EMMPRIN Is Secreted by Human Uterine Epithelial Cells in Microvesicles and Stimulates Metalloproteinase Production by Human Uterine Fibroblast Cells

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Abstract

Endometrial remodeling is a physiological process involved in the gynecological disease, endometriosis. Tissue remodeling is directed by uterine fibroblast production of matrix metalloproteinases (MMPs). Several MMPs are regulated directly by the protein extracellular matrix metalloproteinase inducer (EMMPRIN) and also by proinflammatory cytokines such as interleukin (IL)-1 α / β . We hypothesized that human uterine epithelial cells (HESs) secrete intact EMMPRIN to stimulate MMPs. Microvesicles from HES cell-conditioned medium (CM) expressed intact EMMPRIN protein. Treatment of HES cells with estradiol or phorbol 12-myristate-13-acetate increased the release of EMMPRIN-containing microvesicles. The HES CM stimulated MMP-1, -2, and -3 messenger RNA levels in human uterine fibroblasts (HUFs) and EMMPRIN immunodepletion from HES-cell concentrated CM reduced MMP stimulation ($P < .05$). Treatment of HUF cells with low concentrations of IL-1 β / α stimulated MMP production ($P < .05$). These results indicate that HES cells regulate MMP production by HUF cells by secretion of EMMPRIN, in response to ovarian hormones, proinflammatory cytokines as well as activation of protein kinase C.

Keywords

EMMPRIN, MMPs, microvesicles, interleukin, uterus

Introduction

The uterus is a unique organ that undergoes extensive tissue remodeling with every menstrual cycle as well as throughout pregnancy and after parturition. The process of endometrial remodeling is tightly regulated by the expression of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs). At the initiation of menstruation, several MMPs are highly expressed in the endometrium and their pattern of expression changes throughout the menstrual cycle (reviewed by Curry and Osteen¹). The expression of several MMPs is inhibited by progesterone.²⁻⁴ However, there is also focused expression of MMPs and TIMPs by both fetal and maternal tissue at the time of embryonic implantation⁵ and endometrial decidualization.^{6,7}

Secretion of MMPs is critical for tissue remodeling throughout the menstrual cycle and has been postulated to be a major player in the pathogenesis of the gynecological disease, endometriosis. Endometriosis is associated with severe pelvic pain and infertility affecting 10% of reproductive aged women. Abdominal endometriosis is diagnosed through laparoscopic imaging of the abdominal cavity and is defined as the growth of ectopic endometrial glands and stroma on the mesothelial

surface of abdominal organs.^{8,9} Although the exact mechanisms of disease pathogenesis are unclear, menstrual fragments appear to be dispersed in the peritoneal cavity through the process of retrograde menstruation followed by attachment and invasion into the mesothelial lining of abdominal organs.⁹ Attachment and invasion of ectopic tissue to the mesothelial lining is postulated to occur through the expression of cytokines, interleukin-1 (IL-1) α / β and tumor necrosis factor α (TNF- α).¹⁰⁻¹² These cytokines not only regulate the immune microenvironment at the site of lesion attachment¹³ but also are capable of stimulating MMPs to facilitate lesion invasion.^{14,15}

Invasion of the mesothelial lining of abdominal organs by ectopic endometrium involves many of the same pathways as

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menstruation. Dysregulation of MMPs in both the eutopic and ectopic endometrium in human and animal models of endometriosis has been previously reported.^{16–18} Interestingly, inhibition of MMPs suppresses the development of ectopic endometrial lesions.^{4,19} Therefore factors that can activate local MMP production at the attachment site of ectopic endometrial fragments may enhance remodeling of the mesothelial lining leading to endometrial tissue invasion, while blocking MMP activation through anti-TNF- α compounds reduces lesion invasion.^{10,20} Invasion assays utilizing either menstrual effluent or endometrial fragments have demonstrated that communication between endometrial epithelial and stromal cells is imperative for successful invasion of endometrial fragments as neither cell type alone was sufficient for facilitating invasion into the peritoneal mesothelium.²¹

