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Progesterone Inhibits Activation of Latent Matrix Metalloproteinase (MMP)-2 by Membrane-Type 1 MMP: Enzymes Coordinately Expressed in Human Endometrium[1]

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ABSTRACT

Matrix metalloproteinases (MMP) have specific spatial and temporal expression patterns in human endometrium and are critical for menstruation. Expression and activation mechanisms for proMMP-2 differ from other MMPs; in many cells proMMP-2 is specifically activated by membrane-type (MT)-MMPs. We examined the expression and localization of proMMP-2, MT1-MMP, and MT2-MMP in human endometrium across the menstrual cycle; and we examined the expression of MT1-MMP and activation of proMMP-2 in cultured endometrial stromal cells and their regulation by progesterone. MMP-2 was immunolocalized in 25 of 32 endometrial samples in all cellular compartments but with greatest intensity in degrading menstrual tissue. MT1-MMP mRNA was present throughout the cycle, and immunoreactive protein was detected in 24 of 32 samples, with the strongest staining in subsets of macrophages, neutrophils, and granular lymphocytes (but not mast cells or eosinophils) during the menstrual, mid-proliferative and mid-secretory phases. Patchy epithelial staining and staining of decidual cells, often periglandular in menstrual tissue, were also seen. MT2-MMP was more widespread than MT1-MMP without apparent cyclical variation and with maximal intensity in glandular epithelium. Cultured endometrial stromal cells released proMMP-2, and progesterone treatment significantly reduced the percentage level of its active (62 kDa) form (22.5 ± 1.8% vs. 3.0 ± 1.3%, without and with treatment, respectively, mean ± SEM, $P < 0.0001$). This activation was blocked by a specific MMP inhibitor and restored following inhibitor removal. Progesterone also attenuated cell expression of MT1-MMP mRNA. We postulate that MT1-MMP activates proMMP-2 in endometrium, this activity being increased at the end of the cycle when progesterone levels fall, thus contributing to menstruation.

INTRODUCTION

Matrix metalloproteinases (MMPs), enzymes with the capacity to degrade virtually all components of extracellular matrix, are expressed in human endometrium. Their specific spatial and temporal patterns suggest roles in the extensive tissue remodeling that occurs during the normal menstrual cycle and particularly at menstruation [1,2]. Evidence for the critical nature of this family of enzymes in the menstrual process has been provided by in vitro studies in which specific inhibitors of MMPs, but not other classes of proteinases, completely inhibit the breakdown of menstrual tissue [3].

Most MMPs are secreted as inactive zymogens and require cleavage of the amino-terminal propeptide for activation. The majority of these secreted enzymes can be activated by proteases of other classes, particularly serine proteases. Furthermore, certain MMPs can activate others; and once specific active enzymes, particularly MMP-3, are present, a cascade of MMP activation can be established. Little is yet known of the mechanisms of MMP activation in human endometrium, although a potential role for mast cell tryptase in initiation of an MMP activation cascade has been demonstrated using human endometrial stromal cells, which secrete many of the MMPs, including MMP-1 and MMP-3, that are found in perimenstrual endometrium [4].

MMP-2 (gelatinase A), which is also produced by human endometrial stromal cells, is an unusual MMP in that its expression is not regulated by the same array of steroid hormones, cytokines, and growth factors as are the other secreted MMPs [5,6]. Indeed, the 5′-flanking region of the MMP-2 gene lacks the AP-1 and PEA-3 binding sequences found in most of the other MMP genes [7]. Furthermore, proMMP-2 is ubiquitously released by many types of cells and can be detected in plasma. Its major substrates are collagens type IV, V, and VII, elastin, fibronectin, laminin-5, and the core proteins of proteoglycans. In addition, MMP-2 can process human interleukin-1β precursor into biologically active forms [8]. MMP-2 is also an exception within the MMP family in that it cannot be activated by serine proteinases such as plasmin or by catalytic quantities of other MMPs [9,10]. However, at least in tumor cells, proMMP-2 is uniquely activated by the recently described subfamily of the MMPs, the membrane-type (MT)-MMPs [11,12], which localize the activation to the surface of those cells expressing the enzymes. It has been suggested that cellular activation of proMMP-2 may be initiated by different members of this MT-MMP family depending on tissue distribution [12]. This activation mechanism involves also the tissue inhibitor of metalloproteinase (TIMP)-2 [13]. Different mechanisms may be involved in the activation of MMP-2 produced by normal fibroblasts [14].

MMP-2 mRNA expression has been demonstrated within the stromal compartment in human endometrium [15], and the protein is secreted constitutively by endometrial stromal cells in culture [16,17]. Withdrawal of progesterone from cultures of decidualized endometrial stromal cells results in a small increase in proMMP-2 protein, but this is several orders of magnitude less than the increases in MMP-1 and MMP-3 in the same samples [18]. Nothing is yet known of the location of the enzyme protein in endometrial tissue across the menstrual cycle or of the regulation of its activation in this tissue. This study describes, for the first time, the cellular location of MMP-2 during all phases of the normal menstrual cycle, its production and activation by cultures of endometrial stromal cells, and the regulation of this activation by progesterone. It also demonstrates that both MT1- and MT2-MMPs (MMP-14 and MMP-15, respectively) are present in human endometrium at specific

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cellular locations and that expression of MT1-MMP is attenuated by progesterone in endometrial stromal cells, thus providing an explanation for activation of MMP-2 at menstruation.

MATERIALS AND METHODS

Tissue Collection and Processing

Endometrial tissue was obtained at curettage from women with regular menstrual cycles and no apparent endometrial dysfunction, who gave informed consent for collection of tissue. Approval was given by the Human Ethics Committee at Monash Medical Centre, Melbourne. The women were either of proven fertility and were scheduled for tubal ligation or were undergoing testing for patency of the Fallopian tubes. Patients with leiomyomas or endometriosis, or those who had received any steroid treatment during the past twelve months, were specifically excluded from the study. Tissue samples were divided and fixed in buffered formalin overnight at 4°C, washed in Tris-buffered saline (pH 7.4) (TBS), and processed to paraffin wax blocks. Some tissue was snap frozen and stored at −80°C for RNA extraction, and some were collected into medium for subsequent culture. Tissue was dated initially from the patient's testimony and confirmed histologically.

