong (pro)MMP-9 immunostaining. In addition, the cytoplasm of some stromal cells showed a finely granular (pro)MMP-9 immunostaining exclusively within foci of stromal breakdown, (Figure 1K; this feature was selected for Table 1). For all (pro)MMPs that were immunostained, labeling was most prominent at the margin of cell clusters, ie, at the border of the clefts because of extracellular matrix rupture (Figure 1; F to K, arrows).

Comparison with Bleeding Episodes

By subsequent analysis of the clinical files of the 40 metrorrhagic patients studied, 28 were bleeding at the time of the biopsy, and only these showed foci of stromal breakdown (14 untreated patients, 6 patients on progestin-only treatment, and 2 patients treated with combined estrogen and progestin). In contrast, no stromal breakdown was found in the 12 tissues from metrorrhagic patients who were not bleeding at the time of the biopsy. Association of stromal breakdown with the occurrence of bleeding in metrorrhagic patients was highly significant ($P < 0.002$).

Figure 2. Collagenase activity measured in endometrial tissues. Endometrial biopsies were collected from 18 control women, 27 metrorrhagic patients who were not bleeding at the time of the sampling, and 15 metrorrhagic patients bleeding at the time of sampling. Frozen sections were solubilized and lysates were treated with aminophenylmercuric acetate to artificially activate the zymogen forms. Collagenase activities were normalized to the DNA content. Results are presented as whisker-boxes plots, with the median indicated by the **thick horizontal line**, the 25th to 75th percentiles by the **box**, and range by the **whiskers**. *******, $P < 0.001$ by the two-tailed Wilcoxon two-samples test; **NS**, not significant.

Prospective Study

Collagenase Activities

In the second part of the study, endometrial biopsies were prospectively collected from 42 women complaining of metrorrhagia and from 18 nonbleeding patients investigated for infertility, as negative controls. Metrorrhagic patients were divided into the 15 women who were bleeding at the time of biopsy (9 untreated patients, 6 on progestin-only contraception) and 27 patients who were not. After exclusion of organic lesion in a first frozen section, five further 40- μ m-thick frozen sections were prepared and solubilized for biochemical studies.

The total collagenase activity measured in these endometrial biopsies, which outweighed the inhibitory potential of endogenous TIMPs and α_2 -macroglobulin, was much higher in metrorrhagic women who were bleeding at the time of biopsy than in the nonbleeding metrorrhagic patients or control patients (Figure 2; $P < 0.001$). Difference between nonbleeding metrorrhagic patients and control women was not significant.

Gelatinases and TIMPs

The same tissue lysates were analyzed for gelatinase activities by gelatin zymography and for TIMPs by re-

Figure 3. Representative gelatin zymograms (**A**) and reverse zymograms (**B**). Lysates, that were not treated by aminophenylmercuric acetate, were analyzed by gelatin zymography to study (pro)MMPs (**A**), and by reverse zymography to study TIMPs (**B**). In both gels, gelatinolytic and inhibitory bands were defined by their *Mr*, and further identified by reference to a well-characterized medium conditioned by cultured endometrial explants, as internal standard.¹⁸ In **A**, proMMPs are detected because they become irreversibly activated by SDS. The two **top** bands, identified as NGAL/(pro)MMP-9 complex, reflect specific granules of neutrophils.²³ This covalent complex resists denaturation in SDS and migrates at 135 kd. At **B**, the two **bottom** dark bands indicating protection against added gelatinases identify TIMP-1 and TIMP-2.