Our laboratory has previously reported that extracellular matrix metalloproteinase inducer (EMMPRIN) can stimulate MMP production in human uterine stromal cells.²² The EMMPRIN is a transmembrane glycoprotein identified in several different laboratories and known by various other names including CD147, neurothelin, and basigin. The EMMPRIN is expressed in human endometrium as well as endometriotic lesions.^{22,23} The level of EMMPRIN expression in endometrial epithelial cells appears to be positively regulated by estrogen and suppressed by progesterone, similar to endometriotic lesion growth. Additionally, EMMPRIN functions as its own signaling receptor²⁴ and has been shown to be secreted in its full-length form in microvesicles by tumor cells.^{25,26}

We hypothesized that, similar to tumor cells, EMMPRIN is secreted from human endometrial epithelial cells as a full-length, intact protein in microvesicles and that this soluble form of EMMPRIN is one of the primary factors that can stimulate uterine stromal fibroblast production of MMPs. We also hypothesized that microvesicle release by uterine epithelial cells is a regulated process and can be stimulated in response to specific factors.

Materials and Methods

Cell Isolation, Culture, and Treatments

Human uterine fibroblast (HUF) cells were obtained from the Cell Biology Core Laboratory of the Center for Women's Reproductive Health at the University of Illinois, Chicago, College of Medicine under an approved institutional review board protocol. Cells were isolated and processed as described previously²⁷ and received in our laboratory at the second passage for each individual patient. Although these uterine fibroblast cells are isolated from the maternal–fetal interface at the term of pregnancy, these cells have been characterized as proliferating, nondecidualized uterine fibroblast cells by flow cytometry and their expression of the mesenchymal marker vimentin.²⁸ These cells can, however, undergo decidualization in culture in the presence of estrogen, progesterone and either cyclic adenosine monophosphate or IL-1 β .^{28,29} The HUF cells were grown to confluency in phenol red–free RPMI-1640 culture medium

supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, Georgia), 5% bovine calf serum (BCS; Atlanta Biologicals), 120 U/mL penicillin/streptomycin (Cambrex, Walkerville, Maryland), and 3.2 mmol/L L-glutamine (Cambrex). Once the cells reached confluency, they were transferred to serum-free medium containing only L-glutamine and antibiotics for 24 hours.

Human uterine epithelial cells (HESs) are characterized as a spontaneously immortalized cell line derived from benign proliferating endometrium isolated via hysterectomy and were obtained from Dr Doug Kniss at Ohio State University.^{30,31} The HES cells were cultured, passaged, and maintained in phenol red-free, high glucose Dulbecco Modified Eagle Medium (Invitrogen, Carlsbad, California) supplemented with 10% FBS (Atlanta Biologicals) and 120 U/mL penicillin/streptomycin (Cambrex). Once cells reached confluency, they were transferred to serum-free medium containing only antibiotics for 24 hours and then conditioned medium (CM) was collected. Conditioned medium from HES cells was either frozen unconcentrated or was first concentrated 75-fold (75 $\times$) using Amicon Ultra-10 kD centrifugal filter devices (Millipore, Bedford, Massachusetts). Unconcentrated (1 $\times$) and concentrated (75 $\times$) CM was used for treatment of HUF cells. Additionally, concentrated (75 $\times$) HES CM was used for immunodepletion of EMMPRIN.

The HUF cells were treated with 1 $\times$, 75 $\times$, or immunodepleted 75 $\times$ HES cell CM for 24 hours. After 24 hours of treatment, the CM samples were collected and cells were harvested in TRIzol Reagent (Invitrogen, Carlsbad, California) for RNA isolation. Both cell types were maintained routinely in an incubator with 5% CO₂ at 37°C. Primary HUF cells utilized for these experiments were all at passage 5 and each replicate utilized cells from a different patient (n = 3).

Regulation of EMMPRIN secretion by steroid hormones (estradiol and progesterone) and IL-1 β in HES cells

17 β -Estradiol and progesterone were obtained from Sigma and kept in the dark at 4°C until use. Upon reaching confluency, HES cells were treated with serum-free medium for 24 hours, and then treated for 24 hours with fresh treatment medium containing 0, 10, or 25 nmol/L estradiol alone, 25 or 100 nmol/L progesterone, or 25/25, 25/100 nmol/L of estradiol and progesterone. Additionally, HES cells were also treated with treatment medium containing 0, 0.5, or 2 ng/mL of the proinflammatory cytokine, IL-1 β (R&D Systems, Minneapolis, Minnesota). Conditioned medium was then isolated and concentrated (75 $\times$) and utilized for immunoblotting for EMMPRIN detection.