Isolation and Culture of Human Endometrial Stromal Cells

Cells were prepared from endometrial tissue as described previously [16]. Briefly, chopped tissue was digested with bacterial collagenase type III (Worthington Biochemical Corporation, Freehold, NJ) at a concentration of 45 U/mL, in the presence of 3.5 μg/mL deoxyribonuclease (Boehringer Mannheim Biochemica, Mannheim, Germany) in calcium- and magnesium-free PBS for 40 min at 37°C. The resultant digest was then filtered sequentially through 45- and 10-μm nylon filters to remove glands, and erythrocytes were removed by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). The resulting cells were resuspended in a 1:1 mixture of Dulbecco's Minimum Essential medium and Ham's F-12 medium (Trace Biosciences, Sydney, Australia) with 10% charcoal-stripped fetal calf serum (FCS) and antibiotics (penicillin, streptomycin, and fungizone) and plated in 48-well dishes (2.5×10^5 cells/well) with added estradiol-17β (E_2: 10 nM; Sigma, St. Louis, MO) and with or without the synthetic progestin ORG2058 (P: 100 nM; Organon Laboratories Ltd., Oss, The Netherlands). Cells were changed to serum free conditions at 48 h and cultured for an additional 2 days. Medium was collected, centrifuged to remove cellular debris, and stored at −20°C for subsequent analysis. This experiment was performed with three individual cell cultures. Cells used for isolation of RNA were cultured for six days in the presence of E_2 and P followed by either withdrawal or continuation of P treatment for a further 8 days. Following culture, cells were washed twice with PBS then stored at −20°C until subsequent isolation of RNA. For experiments on MMP inhibition, cultures were established overnight in FCS + E_2, changed to serum-free conditions with added E_2 and with or without the specific hydroxamate MMP inhibitor RO31-4724 (Roche Products Ltd., Welwyn Garden City, UK) for 3 days. Medium was harvested, and cells were washed and reincubated without inhibitor for an additional 4 days when medium was again harvested. This experiment was repeated 4 times.

Gelatin Zymography

Gelatinases in samples of culture medium were analyzed by zymography on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (all reagents from Bio-Rad, North Ryde, Australia) under nonreducing conditions. Loading of samples was normalized according to the DNA content of culture wells [18]. Gelatinase activity was visualized by negative staining, and bands were identified by comparison with samples containing previously identified latent and active MMPs and by their molecular masses determined using molecular weight markers (Bio-Rad) [16]. MMP identity of bands was also confirmed by incubation of parallel gels in the presence of EDTA (5 mM). Relative activities of band intensity between lanes was semiquantitated by densitometric analysis of zymograms [19] using the Hewlett-Packard Scanjet IIp with Deskscan software (Hewlett-Packard, Palo Alto, CA) set on a black-and-white photo with 256 gray shades. The area of the bands was analyzed, using the NIH (Bethesda, MD) Image Version 1.54 equipped with gel-plotting macros, by measuring the area beneath the peaks plotted through the lane profile for the appropriate enzyme band. Comparisons were made only between samples run on the same gel. The percentage of the total MMP-2 (latent plus active) in its active form was calculated for each sample.

RNA Extraction from Endometrial Tissue

Total cellular RNA was extracted from frozen endometrial tissue (60- to 100-mg samples) using a single-step extraction with guanidinium thiocyanate, phenol, and chloroform [20]. Total cellular RNA was extracted from cultured human endometrial stromal cells by a modified miniprep method [21,22]. Briefly, cells were incubated for 5 min at room temperature with 200 μl of 3 M LiCl, 6 M urea. The solution was transferred to a microcentrifuge tube, vortexed vigorously, heated at 50°C for 3 min, and incubated for a further 5 min at room temperature. Extracted RNA was treated with DNase and repurified because substantial amounts of DNA were often detectable. RNA was quantitated by its absorbance at 260 nm.

Northern Blot Analysis

Ten micrograms of total RNA from endometrial samples taken across the normal menstrual cycle was denatured in 1 M glyoxal with 50% dimethyl sulfoxide, electrophoresed in a 1.2% agarose gel, transferred to Hybond nylon membranes (Amersham International, Sydney, Australia), baked at 80°C for 2 h, UV cross-linked for 5 min, and prehybridized for 4 h in hybridization buffer (6-strength saline-sodium phosphate-EDTA [SSPE; single strength is 0.15 M NaCl, 10 mM $NaH_2PO_4.2H_2O$, and 1 mM EDTA, pH 7.4], 5-strength Denhardt's solution [single strength is 0.0004% each of Ficoll, polyvinylpyrrolidone, and BS4], 0.5% SDS, 0.1 mg/ml herring sperm DNA, and 50% formamide) at 42°C. The blot was then hybridized with a ^{32}P-labeled cDNA probe for MT1-MMP (accession no. D26512, base pairs [bp] 1647–2889; a generous gift of Dr. M. Seiki, University of Tokyo, Japan) for 16 h at 42°C and washed at room temperature in buffer containing double-strength SSC with 0.1% SDS (single-strength SSC is 0.15 M sodium chloride and 15 mM sodium citrate, pH 7.0) and then in a final washing stringency of 0.5-strength SSC with 0.1% SDS at 55°C. For autoradiography, we used Kodak X-OMAT(AR) film (Kodak, Melbourne, Australia) and an in-

tensifying screen at −80°C for 2 h. Significant nonspecific binding to 18S and 28S was observed in tissue and cellular RNA extracts with the full-length probe, so a 407-bp shortened form was used to probe a smaller selection of samples from across the cycle to confirm specific hybridization to a single band at 3.9 kilobases (kb). After hybridization, the blot was washed at room temperature in double-strength SSC with 0.1% SDS, and then in double-strength SSC with 0.1% SDS at 42°C, followed by autoradiography for 10 days. After boiling in 0.1% SDS for 3 min, the membranes were subsequently probed with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe as previously described [18]. Autoradiographs were scanned using the Hewlett-Packard Scanjet IIp with Deskscan software, and the band intensities were determined using NIH Image Version 1.54. Specific binding of the MT1-MMP cDNA probe generated from the 1.24 kb full-length sequence to the 3.9 kb mRNA was further confirmed by Northern blot hybridization to 1 μg of RNA extracted from MT1-MMP transfected Cos cells (MT1-MMP construct-free plasmid transfected cells used as negative control; both generous gifts of Dr. E. Thompson).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Twenty samples of endometrial RNA were prepared from samples taken across the menstrual cycle (menstrual, early secretory, and premenstrual phases, n = 2–3; early and mid proliferative and mid-late secretory phases, n = 4) along with a sample of mRNA derived from endometrial stromal cells cultured in the presence of phorbol myristate acetate (PMA: 40 nM) as a positive control for both MMP-2 and TIMP-2. These were subjected to RT-PCR using forward and reverse primers as previously described for MMP-2 and TIMP-2 along with GAPDH [4]. The PCR products were separated on a 1.8% TBE (0.045 M Tris base, 0.045 M boric acid, 1 mM EDTA, pH 8.0) agarose gel, stained with ethidium bromide, and photographed using a Polaroid system.