verse zymography. Despite considerable variations between patients as for collagenase activities, gelatin zymograms showed that latent and active forms of both gelatinases A (MMP-2) and B (MMP-9) were more abundant in metrorrhagic patients, in particular at the time of bleeding (Figure 3A). The complex of proMMP-9 with NGAL, that is stored in lactoferrin-containing specific granules of neutrophils,^{23,24} was also most abundant and found to be partially activated in metrorrhagic women at bleeding (Figure 3A). In contrast to gelatinases, much less TIMP-1 was observed by reverse zymography in the endometrium of bleeding metrorrhagic women than in the two other groups. There was no difference for TIMP-2; TIMP-3 was not detected (Figure 3B). Densitometric analysis confirmed the significant increase of all forms of gelatinases in metrorrhagic women, and the further striking increase at bleeding, except for latent gelatinase A (proMMP-2) (Figure 4). Instead, activation of proMMP-2 that already occurs in nonbleeding metrorrhagic women is most prominent during bleeding episodes, as high-

Figure 4. Quantification of gelatinases (zymographic activities). Latent and active forms of NGAL/MMP-9, MMP-9, and MMP-2 were quantified by densitometric analysis of gels illustrated by Figure 3A. Results are presented as in Figure 2. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$ by the two-tailed Wilcoxon two-samples test; NS, not significant.

lighted at Table 2. Densitometry also confirmed that TIMP-1 decreased only at bleeding (Figure 5).

In Situ Gelatin Zymography

To localize gelatinolytic activities at the tissue level, additional frozen sections were prepared from the endo-

Figure 5. Quantification of TIMPs (reversed zymographic activities). TIMPs-1 and -2 were quantified by densitometric analysis of gels illustrated by Figure 3B. Results are presented as in Figure 2. *, $P < 0.05$; **, $P < 0.005$ by the two-tailed Wilcoxon two-samples test; NS, not significant.

metrium of 14 metrorrhagic women (9 bleeding, 5 non-bleeding), spread on gelatin-coated slides, incubated for a few hours at 20°C, and examined for gelatin degradation beneath the sections. This method detects only the active forms of gelatinases that override the inhibitory potential of the locally present endogenous inhibitors. Whereas no appreciable gelatin degradation was found for the five metrorrhagic patients who were not bleeding at the time of biopsy (Figure 6A), gelatin was degraded in large areas ($> 4 \text{ mm}^2$; seven of nine) (Figure 6B) or completely degraded (two of nine) by the endometrium of bleeding metrorrhagic women ($P = 0.01$). Gelatinolysis was completely prevented by treating the slides with a general inhibitor of metalloproteinases (o-phenanthroline) or a specific inhibitor of MMPs (RP), whereas an inhibitor of both serine and cysteine proteinases (leupeptin) was ineffective (Figure 6; C to F).

Morphological Analyses

The remaining tissue was fixed in formalin and processed exactly as for the retrospective study. Morphological results of this prospective study, summarized in Table 3, were entirely consistent with those of the retrospective study. Furthermore, the fractional area of stromal breakdown in H&E-stained sections of the 15 bleeding patients was estimated by two independent

Table 2. Fraction of Active Forms of Gelatinases

	Control nonbleeding (n = 18)	Metrorrhagic	
		Nonbleeding (n = 27)	Bleeding (n = 15)
NGAL/MMP-9	0.01 (0–0.34)	0.02 (0–0.32)	0.08 (0–0.55)*
MMP-9	0.14 (0–0.59)	0.11 (0–0.60)	0.29 (0–0.76)
NGAL/MMP-9 + MMP-9	0.11 (0–0.49)	0.09 (0–0.47)	0.29 (0.03–0.73)†
MMP-2	0.00 (0–0.50)	0.13 (0–0.60)‡	0.44 (0.10–0.56)§,¶

The table shows the medians (ranges) of the fraction of the active form to the total (ie, sum of latent and active) for each gelatinase, as measured by densitometry of zymograms.

* $P = 0.02$ and §, $P < 0.001$ when comparing bleeding metrorrhagic patients to control women; †, $P < 0.05$ and ¶, $P < 0.005$ when comparing bleeding to nonbleeding metrorrhagic patients; ‡ $P < 0.005$ when comparing nonbleeding metrorrhagic patients to control women.