Microvesicle Isolation

The HES cells were grown to confluency and serum starved for 24 hours. At this time, fresh serum-free medium was applied and collected at 0, 8, 12, and 24 hours. The CM was centrifuged at 1500g for 10 minutes at 4°C with the supernatant collected and centrifuged at 1500g for an additional 15 minutes to ensure complete removal of cell fragments. Microvesicles were

isolated by ultracentrifugation of the supernatant at 150 000g for 1 hour at 4°C. The supernatant (1×) was collected and the microvesicle pellet was resuspended in 1 mL PBS pH 7.4 (80×), both fractions were then stored at −20°C until the time of analysis. This methodology has been utilized extensively to isolate microvesicles from CM.^{25,32}

Phorbol 12-Myristate-13-Acetate (PMA) Treatment on Shedding of EMMPRIN Containing Microvesicles in HES Cells

Phorbol 12-myristate-13-acetate was obtained from Sigma (St Louis, Missouri), dissolved in 100% ethanol and stored in the dark at −20°C as a 1 mg/mL stock. Confluent HES cells were serum starved for 24 hours and then treated with 0, 25, 50, 100, or 200 ng/mL of PMA for 2 or 4 hours for initial dose-response studies and then treated with 100 ng/mL PMA for 15 minutes, 30 minutes, 2 hours, or 4 hours for timecourse experiments. Conditioned medium was collected and microvesicles were isolated as described above.

Immunoblotting

For all immunoblot analyzes of CM, we loaded equal volumes of sample instead of equal mass of protein due to the fact that we were comparing unconcentrated versus concentrated CM and non-cellular lysate. To ensure that we did not negate the purpose of concentrating the CM, we loaded equal volumes listed for each experiment from each CM sample. Equal volumes (15 µL) of HES cell 1×, 75×, or 75× immunodepleted CM and 10 µg of cell lysates were denatured in laemmli sample buffer (50 mmol/L Tris, pH 6.8, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol) at 95°C for 5 minutes. Proteins were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and transferred onto 0.45 µm Protran nitrocellulose membranes (Schleicher & Schuell, Keene, New Hampshire) in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, and 200 mmol/L methanol, pH 7.5) overnight at 4°C. Membranes were incubated in Tris-buffered saline ([TBS] 100 mmol/L Tris, 154 mmol/L NaCl, pH 8.0) containing 0.1% Tween 20 (TTBS) with 5% nonfat dry milk for 2 hours at 25°C to block nonspecific binding. The EMMPRIN N-terminal antibody was from BD Biosciences (Franklin Lakes, New Jersey). The C-terminal antibody was from Santa Cruz Biotechnology (Santa Cruz, California). Membranes were incubated with 0.1 to 1 µg/mL of specific antibody in TTBS containing 2.5% nonfat dry milk for 90 minutes at 25°C and washed 5 times for 3 minutes each in TTBS. Membranes were incubated with a 1:15 000 dilution of anti-mouse immunoglobulin G (IgG)–horseradish peroxidase (HRP) in TTBS containing 2.5% nonfat dry milk for 45 minutes at 25°C.

For immunoblot analysis of microvesicles, 15 µL of the microvesicle fraction (80× concentrated) was used. As stated previously, since CM samples were concentrated during the microvesicle preparation, we loaded equal volumes not equal

mass to be sure not to negate the purpose of concentrating the samples. A highly sensitive anti-EMMPRIN (BD Pharmingen, San Diego, California) antibody was used to detect EMMPRIN in microvesicles. This antibody was used at 0.1 µg/mL in 2.5% milk and incubated on the membranes for 1.5 hours. The membranes were washed with TTBS (pH 7.4), incubated with anti-mouse-HRP secondary antibody for 1 hour, and washed. For identification of microvesicle fractions, we utilized a specific antibody to recognize integrin β-1 (Millipore, Billerica, Massachusetts), which has been identified as a marker for microvesicles, specifically membrane shedding vesicles.³³ Primary antibody was used at 1 µg/mL in 2.5% milk and detected by anti-mouse IgG-HRP similar to methods described above.