Immunohistochemistry

Sections of endometrial tissue were cut at 5 μm, dewaxed, and hydrated. One section from each subject was stained with hematoxylin for histological dating of the menstrual cycle by an experienced gynecological pathologist according to the method of Noyes et al. [23]. Specimens were classified according to an idealized 28-day reproductive cycle and then grouped into early, mid, and late proliferative phases; early, mid, and late secretory phases; and menstrual phase. A total of 32, 36, and 35 tissues were examined for MMP-2, MT1-MMP, and MT2-MMP, respectively, with a minimum of 3 and maximum of 7 tissues in each phase. Where any results were equivocal, the staining was repeated and additional samples from the same phase examined. Some sections were double stained for leukocyte identification and MT1-MMP.

Mouse anti-human MMP-2 (clone 42-5D11), anti-human MT1-MMP (clone 114-6G6), and anti-human MT2-MMP (clone 1.62-22G5) were purchased from Oncogene Research Products (Cambridge, MA). All were at initial protein concentrations of 100 μg/ml. Antisera used for detection of leukocyte subtypes were mouse monoclonals: anti-human CD68 (KP1; Dako, Glostrup, Denmark, detects monocyte/macrophage lineage); anti-human neutrophil elastase, (Dako); anti-human eosinophil cationic protein, (Pharmacia, Uppsala, Sweden); anti-human CD43 (clone DFT1; Novocastra Laboratories Ltd., UK; detects granulated lymphocytes and some B cells) and anti-human mast cell tryptase (MAB1222: Chemicon Int., Temecula, CA).

Five-micrometer sections of endometrial tissues were subjected to immunohistochemistry. One section from a single block of premenstrual endometrium was included in every staining run to provide quality control. For every tissue, a second section on the same slide was used as a negative control with normal mouse IgG diluted to the same final protein concentration as the primary antibody and substituted for primary antibody. Immunohistochemical detection of MMP-2 and MT2-MMP, used the Strept-ABC system with New Fuchsin (Dako) as chromogen, whereas the detection of MT1-MMP used the liquid DAB kit from Zymed Laboratories Inc. (San Francisco, CA) as chromogen. All procedures were performed at room temperature unless otherwise stated. For MT1-MMP staining, tissues were pretreated with 0.3% hydrogen peroxide in methanol for 30 min. For detection of all three MMPs, the tissues were washed with 0.6% Tween-20/TBS for 20 min and then with TBS for 3 min before blocking for nonspecific staining with 10% normal horse serum in TBS for 20 min. Primary antisera (diluted 1:50 in 10% normal horse serum/TBS) or a similar concentration of normal mouse IgG were applied and incubated for 2 h (MMP-2) or overnight at 4°C (for MT-MMPs). After washing, slides were incubated in biotinylated horse anti-mouse serum (Vector Laboratories, Burlingame, CA; 1:200) for 30 min followed by the detection system used according to the manufacturer's instructions and with the appropriate chromogen. Sections were counterstained with Harris hematoxylin (1:10), dehydrated, and mounted. Microscopy was performed using an Olympus BH2 microscope and photographed using an Olympus camera with ND and LBD2N filters (Olympus Optical Co., Hamburg, Germany). Immunostaining intensity in individual cellular compartments within each section was scored by two independent observers from 0 (negative) to 4 (maximal staining intensity) in relation to the positive and negative controls. These controls were sections from a single block of endometrium included in each immunostaining run and replacement of primary antibody with appropriately diluted IgG positive and negative controls, respectively. For the analysis, these values were transformed into a percentage immunostaining intensity where negative = 0% and maximal staining intensity = 100%. Results are presented as mean ± SEM.

Double staining was also performed for MT1-MMP and markers for monocyte/macrophage, neutrophils, eosinophils, mast cells, and endometrial granular lymphocytes. In these cases, the EnVision Doublestain kit (Dako) was used with BCIP/NBT as chromogen for individual cell markers (purple stain). These primary antibodies were all used at 1:10 except anti-tryptase, which was at 1:40. MT1-MMP staining was performed as above but with the Strept-AB-Complex/alkaline phosphate system with New Fuchsin (red stain) as chromogen and with the primary antibody at 1:40. Following color development of the BCIP/NBT reagent, the sections were washed with distilled water followed by the double-stain block provided with the kit. The second primary antibody was applied, incubated at room temperature for 30 min, and washed; and the labeled polymer-alkaline phosphatase reagent was applied for 30 min followed by the New Fuchsin chromogen, mixed according to instructions with activating agent and levamisole (1 mM).

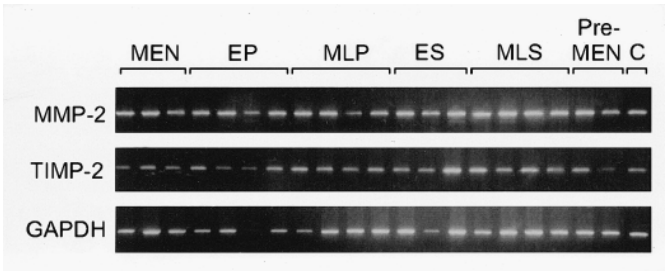


FIG. 1. RT-PCR analysis of RNA derived from endometrial tissue taken across the cycle using primers designed to detect matrix metalloproteinase (MMP)-2, tissue inhibitor of matrix metalloproteinases (TIMP)-2, and glyceraldehyde phosphate dehydrogenase (GAPDH). Phases of the cycle labeled as follows: MEN, menstrual; EP, early proliferative; MLP, mid-late proliferative; ES, early secretory; MLS, mid-late secretory; PreMEN, premenstrual; C, endometrial stromal cells stimulated in culture with phorbol myristate acetate (positive control).