Figure 6. Evidence of focal gelatinase activity. Representative frozen sections of endometrial biopsies from two metrorrhagic patients, one not bleeding (A) and one bleeding at the time of sampling (B), were spread on gelatin-coated slides and incubated at 20°C for 4 hours. Substrate lysis is evidenced by local loss of gelatin staining with amido black 10B (*). Notice its sharp boundaries (indicated by the dotted line). This feature was found only in bleeding metrorrhagic patients. In a control experiment, slides pre-treated or not with the inhibitor indicated below were covered with serial frozen sections of a bleeding metrorrhagic endometrium and incubated at 37°C for 8 hours with vehicle alone (C), or with 1 mmol/L of *o*-phenanthroline (D), 20 μ mol/L leupeptin (E), or 25 μ mol/L RP (F). Notice that total gelatin degradation under the control section is fully prevented by inhibition of MMPs, but not by inhibition of both serine and cysteine proteinases. Scale bars, 100 μ m.

investigators and averaged. The extent of stromal breakdown correlated with the level of collagenase activity in the corresponding tissue lysates ($r = 0.76$, $P < 0.01$).

Discussion

This report demonstrates that the occurrence of irregular dysfunctional bleeding episodes is associated with focal breakdown of the endometrial extracellular matrix, linked with the locally restricted expression, activation, and uncontrolled activity of several menstrual-associated MMPs: collagenase-1 (MMP-1), stromelysin-1 (MMP-3), and both gelatinases A (MMP-2) and B (MMP-9), as well as with a decreased expression of their inhibitor, TIMP-1. Acting together, these proteinases are able to degrade endometrial collagens, not only fibrillar type I, III, and V collagens that constitute the argyrophilic interstitial network, but also type IV collagen in basement membranes that line surface and glandular epithelia, surround decidualized stromal cells, and stabilize vessel walls.²⁵ These MMPs can also degrade proteoglycans as well as laminin and fibronectin. Foci of extensive extracellular matrix lysis, demonstrated in this work by the loss of silver staining and by *in situ* gelatinolysis, can readily account for the characteristic shrinkage of endometrial stroma, clustering of the stromal cells, tissue fragmentation, and shedding, altogether referred to as "stromal breakdown."⁶ Rupture of the blood vessels wall and sloughing of the surface epithelium will also occur, leading to interstitial hemorrhages and luminal bleeding. Because necrosis was never seen at histological examination,⁶ and because bleeding endometrium is viable and can be cultured,^{7,19} endometrial necrosis has no place in this sequence of events.

Although considered a hallmark of dysfunctional endometrial bleeding,⁶ stromal breakdown is not recognized as being restricted in time to bleeding episodes. This association is demonstrated in the present report, because stromal breakdown was observed exclusively in the endometrium of patients at bleeding, both in the retrospective and in the prospective parts of the study. One could argue that, although stromal breakdown was detected in all 15 bleeding patients prospectively recruited (as in all 6 menstrual tissues examined), it was not detected in 6 of the 28 bleeding patients of the retrospective study. However, the focal appearance of stromal breakdown, as shown in this and previous reports,^{19,21} can readily explain that small endometrial biopsies may miss this feature (negative results because of low probability of sampling). We were actually surprised to find stromal breakdown in such a high percentage of the biopsies performed during a bleeding episode, even though its extent was very limited in some samples, indicating that tissue lysis occurs at multiple places and at a short interval in the superficial portions of the bleeding endometrium. In addition, it is likely that biopsy oriented by hysteroscopy in the prospective study favored sampling of broken down tissue, as compared to blind sampling in the retrospective study.