For detection of MMPs in response to treatment with ILs, 15 µL of CM samples were denatured and prepared for immunoblotting as described previously. Membranes were probed with specific MMP-1 and MMP-3 antibodies (R&D Systems, Minneapolis, Minnesota). All antibodies were detected using the Super Signal chemiluminescence system (Pierce, Rockford, Illinois). Unstained protein molecular weight markers were used as standards (BioRad Laboratories, Hercules, California) and detected with Ponceau staining.

Immunoaffinity Depletion of EMMPRIN

Following 24 hours of HES cell culture, CM was collected, concentrated (75×), and depleted of EMMPRIN protein by immunoprecipitation using the Seize Primary Immunoprecipitation Kit (Pierce). Briefly, 200 µg of EMMPRIN-specific antibody, created in our laboratory,²⁴ was coupled to an AminoLink support gel (Pierce). The HES cell CM (0.5 mL of 75×) was incubated in the affinity column for 3 hours at 4°C. This was repeated twice to obtain a larger volume of immunodepleted CM. After 3-hour incubation, 15 µL of the unbound fraction in the CM was analyzed by immunoblotting and the membranes were exposed to film overnight for chemiluminescence detection of EMMPRIN protein. After confirming the complete removal of EMMPRIN from unbound fractions, these fractions were pooled and used for cell culture treatments.

Interleukin-1β/α Treatment of HUF Cells

The HUF cells were grown to confluence in RPMI-1640 culture medium (without phenol red) supplemented with 5% FBS, 5% BCS (Atlanta Biologicals), 120 U/mL penicillin/streptomycin, and 3.2 mmol/L L-glutamine (Cambrex). Once confluent, cells were subjected to a 24-hour serum starvation in RPMI-1640 medium (without phenol red) containing 3.2 mmol/L L-glutamine and 120 U/mL penicillin/streptomycin and then treated for 24 hours with 0, 10, 25, or 100 pg/mL of recombinant human IL-1α or -1β (R&D Systems) dissolved in 0.1% bovine serum albumin (BSA). Control groups received the same amounts of BSA as the 100 pg/mL treatment groups. Conditioned medium from treated HUF cells was collected, centrifuged at 2000g for 5 minutes to remove cell fragments,

concentrated (75×) in Amicon Ultra 10 000 MWCO concentrator tubes (Millipore) at 4000g for 30 minutes and stored at -20°C until processed. Cells were collected with TRIzol (Invitrogen) for RNA isolation.

RNA Isolation and Quantitative Reverse Transcription-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was used in 20 μL of reverse transcription (RT) reactions using the iScript First Strand Synthesis Kit for RT-PCR following manufacturer's instructions (BioRad Laboratories). Synthesized complementary DNA (cDNA) was then used for real-time PCR analysis.

Real-time PCR analyses were performed in 10 μL volume containing 1× TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Atlanta, Georgia), diluted cDNA, and RNase-free water. Genes were amplified using 20× Assays-on-Demand Gene Expression Assays (Cat #; MMP1 = Hs00233958_m1, MMP2 = Hs00234422_m1, MMP3 = Hs00233962_m1, EMMPRIN = Hs00174305_m1, and glyceraldehyde 3-phosphate dehydrogenase [GAPDH] = Hs99999905_m1). Real-time PCR amplification and detection were performed in MicroAmp optical 384-well reaction plates using the ABI Prism 7900 HT sequence detection system. Amplification conditions included: hold 10 minutes at 95°C , 40 thermal cycles of denaturing for 15 seconds at 95°C , and anneal/extension for 1 minute at 60°C . Relative fold induction levels were calculated using the comparative C_T Method for separate tube amplification. The GAPDH gene expression served as an endogenous control.