After washing, slides were mounted with Faramount (Dako) and examined as above.

Statistical Analyses

Data from cell culture studies were expressed as mean percentage of control ± SEM where cells treated in the presence of progestin were taken as 100%. All treatments were in triplicate or quadruplicate wells. Data were analyzed by paired Student's *t*-test, and differences were considered significant at the 0.05 level.

RESULTS

MMP-2 mRNA Is Expressed in Human Endometrium Throughout the Menstrual Cycle

RT-PCR demonstrated that both MMP-2 and TIMP-2 mRNA were expressed in every sample of endometrial tissue examined as well as in the PMA-stimulated, cultured endometrial stromal cells (Fig. 1). These data compare with previous Northern analysis for TIMP-2 mRNA, which was also present in every sample tested, whereas MMP-1 and MMP-3 mRNA were expressed in only a proportion of samples from the premenstrual and menstrual phases [24].

MMP-2 Is Localized in Human Endometrium Across the Menstrual Cycle

MMP-2 protein was positively immunolocalized in 25 of the 32 endometrial samples tested (Figs. 2 and 3). It was seen variously in the different cellular compartments of the tissue: luminal epithelium (in 4 of 22 samples with this feature), glandular epithelium (18 samples), stroma (24 samples), endothelium (20 samples), vascular smooth muscle (16 samples), decidual cells (all 9 samples with this feature), and in leukocytes during the menstrual phase, supporting the previously described wide cellular distribution and constitutive production of this enzyme [5]. The relative intensity of staining in the different cellular compartments in the different phases of the cycle is shown in graphical form in Figure 2. The highest intensity of staining was observed in menstrual tissue that was breaking down (Figs. 2 and 3A). Much of this was extracellular within the stroma, and its cellular source was not clear. Similar strong staining was observed also within leukocytes at this time (Figs. 2 and 3C), and this was of greater intensity than the staining in the surrounding decidualized cells. Epithelial staining, when present, was of low intensity (Fig. 3B), as was staining in the stroma outside of the menstrual phase. Staining of both endothelial cells and smooth muscle cells was also observed in many sections (Fig. 3B).

MT1-MMP mRNA Is Expressed in Human Endometrium Throughout the Menstrual Cycle

Positive hybridization of the cDNA for MT1-MMP was seen in 16 samples of dated endometrial RNA, taken across the menstrual cycle, with no apparent cyclical variability. An additional blot containing four samples, representative of the different cycle phases, was probed with the 407-bp probe, and it shows a single band of hybridization at 3.9 kb (Fig. 4). This is the identical band size to that seen when the full-length probe was hybridized to RNA derived from the transfected cos cells used as positive control (not shown) and was also present in all 16 endometrial samples.

MT1-MMP Is Localized in Human Endometrium Across the Menstrual Cycle

MT1-MMP was detected in one or more cellular compartments in 22 of the 36 samples examined by immunohistochemistry (Figs. 2 and 3). The strongest staining was seen in leukocytes, and although these were predominant during the menstrual phase, strongly staining leukocytes were also seen during other phases of the cycle, in particular the mid-proliferative and mid-secretory phases when tissue edema is indicative of some degradation of extracellular matrix. No leukocytes positive for MT1-MMP were found during the early proliferative and early secretory phases. Epithelial staining was observed predominantly during the mid-proliferative and mid-late secretory phases, being found in the glands in 14 of the 17 tissues examined at these times (Figs. 2 and 3E). This was often patchy, with only some areas of luminal epithelium showing positive staining, and it never reached the intensity of staining seen in leukocytes. Stromal staining was also of low intensity and was observed in many of the tissues in which epithelial staining was present. Some staining of decidualized stromal cells was also seen (Fig. 3F). During the menstrual phase, MT1-MMP was often located in the periglandular regions of the stroma (Fig. 3D). During the early proliferative and early secretory phases, no MT1-MMP staining was found in any cellular compartment.

Subsets of Leukocytes Express MT1-MMP

Double staining for MT1-MMP and leukocyte type (CD68+ monocyte/macrophages, neutrophils, eosinophils, CD43+ endometrial granular lymphocytes, and mast cells) in menstrual tissue demonstrated that a number of these cell types produced the enzyme. These were monocytes/macrophages, neutrophils, and granular lymphocytes but not mast cells or eosinophils (Fig. 3, J–Q). The MT1-MMP staining was often seen around the periphery of the cell, indicative of its location in the cell membrane. Only subsets of each cell-type were positive for MT1-MMP; in all cases cells identified as of the same cell type were clearly negative for the enzyme.

MT2-MMP Is Localized in Human Endometrium Across the Menstrual Cycle

MT2-MMP was detected in one or more cellular compartments in 32 of the 34 samples examined by immunohistochemistry (Fig. 2 and Fig. 3, H and I). The strongest staining was seen in glandular epithelium throughout the