The focal distribution of stromal breakdown in the endometrium appears to be related to a heterogeneous decrease in the abundance of ovarian steroid receptors, both in epithelial and stromal cells. Expression of these receptors in irregular dysfunctional bleeding is not clear, with a limited number of studies showing decreased lev-

Table 3. Morphological Results of the Prospective Study

Patients	Control nonbleeding (n = 18)	Metrorrhagic	
		Nonbleeding (n = 27)	Bleeding (n = 15)
Stromal breakdown	0/18	0/27	15/15
Lysis of argyrophilic fibers	0/18	0/27	15/15
Estrogen receptor- α	18/18	25/25	0/15
Progesterone receptors-A and -B	15/18	19/25	0/15
proMMP-1 mRNA	0/1	0/2	5/8
(pro)MMP-1	0/18	0/27	10/15
(pro)MMP-2*	0/18	0/25	10/15
(pro)MMP-3	0/18	0/25	8/15
(pro)MMP-9†	0/18	0/25	9/15

Same analysis as for Table 1 in this prospective study. For metrorrhagic patients who were bleeding at the time of the biopsy, features exclusively refer to the areas of stromal breakdown.

*Only strong (pro)MMP-2 immunolabeling as illustrated in Figure 11.

†Only immunolabeling of stromal cells. Stromal breakdown, lysis of the argyrophilic fibers, disappearance of the receptors and MMPs expression were significantly associated with bleeding ($P < 0.002$ for stromal breakdown, lysis of the fibers, disappearance of ER and PR, detection of (pro)MMP-1, (pro)MMP-3, and (pro)MMP-9, and strong (pro)MMP-2 immunolabeling).

els of PR mRNA and protein in stromal cells,^{26,27} or a decreased PR-to-ER ratio with no difference in the separate levels of PR and ER.²⁸ In the present study, immunostaining of ER- α and PR was generally decreased in breaking down areas as compared to preserved tissue, in agreement with our detailed analysis of Norplant users.¹⁹ However, the relationship between stromal breakdown and decreased ER and PR immunostaining was not entirely consistent, because decreased immunostaining was also occasionally observed in preserved tissues surrounding areas of breakdown, or in some areas showing no breakdown at all. In our view, it is the heterogeneity of the ER and PR immunostaining pattern that is most characteristic of irregularly bleeding endometrium, as it contrasts with the homogeneous labeling in the preserved areas of normal menstrual endometrium. Further studies are clearly needed to explain the mechanisms responsible for the heterogeneous expression of ER- α and PR in the endometrium during irregular dysfunctional bleeding and their relation with stromal breakdown.

The focal decrease of PR could partially explain the focal appearance of MMP-1, -3, and -9 and increase of proMMP-2 expression by stromal cells in the irregularly bleeding endometrium, as previously reported in cultured explants of normal nonmenstrual endometrium.^{14,18,21,29} As an additional level of control, cytokines are likely to exert a paracrine regulation, because MMPs were not expressed in all areas depleted of PR. For instance, interleukin-1 α and lefty A (also called EBAF, for endometrial bleeding-associated factor) increase the expression of proMMP-1 and proMMP-3 by stromal cells, respectively, whereas transforming growth factor- β decreases the expression of proMMP-3.^{30–33} Moreover, bleeding episodes in patients treated with Norplant were generally associated with a strong increase of interleukin-1 α release.¹⁹ Further investigations are thus needed to address the contribution of these and other cytokines in the focal induction of MMPs in the stromal cells.

Neutrophils and other inflammatory cells infiltrating the areas breaking down were strongly immunolabeled for (pro)MMP-9, in agreement with previous studies showing its occurrence in some, but not all, endometrial inflammatory cells.³⁴ Moreover, neutrophils and eosinophils are