Statistical Analysis

An analysis of variance (ANOVA) model was used to evaluate the experimental variability between individual experiments. The difference between the threshold cycle of the target gene and GAPDH ($2^{-\Delta C_T}$) was used to determine statistically significant changes in messenger RNA (mRNA) levels for each treatment. The threshold cycle was defined as the cycle number where all transcripts were in the linear phase of amplification. The difference between the target gene and GAPDH was then normalized to control treatment (no treatment) expression and expressed as a relative fold difference. Statistical significance was measured to identify treatment effects within each gene evaluated by post hoc orthogonal contrast statements.

For immunoblots, densitometric analysis was performed using Image J software (NIH). To calculate the fold change between the different groups, the densitometry values for treatments were normalized to the average of the controls. An ANOVA test followed by secondary post hoc orthogonal contrast statements were used to determine statistical significance of the differences between treatment and control groups for each given protein. Different letters on the graphs indicate statistical differences.

Figure 1. Secretion of intact full-length extracellular matrix metalloproteinase inducer (EMMPRIN) from human uterine epithelial (HES) cells. Both N- and C-termini of EMMPRIN are detectable in concentrated (75×) but not in unconcentrated (1×) HES conditioned medium (CM) after 24 hours of cell culture. Both HeLa and HES cell lysates (CL) were used as positive controls.

Results

The EMMPRIN Protein Is Secreted by HES Cells via Microvesicle Shedding

The EMMPRIN protein was detected in both HES cell lysates and concentrated (75×) CM from HES cell cultures (Figure 1). The EMMPRIN immunoblot analysis using antibodies specific for both the extracellular (N-terminal) and intracellular (C-terminal) demonstrated that full-length EMMPRIN was present within the HES cell CM.

We then targeted specific factors present in the uterine microenvironment to determine what regulates the physiologic release of EMMPRIN from uterine epithelial cells. The release of EMMPRIN from HES cells into the CM was stimulated upon treatment with ovarian hormones or the proinflammatory cytokine, IL-1 β . Treatment of HES cells for 24 hours with estradiol (10 or 25 nmol/L) or progesterone (25 or 100 nmol/L) stimulated the release of EMMPRIN into the CM (Figure 2A). Stimulation of HES cells with both estradiol and progesterone did not increase EMMPRIN secretion above that of either steroid hormone alone, indicating a lack of synergism between estrogen and progesterone (Figure 2A). Treatment of HES cells with IL-1 β , for 24 hours stimulated EMMPRIN release into the CM and this stimulatory effect was evident at our lowest treatment concentrations of 0.5 ng/mL (Figure 2B). These data indicate that uterine epithelial cells are responsive to low levels of inflammatory cytokines and that EMMPRIN secretion is part of an inflammatory response.

We found that EMMPRIN secretion by HES cells occurred through microvesicle (specifically membrane shedding

Figure 2. Steroid hormone and cytokine stimulation of extracellular matrix metalloproteinase inducer (EMMPRIN) release by human uterine epithelial (HES) cells. A, The HES cells were treated with estradiol (0, 10, or 25 nmol/L), progesterone (25 or 100 nmol/L) or estradiol + progesterone (25/25 or 25/100 nmol/L) or B, with interleukin (IL)-1 β at 0.5 or 2 ng/mL for 24 hours and then the conditioned medium was concentrated 75-fold and assayed for EMMPRIN by immunoblotting. The HES cell lysates served as the positive control (+); $n = 3$.

vesicles) release. Intact EMMPRIN protein was present within the microvesicle (80 $\times$) fractions (P) of the CM, and the amount of protein within the microvesicle fraction increased with increased time of exposure to the HES cells in culture (Figure 3; top panel). The identification of integrin β -1 within the microvesicle fractions (Figure 3; lower panel) indicated that EMMPRIN release occurred through membrane shedding of vesicles and not exocytosis. After 24 hours of culture, detectable amounts of EMMPRIN were also found within the supernatant (1 $\times$) fraction of the CM (Figure 3, circled band) and not associated with microvesicles, as indicated by lack of integrin β -1 signal. The presence of full-length EMMPRIN unassociated with microvesicles in the CM suggested that the microvesicles undergo a time-dependent release of EMMPRIN.