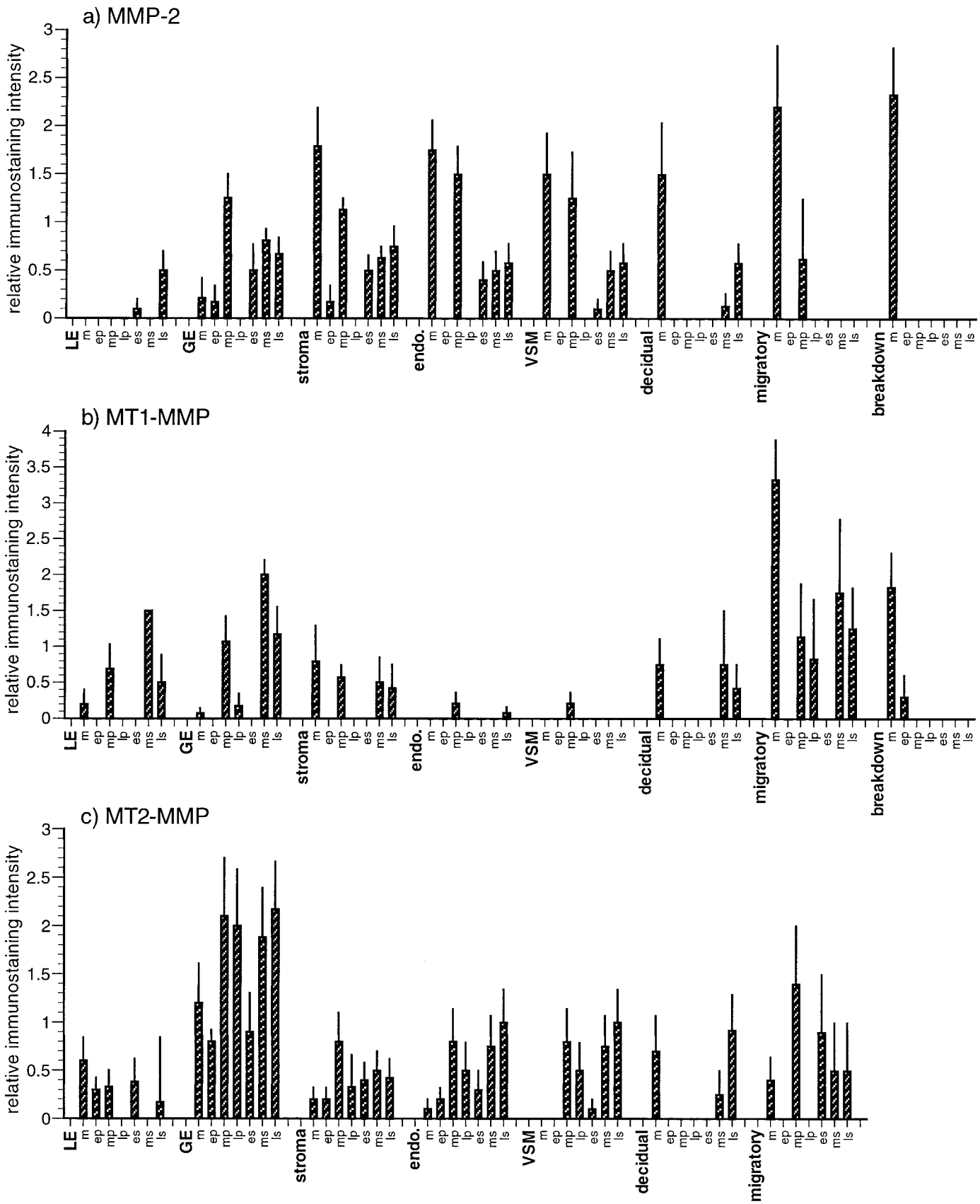
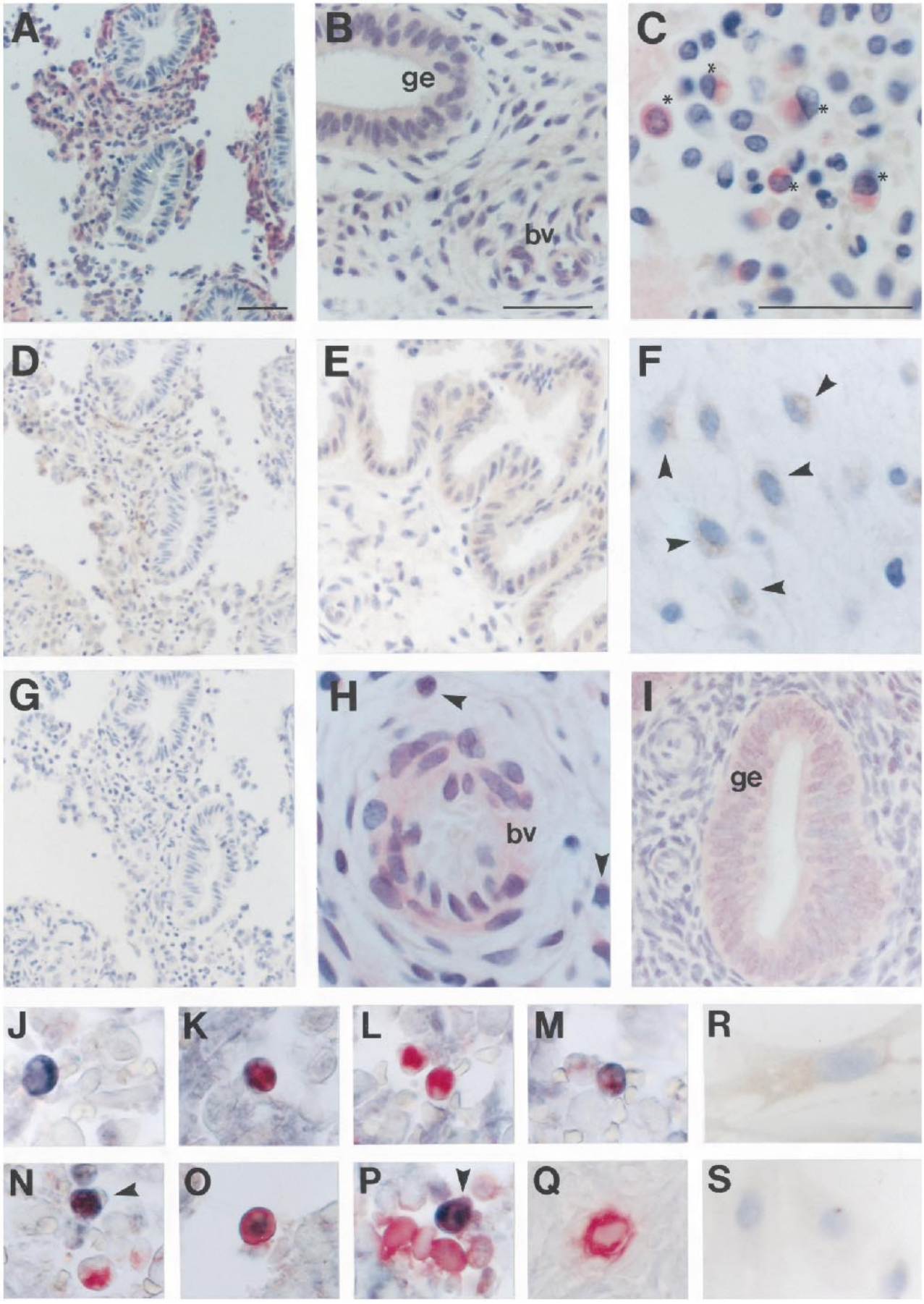