more abundant in biopsies of Norplant users with endometrial shedding features, as compared to tissues devoid of stromal breakdown.³⁵ Inside neutrophils, approximately half of the proMMP-9 is stored in the specific granules,²³ where it is covalently linked to the NGAL.^{11,24} The other half of proMMP-9, not complexed to lipocalin, is stored in the easily mobilizable gelatinase granules and therefore, may contribute to the overall 92-kd band of zymographic activity. Because median amounts of NGAL/(pro)MMP-9 in tissue lysates from bleeding metrorrhagic patients correspond to ~25% of total (pro)MMP-9 content (ie, sum of linked and not linked to NGAL), endometrial neutrophils represent an important source of gelatinase, contributing to half the tissue content of total (pro)MMP-9. Observations on NGAL/(pro)MMP-9 call attention to the fact that specific granules of neutrophils also contain collagenase-2 (MMP-8), which could contribute to the measured collagenase activity; this was not addressed in the present work. Interestingly, the fraction of activated NGAL/MMP-9 tended to be lower than that of MMP-9 (Table 2; $P < 0.10$), suggesting that the activation process of NGAL/proMMP-9 is slower or different. Despite its abundance, the fraction of total (pro)MMP-9 contributed by neutrophils did not vary between controls, nonbleeding, and bleeding metrorrhagic women, arguing against a role of these cells in triggering tissue degradation. Because scattered inflammatory cells are constantly observed in endometrial tissues, irrespective of bleeding, we feel it unlikely that they induce stromal breakdown by releasing active MMPs. Instead, we propose an amplification role whereby neutrophils are recruited to areas where breakdown has been initiated. There, they release proMMPs that become activated by other MMPs previously released from, and activated under the control of, local stromal cells.

How do stromal MMPs become activated in the first place? Endometrial stromal cells produce proMMP-3, which, once activated, will in turn activate proMMP-9,¹⁷ and possibly other proMMPs including proMMP-1. ProMMP-2, once complexed by TIMP-2, is activated by a membrane-type MMP, probably MMP-14 (MT1-MMP) in the endometrium.^{10,36} Active MMP-2 was significantly more abundant in tissues of metrorrhagic patients at

bleeding than between bleeding episodes whereas the total amount of (pro)MMP-2 was not increased. Moreover, the membranous pattern of (pro)MMP-2 immunostaining in bleeding endometria points to binding and activation of proMMP-2 at the plasma membrane of stromal cells. Although these results suggest that activation rather than increased production of proMMP-2 contributes to the proteolytic activity in the areas of tissue degradation, *in situ* hybridization and immunohistochemistry indicate that (pro)MMP-2 expression is in addition specifically increased at sites of stromal breakdown in the bleeding endometrium. Whereas expression of TIMP-1 increases at menstruation,^{12,16} it showed a striking decrease in bleeding metrorrhagic patients, as already observed in patients treated with Norplant.¹⁹ Further studies are needed to understand the differential regulation between MMPs and TIMPs in irregular bleeding episodes compared to normal menstruation.

In summary, we propose that irregular dysfunctional bleeding, accounting for more than 70% of the metrorrhagias in our prospective study, is induced by the untimely but coordinated activity of several MMPs, including MMP-1, -2, -3, and -9. The heterogeneous loss of ovarian receptors in the endometrium presumably leads to the focal derepression of MMPs that are conversely induced in stromal cells by cytokines such as interleukin-1 α .³¹ Some changes, in particular an increased expression of proMMP-2, precede matrix breakdown because they are detected in nonbleeding metrorrhagic patients. The threshold for matrix breakdown would be focally reached on further rise of MMP expression and activation together with decreased production of TIMP-1, when the resulting increased proteolytic capacity exceeds the buffering potential of α_2 -macroglobulin and TIMPs present in the tissue. We also suggest that inflammatory cells, in particular neutrophils bearing abundant proMMP-9 and -8, are secondarily recruited at the same sites and release these zymogens, which, once activated, amplify extracellular matrix degradation. It is likely that other MMPs, such as MMP-14 involved in the activation of proMMP-2, and other families of proteinases, including lysosomal enzymes, eventually participate in tissue breakdown. The menstrual-like degradation of interstitial matrix and basement membranes that ensure cohesion of the vessels and of the surface epithelium will induce bleeding. This will become a clinically noticeable episode when many superficial areas of the endometrium simultaneously undergo lysis. New therapeutic prospects for irregular dysfunctional bleeding will emerge from further knowledge of the cellular and molecular mechanisms controlling the expression, activation, and inhibition of MMPs in the endometrium, and better understanding of the similarities and differences with their physiological control at the start of menstruation.

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