To determine whether activation of the protein kinase C (PKC) signaling pathway could modulate the release of

membrane microvesicles, HES cells were treated with the phorbol ester PMA. The results demonstrated both a dose- and time-dependent increase in PMA-induced microvesicle release from HES cell membranes (Figure 4A and B).

HES Cell-CM Stimulates MMP Expression

The release of EMMPRIN from HES cells suggested that EMMPRIN might play a role in uterine remodeling by stimulating MMP production in underlying HUFs. To test this, HES cell-CM and EMMPRIN-immunodepleted HES cell CM were used to treat HUF cells. Following 2 rounds of immunodepletion of HES CM, we were no longer able to detect any EMMPRIN protein indicating that the majority of EMMPRIN had been successfully removed (Figure 5A). Treatment with 1 $\times$ or 75 $\times$ concentrated HES cell CM stimulated MMP-1 (11.53- [1 $\times$] vs 81.38- [75 $\times$] fold), MMP-2 (12.26- [1 $\times$] vs 82.73- [75 $\times$] fold), and MMP-3 (3.94- [1 $\times$] vs 28.09- [75 $\times$] fold) mRNA levels compared with untreated cells (Figure 5B; $P < .05$). Treatment with EMMPRIN-immunodepleted 75 $\times$ HES cell CM resulted in a reduced MMP stimulation in HUF cells compared with HUF cells treated with 75 $\times$ non-immunodepleted CM (MMP-1 [33.85- vs 81.38-fold], MMP-2 (23.33- vs 82.73-fold), and MMP-3 (2.33- vs 28.09-fold; Figure 5B; $P < .05$).

IL-1 α -IL-1 β Stimulation of MMPs by HUF cells

The evidence that EMMPRIN-depleted medium was still able to stimulate MMP mRNA levels led us to investigate other epithelial secreted factors, such as IL-1 α / β . Peritoneal fluid levels of IL-1 are reported to range from 11.4 to 16 pg/mL in endometriotic patients.³⁴ We therefore tested the ability of picogram concentrations of IL-1 α / β on MMP stimulation in HUF cells. We previously reported that MMP-2 mRNA levels were not stimulated by IL treatment of HUF cells²² and therefore only tested MMP-1 and MMP-3 mRNA and protein levels. Treatment with IL-1 α / β at concentrations ranging from 10 to 100 pg/mL indicated that IL-1 α increased MMP-1 and MMP-3 mRNA levels 12-fold over control BSA-treated cells at the highest concentration (100 pg/mL; Figure 6A, top panel).

Figure 3. The human uterine epithelial (HES) cells release extracellular matrix metalloproteinase inducer (EMMPRIN) by microvesicle shedding. The HES cells were serum starved for 24 hours, then received fresh medium which was collected at 0, 8, 12, or 24 hours of cell culture and then ultracentrifuged for microvesicle isolation. Supernatant ([S] 1 $\times$) and microvesicle pelleted ([P] 80 $\times$) fractions were analyzed by immunoblotting for EMMPRIN (top panel) and integrin β -1 (bottom panel). The EMMPRIN was detected in the microvesicle pellet fraction at 8, 12, and 24 hours but only at 24 hours in the supernatant (soluble) fraction (see circled band). The HES cell lysate was used as a positive control (+) for EMMPRIN.

Figure 4. The phorbyl 12-myristate-13-acetate (PMA) stimulates shedding of extracellular matrix metalloproteinase inducer (EMMPRIN) containing microvesicles. A, Treatment of human uterine epithelial (HES) cells with PMA for 2 or 4 hours in a dose-dependent manner did not alter EMMPRIN protein expression in HES cell lysates (CL) but did stimulate EMMPRIN shedding into the conditioned medium (CM). B, Treatment of HES cells with 100 ng/mL of PMA increased the shedding of EMMPRIN-containing microvesicles such that EMMPRIN was detected in the supernatant (S) and microvesicle pellet (P) fraction after 2 and 4 hours of treatment. CL+, HES cell lysates; CM+, HES conditioned medium. Both treatments served as positive controls.