FIG. 2. Cellular localization and relative intensity (mean ± SEM) of immunostaining for **a**) matrix metalloproteinase (MMP)-2, **b**) membrane-type (MT)1-MMP, and **c**) MT2-MMP in endometrium during the menstrual cycle. Histogram bar shading is in the following order: menstrual (m); early, mid, and late proliferative (ep, mp, lp, respectively); early, mid, and late secretory (es, ms, ls, respectively). Cellular compartments are luminal epithelium (LE), glandular epithelium (GE), stroma, endothelial cells (endo) vascular smooth muscle (VSM), decidual cells, and migratory cells (leukocytes, including macrophages, eosinophils, neutrophils, endometrial granular lymphocytes, and mast cells). Areas of tissue degradation are shown separately (breakdown). Numbers of samples represented by the seven histograms, in order across the stages of the cycle as indicated above, are as follows: MMP-2: n = 7,3,4,3,5,4,6; MT1-MMP: n = 7,5,7,3,4,4,6; MT2-MMP: n = 5,5,5,3,5,4,6. Where no error bars are seen, staining was completely consistent within the group.



cycle: some staining of glands was observed in 32 of the 34 samples though the intensity varied between samples with no apparent cyclicity (Fig. 3I). Much less intense staining was seen in luminal epithelium and was not present in all samples (16 of 24 samples with this feature). Low intensity of staining was also seen in stroma (18 of 34 samples) and in endothelium and/or vascular smooth muscle (15 and 19 of 34 samples, respectively; Fig. 3H). Where decidual cells were present, staining for MT2-MMP was moderate. Some leukocytes stained moderately (in 9 of 34 samples), though this was not predominant as for MT1-MMP. Thus MT2-MMP is more widely distributed than MT1-MMP and with no observable cyclical variation.

MMP-2 Is Produced and Activated in Endometrial Stromal Cells: Regulation of Activation by Progesterone

When the active MMP-2 produced by the endometrial stromal cells in culture was expressed as a percentage of total MMP-2 (latent plus active), there was a highly significant difference between the cells treated with E_2 alone and those treated with E_2+P, the cells treated with P producing significantly less active MMP-2 than those without P ($P = 0.0001$) (Fig. 5). This difference held also when just the active MMP-2 in the cultures was compared between the two treatment groups ($P = 0.002$). There was no difference between the amount of latent MMP-2 in the two sets of cultures ($P = 0.39$).

Cultured Endometrial Cells Positively Immunostain for MT-MMP

Staining for MT1-MMP was also detected in endometrial stromal cells that had been maintained in culture for 3 days with fetal calf serum followed by three days in serum-free medium with added E_2 and either in the presence or absence of P (Fig. 3, R and S). In the cells treated with E_2, alone there was substantial staining both of cytoplasm and also of the cell membranes; whereas in the cells that had received P, the staining was markedly reduced and appeared to be predominantly cytoplasmic, possibly concentrated in the Golgi apparatus. No positive staining was detectable in cultured endometrial epithelial cells.

Progesterone Attenuates Expression of mRNA for MT1-MMP in Endometrial Stromal Cells

Northern blot analysis and densitometric analysis of MT1-MMP expression in cultured endometrial cells treated with E_2 alone compared with E_2+P showed attenuation of MT1-MMP expression by P in three of four separate cell preparations (Fig. 6). Analysis of combined data from the three positive experiments showed this attenuation to be significant ($P < 0.05$).

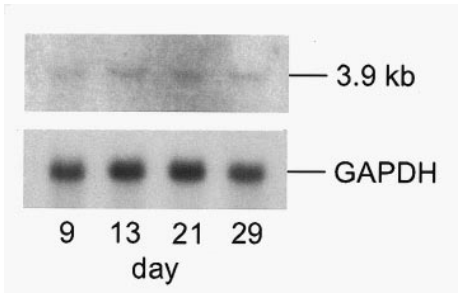


FIG. 4. Northern analysis of MT1-MMP mRNA in dated samples of human endometrium. The blot was probed using a 400-bp fragment from the cDNA for MT1-MMP, which hybridized to a 3.9-kb transcript, and also using a probe against glyceraldehyde phosphate dehydrogenase (GAPDH) as a loading control.

Specific MMP Inhibitors Negate the Activation of proMMP-2 in Endometrial Stromal Cells

When stromal cells in culture with E_2 alone were treated with the specific MMP inhibitor RO31-4724 (5 μM) and the culture medium was subjected to gelatin zymography, the active form of MMP-2 in the medium was reduced to 39 ± 9% of that in medium from cultures that had not received inhibitor ($P = 0.0007$); removal of the inhibitor resulted in restoration of activation to 62 ± 15% of that seen in medium from wells that had been cultured under the same conditions but without the inhibitor added between Days 1–3 ($P > 0.05$) (Fig. 7). This study established that activation was due to the presence of an MMP rather than another class of protease.

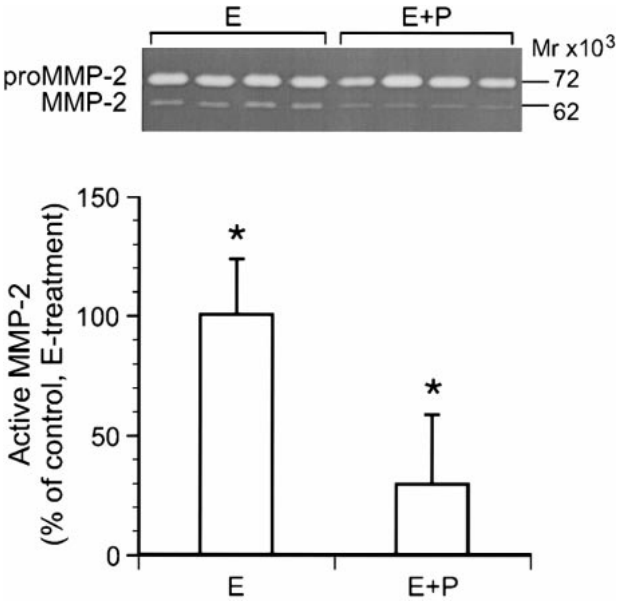


FIG. 5. **Upper**) A representative zymogram of culture medium from endometrial cells cultured with either E_2 or E_2 + P. The band at 72 kDa represents the latent form of MMP-2 and that at 62 kDa, the active form. **Lower**) Combined data from densitometric analysis of three separate cell cultures. Active MMP-2 is expressed as a percentage of the total MMP-2 produced by cells treated with E_2 or E_2 + P. The P-treated cells produce significantly less ($P = 0.0001$) active MMP-2 than the cells treated with E_2 alone. *Represents significant difference between groups.