Interestingly, while IL-1 α treatment did not affect MMP-1 protein secretion, treatment of HUF cells with either 25 or 100 pg/mL of IL-1 α stimulated MMP-3 protein secretion (Figure 6A, bottom panel).

Treatment of HUF cells with IL-1 β increased MMP-1 and MMP-3 mRNA levels at each concentration tested with a 15- to 30-fold increase for MMP-1 and a 5- to 17-fold increase for MMP-3 (Figure 6B, top panel). Accordingly, we found that IL-1 β treatment significantly increased MMP-1 (42- to 80-fold) and MMP-3 (30- to 100-fold) protein secretion at the same concentrations (Figure 6B, bottom panel). Whether IL-1 α and -1 β would act synergistically to stimulate MMP production by HUF cells was tested but neither MMP-1 nor MMP-3 mRNA or protein secretion was further increased when IL-1 α and - β were used together over the levels when either IL was used alone (data not shown).

Discussion

From these data presented, we can conclude that the transmembrane glycoprotein EMMPRIN is secreted by uterine epithelial cells through microvesicle shedding and stimulates MMP production by uterine stromal cells. The secretion of EMMPRIN is positively regulated by ovarian hormones estradiol and progesterone but also the proinflammatory cytokine IL-1 β . Upon epithelial cell stimulation, EMMPRIN release occurred through a PKC-dependent pathway. These data also suggest that uterine stromal cells secrete other factors (ie, ILs) that also stimulate MMP production suggesting that lesion remodeling is regulated through multiple mechanisms.

Regulation of EMMPRIN secretion by ovarian hormones support previous immunohistochemical evidence in both cycling human and murine endometrium that EMMPRIN

Figure 5. Immunodepletion of extracellular matrix metalloproteinase inducer (EMMPRIN) from human uterine epithelial (HES) cell conditioned medium reduces matrix metalloproteinase (MMP) stimulation in human uterine fibroblast (HUF) cells. A, Immunodepletion of EMMPRIN (75 $\times$ -ID) from 75-fold concentrated HES cell conditioned medium (75 $\times$ CM) was monitored by immunoblotting of fractions (1, first round of depletion; 2, second round depletion) and then used for (B) treatment of HUF cells to determine relative fold induction of MMP messenger RNAs (mRNAs). All samples were normalized to control untreated cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Statistical significance was compared relative to controls ($P < .05$), $n = 4$. 1 $\times$, unconcentrated HES-conditioned media.

Figure 6. Interleukin-1 α / β increases matrix metalloproteinase (MMP) expression and secretion in human uterine fibroblast (HUF) cells. The HUF cells were treated with 0, 10, 25, or 100 pg/mL of either interleukin (IL)-1 α or -1 β for 24 hours and MMP messenger RNA (mRNA) levels and protein expression were analyzed by qualitative polymerase chain reaction (q-PCR) and immunoblotting, respectively. A, The IL-1 α increased MMP-1 and -3 mRNA levels (top panel) but only MMP-3 protein secretion (bottom panel) at 25 and 100 pg/mL. B, IL-1 β increased MMP-1 and -3 mRNA levels and protein secretion in a dose-dependent manner. All samples were normalized to control untreated samples. Statistical significance was compared relative to control values for each gene or protein ($P < .05$), $n = 4$. 0 hrC, 0 hour of culture; UT, untreated 24 hour of culture; BSA, vehicle treatment.

localization is abundant during the proliferative or estrus phases of the reproductive cycle.^{6,22,23} Cytokine regulation of EMMPRIN is supported by EMMPRIN's dual role as an immune modulator³⁵ by suppressing activated T-cell responses³⁶ and serving as an anti-inflammatory mediator.³⁷ Ovarian hormone and cytokine regulated release of EMMPRIN through microvesicle shedding is consistent with communication modality utilized by both reproductive and nonreproductive tissues. Numerous cell types (fibroblasts, neuronal, immune, endothelial, and tumor) release microvesicles to coordinate normal and pathological physiological processes (reviewed by Cocucci et al³³). Additionally, microvesicle release is critical for proper placental function^{32,38} and more importantly, dysregulation of microvesicle release is implicated in obstetrical disorders such as preeclampsia.^{39,40}

Our data indicate that full-length EMMPRIN protein released from endometrial epithelial cells is a component of membrane microvesicles not exocytosis of endosomes. Following release from the cell membrane, the shed microvesicles degrade over time and release EMMPRIN as a soluble protein into the extracellular fluid. Soluble EMMPRIN was first detectable 8 hours after microvesicle release from the

cells and increased with time in a manner similar to a previous report by Sidhu et al.²⁵ Furthermore, microvesicle shedding from uterine epithelial cells can be regulated by the PKC signaling pathway as shown in other cell lines.^{25,41} The ability of HES cells to secrete a soluble, intact form of EMMPRIN supports the premise that epithelial cells can communicate with neighboring stromal cells without requiring direct cell-to-cell contact.

Although the downstream targets of EMMPRIN's receptor are relatively unknown, EMMPRIN stimulates MMP production in mesenchymal cells through the phospholipase A₂/5-lipoxygenase catalyzed pathways.⁴² In contrast, EMMPRIN stimulated MMP production in human endometrial stromal cells through activation of extracellular signal related kinases (ERK-1/2).²⁴ To demonstrate the necessity of EMMPRIN for stimulation of MMP expression in HUF cells, EMMPRIN was depleted from HES cell-CM prior to treatment of the HUF cells. While EMMPRIN immunodepletion reduced the ability of condition medium to stimulate MMP expression in HUF cells, it did not completely abolish it. These results suggested the presence of additional soluble factors in epithelial cell-CM capable of stimulating HUF cell MMP production. Candidates for such factors include cytokines, chemokines, and

antifibrogenic factors (stratifylin)^{43,44} all well-known regulators of MMPs.

Secretion of interleukin-1 β / α protein is increased in women with endometriosis,^{34,45} and these cytokines are also well-known positive regulators of MMPs in several cell types including uterine cells.^{22,46-48} Furthermore, IL-1 α is secreted specifically by uterine epithelial cells and regulates both IL and MMP production by uterine stromal cells.^{49,50} In our study, IL-1 α increased MMP-3 protein at the 10 pg/mL treatment dose and higher. Matrix metalloproteinase bioactivity was not examined in the studies presented here, but the secretion and expression increases are consistent with previous MMP studies.^{4,22,51} Therefore, IL-1 likely contributes to the ability of HES cell-CM to stimulate MMP expression and secretion by HUF cells. In the context of endometriosis, a small increase in IL-1 α or - β due to a mild inflammatory response in the peritoneum could increase MMP levels enough to allow the initial stages of endometriotic lesion invasion. The current work indicates that HES cells secrete low concentrations of IL-1 α and IL-1 β and that these concentrations increase MMP mRNA levels and protein secretion by uterine fibroblasts. Taken together, these data suggest a mechanism of action explaining how epithelial cells present within an endometrial lesion might instruct stromal cells, via EMMPRIN and IL signaling, to invade into tissues in the extra-uterine environment.

In conclusion, the findings presented in our studies provide new information to help elucidate the mechanisms involved in paracrine communication between endometrial epithelial and stromal cells. Shedding of EMMPRIN-containing microvesicles by uterine epithelial cells leads to stimulation of stromal cell MMP production and increased tissue remodeling within the endometrium. The shedding of microvesicles can be regulated and allows for direct communication between epithelial and stromal cells to coordinate extracellular matrix degradation and remodeling which is required for the initiation of menstruation, regeneration of the endometrial lining during the menstrual cycle, and also for ectopic endometrial invasion in endometriosis. Understanding the regulatory mechanisms involved in microvesicle shedding may lead to potential therapeutic targets for treatment and prevention of pathogenic conditions such as endometriosis and cancer.

Authors' Note

A. G. Braundmeier: study design, execution, manuscript drafting, and critical discussion. C. A. Dayger: study design, execution, analysis, manuscript drafting, and critical discussion. P. Mehrotra: execution, analysis, manuscript drafting. R. J. Belton, Jr: manuscript editing, critical discussion. R. A. Nowak study design, analysis, manuscript drafting, and critical discussion.

Declaration of Conflicting Interests

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