FIG. 3. Photomicrographs showing immunolocalization of MMP-2 (**A–C**), MT1-MMP (**D–F**), and MT2-MMP (**H,I**) in endometrial tissue across the menstrual cycle. **A,C,D,G**) Tissue during menstruation; **B,I**) mid-proliferative tissue; **E,F,H**) late secretory tissue. Decidual cells are marked with arrowheads (**F,H**) and leukocytes with * (**C**). **J–Q**) Double staining for leukocyte markers (red) and MT1-MMP (purple). **J**) MT1-MMP+, eosinophil cationic protein−; **K**) MT1-MMP+, neutrophil elastase+; **L**) MT1-MMP−, elastase+; **M**) MT1-MMP+, CD68+; **N**) individual cells positive for CD68, MT1-MMP, and both (arrowhead); **O**) MT1-MMP+, CD43+; **P**); cells positive for CD43 alone and both MT1-MMP+ and CD43+ (arrowhead); **Q**) mast cell tryptase+, MT1-MMP−. **R,S**) MT1-MMP in cultured endometrial stromal cells without P (**R**) and with P (**S**). **G**) Negative control, normal mouse IgG substituted for monoclonal antibody. Bars = 10 μM. The scale bar in **A** also refers to **D** and **G,** that in **B** also to **E, I, R,** and **S,** and that in **C** also to **F, H,** and **J–Q.**

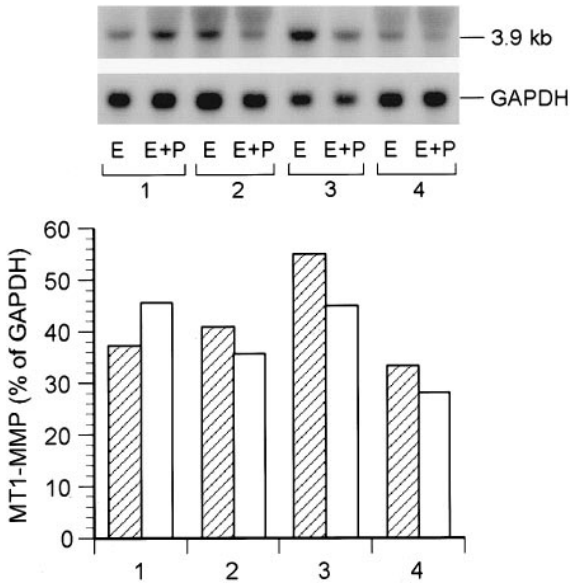


FIG. 6. **Upper**) Northern analysis of MT1-MMP mRNA in endometrial stromal cells cultured with either E_2 or E_2 + P. The blot was probed using a 400-bp fragment from the cDNA for MT1-MMP, which hybridized to a 3.9-kb transcript, and also using a probe against glyceraldehyde phosphate dehydrogenase (GAPDH) as a loading control. Lanes 1–4 represent data from four separate cell preparations analyzed. **Lower**) Densitometric analysis of the data after correction for loading. There is a significant ($P < 0.05$) attenuation of mRNA for MT1-MMP in the P-treated cultures.

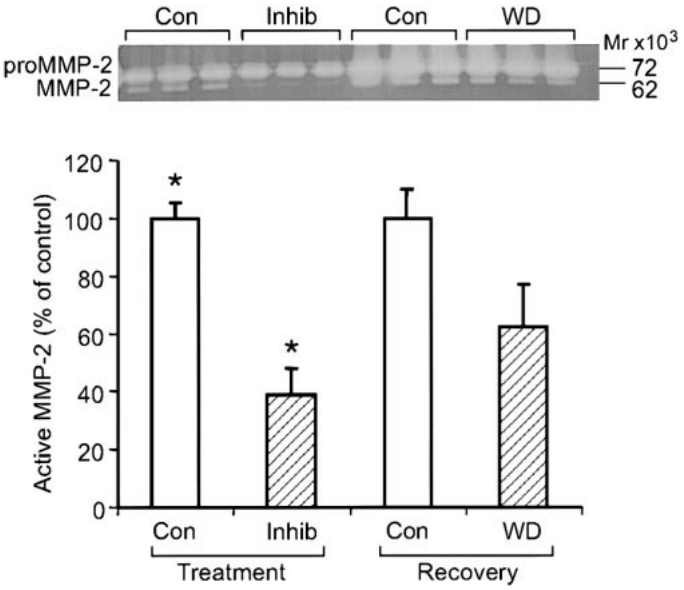


FIG. 7. **Upper**) A representative zymogram of culture medium from endometrial cells cultured with E_2, and without (CON) or with (INHIB) the MMP inhibitor RO31-4724. The band at 72 kDa represents the latent form of MMP-2 and that at 62 kDa, the active form. The zymogram shows culture medium taken at the end of the inhibitory (treatment) phase and the recovery phase of the experiment. WD represents samples following withdrawal of inhibitor for 4 days. **Lower**) Data from densitometric analysis of these cultures expressed as percentage of control in either the treatment or recovery phase. The inhibitor-treated cells produce less active MMP-2 than the untreated cells (*$P = 0.0007$). Following recovery, no significant difference was seen between the two treatment groups ($P > 0.05$). Representative one of four separate experiments.

DISCUSSION

The data presented here demonstrates firstly that not only MMP-2 but also its likely activators, MT1-MMP and MT2-MMP, are present in human endometrium in a range of cellular compartments across the menstrual cycle. Furthermore, a role for progesterone as an inhibitor of proMMP-2 activation is shown using cultured endometrial stromal cells. In these cells, activation of proMMP-2 and expression of MT1-MMP mRNA were coordinately attenuated by P, strongly suggesting a role for MT1-MMP as an activator of proMMP-2 in endometrium. Given that MT-MMPs are the major known MMP activators of proMMP-2, the inhibition of active MMP-2 production by a specific inhibitor of MMP activity supports the contention that MT-MMPs are responsible for this activation. Such findings fit well with the postulated physiological role for MMP-2 at menstruation, a process of tissue destruction that occurs only after progesterone is withdrawn from secretory endometrium [2]. The increase of MT1-MMP protein in menstrual endometrium also supports its role as activator of proMMP-2 at this time.

Progesterone (along with other steroid hormones) is a known transcriptional regulator of most MMPs, but the gene encoding MMP-2 lacks the AP-1 and PEA-3 binding sequences in the 5′ region, which are found in the majority of MMP genes [25]. To our knowledge, this is the first demonstration of the negative regulation of MT-MMPs by progesterone, although their up-regulation by cytokines such as TNF-α has been documented [26]. Given the expression of MT1-MMP in placental biopsies [27] and in invading trophoblast [28], situations in which plasma progesterone levels are elevated, it is likely that, as shown for MMP-1 and MMP-3, other factors such as cytokines can override the repressive effect of progesterone on MT-MMP gene expression [4,29]. Alternatively, cell-specific regulation may occur, perhaps reflecting expression of the progesterone receptor.

The wide cellular distribution of MMP-2 protein (probably in its latent form) is in accord with its constitutive production by a variety of cell types and tissues [5]. Its strong extracellular staining in the stroma during the menstrual phase is indicative of a role for the enzyme in the tissue degradation that is central to this process. However, it is still not entirely clear which cells are synthesizing the MMP-2. Studies using in situ hybridization on endometrial tissue demonstrated mRNA for MMP-2 predominantly in endometrial stromal cells, with epithelial expression in only two cases of menstrual tissue [22]. Likewise, in the present study, epithelial staining for the protein was very variable, with 4 of 22 samples having positivity in luminal epithelium while 18 of 32 samples showed positive staining in the glands. MT-MMPs have a similar distribution in epithelium, and it could be that at least some of the MMP-2 detected by immunostaining in these cells is bound to MT-MMPs. Recruitment of proMMP-2 to the cell surface requires its interaction as a ternary complex bound both to TIMP-2 and MT-MMP [13]. We have previously demonstrated an abundance of TIMP-2 in virtually all cellular compartments in the endometrium throughout the menstrual cycle [30], so this would be available for formation of such complexes.

MT1-MMP has a somewhat more limited pattern of distribution than MMP-2, suggesting cell-specific mechanisms for the activation of proMMP-2. MT2-MMP is more widely distributed than MT1-MMP. Staining for all enzymes is seen in the decidual cells that differentiate from stromal cells late in the cycle, while in the rest of the stroma, MT1-MMP, MT2-MMP, and MMP-2 all stain only lightly. In the

endometrium, MT1-MMP is virtually absent from vascular smooth muscle cells and endothelial cells, both of which stain positively for MMP-2 and sometimes for MT2-MMP. A recent study using a model for microvascular cell angiogenesis [31] showed that activation of proMMP-2 released from endothelial cells was due to collagen induction of MT1- but not MT3-MMP. Other mechanisms of proMMP-2 activation in endothelial cells include actions of vascular endothelial cell growth factor (VEGF), thrombin, and endothelial cell-stimulating angiogenesis factor [32–34]. All such activation is likely to occur independently of the plasmin/plasminogen system, as mice with targeted disruption of various components of this system are still able to activate proMMP-2 in all cell types examined [35].

The strongest staining for MT1-MMP in endometrium was observed in leukocytes that are extravascular within the tissue, particularly during the menstrual phase, but also in lesser numbers during the mid-proliferative and mid-secretory phases. MMP-2 is detectable also in some of these cells during the menstrual phase. Double-staining using specific cell-type markers identified the MT1-MMP positive cells as macrophages, neutrophils, and endometrial granular leukocytes but not eosinophils or mast cells. Interestingly, only subsets of each of these cell types expressed MT1-MMP, suggesting variable differentiation or activation of these cells within the tissue. Subtypes of endometrial leukocytes expressing other regulatory molecules have recently been described in our laboratory (for example, activin [36] and MMP-9 [37]): the mechanisms by which certain cells within the tissue come to express different phenotypes is not known. Such leukocytes, by virtue of the plethora of regulatory molecules they produce, are postulated to play an important role in MMP regulation at menstruation [38]. To our knowledge, expression of MT1-MMP has not previously been demonstrated in leukocytes, and whether this phenotype is found in similar cells at sites of inflammation remains to be established.

It has been suggested that cellular activation of pro-MMP-2 may be initiated by different members of the MT-MMP family depending upon tissue distribution [12]. For example, in the brain, MT2-MMP is more highly expressed than MT1-MMP and induces activation of proMMP-2 [39]. We have demonstrated that at least two members of the family, MT1-MMP and MT2-MMP, are present in human endometrium. Whether MT3- and MT4-MMP are also expressed and the relative physiological importance of different members of the family in this tissue remains to be established.

The physiological importance of MT1-MMP during the mid-proliferative and mid-secretory phases is not known, although during both phases the tissue becomes edematous, changes in extracellular matrix are detected [40], and MMP-3 is seen extracellularly [41]. It may be that MT1-MMP is active at these times, not only activating MMP-2 but also degrading specific components of extracellular matrix in its vicinity [42]. Further, MT1-MMP expression is associated with an epithelial-mesenchymal transition in cancer cells [43], a transitional state also suggested to be a feature of endometrial epithelium at the time of embryo implantation [44], the mid-secretory phase in the human.

In conclusion, these studies have demonstrated that MMP-2 and its activators, MT1- and MT2-MMP, are expressed with cell type specificity in human endometrium and with increased expression in the late secretory and menstrual phases, and that progesterone can regulate proMMP-2 activation by an MMP-dependent mechanism. In particular, evidence suggests important roles at menstruation, when withdrawal of progesterone would lead to up-regulation of MT-MMP expression and consequent pro-MMP-2 activation. Further studies are required in particular to establish the likely cellular interactions, particularly between leukocytes and endometrial stromal and epithelial cells, and to determine in more detail the transcriptional regulation of the MT-MMPs.

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