Epithelial cell protein milk fat globule–epidermal growth factor 8 and human chorionic gonadotropin regulate stromal cell apoptosis in the human endometrium

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Objective: To study the regulation of apoptosis in human endometrial cells. The specific aims were to determine whether milk fat globule–epidermal growth factor 8 (MFG-E8), a novel endometrial epithelial protein, modulates caspase activation and DNA fragmentation; and to examine whether hCG, an early embryonic product, regulates Bax and Bcl-*2* equilibrium, as well as MFG-E8 expression.
Design: Primary cultures of human endometrial epithelial cells (EECs) and endometrial stromal cells (ESCs).
Setting: Academic center.
Patient(s): Ovulatory women aged 21–30 years.
Intervention(s): Treatment with MFG-E8 and hCG.
Main Outcome Measure(s): Apoptotic activity was quantified using a luciferase assay. Deoxyribonucleic acid fragmentation was detected by TUNEL assay. Bax, Bcl-2, and MFG-E8 messenger RNA expression levels were determined by quantitative reverse transcription–polymerase chain reaction. Immunocytochemistry was used to establish cell purity and presence of MFG-E8 and hCG-R (receptor) proteins.
Result(s): Endometrial epithelial cells were cytokeratin^{+}, vimentin^{-}, MFG-E8^{+}, and hCG-R^{+}, whereas ESC were vimentin^{+}, cytokeratin^{-}, MFG-E8^{-}, and hCG-R^{+}. Treatment of ESC with MFG-E8 resulted in a 13-fold increase in caspase activity and a 30-fold increase in TUNEL. On the other hand, hCG decreased messenger RNA expression of Bax in ESC.
Conclusion(s): Milk fat globule–epidermal growth factor 8 has proapoptotic activity, suggesting participation in endometrial remodeling via an epithelial–stromal cell paracrine effect. Conversely, pregnancy levels of hCG has opposite effects on stromal cells. (Fertil Steril® 2012;98:1549–56. ©2012 by American Society for Reproductive Medicine.)
Key Words: Apoptosis, caspase activity, endometrium, hCG, human, MFG-E8

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The human endometrium is a highly regulated tissue, undergoing predictable phases of growth and proliferation followed by secretory changes in preparation for pregnancy (1). Classic histologic findings highlight the significant morphologic changes that occur in the distinct phases of the menstrual cycle (2). If pregnancy occurs, the endometrium is globally stabilized, allowing for the blastocyst to juxtapose and attach to the endometrial epithelium (3).

The endometrium is receptive to embryo attachment and invasion during a critical period of the menstrual cycle known as the "window of implantation" (4). The gene expression profile of the prereceptive and receptive endometrium during normal natural cycles has been

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studied by several investigators, primarily using microarray technologies, demonstrating a number of up- and down-regulated genes during the window of implantation (5). Our group demonstrated the up-regulation of 107 genes during the window of implantation (6). Among them, milk fat globule–epidermal growth factor (EGF) 8 protein (MFG-E8), a novel endometrial protein not previously linked to the endometrium or implantation, was found to be significantly up-regulated during the receptive phase (6).

The secreted glycoprotein MFG-E8 (also known as lactadherin, SED1, and breast antigen 46 [BA46]) is a component of the milk fat globule membrane (7). Milk fat globule–E8 has been isolated in the human, mouse, rat, cow, and monkey. Human MFG-E8 is a 46-kDa glycoprotein peripherally associated to the cell membrane with two N-terminal EGF-like repeats, one of which includes an arginine-glycine-aspartic acid (RGD) motif that serves as the ligand to its $\alpha v\beta 3$ integrin receptor. Milk fat globule–E8 has a variety of functions related to apoptosis, cell adhesion and remodeling, neovascularization, and immunomodulation in extrauterine tissues (8–12). Milk fat globule–E8 promotes RGD-dependent cell adhesion via integrins (10), and MFG-E8, secreted by macrophages, specifically binds to apoptotic cells by recognizing aminophospholipids to facilitate engulfment by phagocytes (11). Additionally, MFG-E8, found in significant quantities in human breast milk, plays a role in breast tissue remodeling during lactation and the prevention of rotavirus infection in pediatric populations (8, 9).

We recently demonstrated that MFG-E8 intracellular protein is predominantly localized to the epithelial compartment of the human endometrium (both in luminal and glandular epithelial cells, and with intense staining at both apical and basal cellular compartments), whereas its receptor, integrin $\alpha v\beta 3$, is present in both the epithelial and stromal compartments, both with cycle-dependent and coincident peak expression at midsecretory phase (13). We also showed that intracellular MFG-E8 protein was up-regulated after 72 hours of PRL treatment, providing support for a modulatory role for PRL as a stromal–epithelial paracrine factor controlling MFG-E8 production (13). Furthermore, we reported that MFG-E8 protein is highly expressed in human chorionic villi at all trimesters of gestation (in both syncytio- and cytotrophoblast cells) and in murine implantation sites (in surface epithelial, decidual, and vasculature cells) (14). Finally, we presented new evidence that MFG-E8 can also be immunolocalized in endometrial endothelial cells and that recombinant MFG-E8 modulates endometrial endothelial cell proliferation and adhesion under in vitro conditions (15).

Human chorionic gonadotropin is a highly conserved glycoprotein hormone; its gene expression can be detected in the oocyte, zygote, and early-cleavage-stage embryos, and the protein is secreted by the early blastocyst (16–19). Before hCG can be measured in peripheral circulation and around the time of implantation, the embryo secretes hCG within the uterus. The presence of hCG receptors (hCG-R) has been demonstrated throughout the endometrial cycle (20), which strongly suggests that hCG may play a role in the early "dialogue" between the embryo and uterus to facilitate embryo implantation.

Although a luteotropic role for hCG in the corpus luteum is well established, the significance of hCG-R in the endometrium has not yet been defined. It has been proposed that hCG, as an early embryonic signal, rescues the stromal fibroblasts from normal regression before the end of the menstrual cycle at the implantation site. Endometrial Bax and/or Bcl-2, two well-characterized apoptotic regulators, can be modulated by hCG (21, 22). We speculated that epithelial MFG-E8 may also be involved in reorganization of the endometrium via apoptosis control.

This study hypothesized that MFG-E8, a marker of epithelial function, and embryonic hCG may act in a paracrine fashion, to control the cellular fate of endometrial stromal cells and thus participate in endometrial remodeling and stromal invasion during the critical stages of early implantation. The objectives of this study were to determine whether MFG-E8 modulates activation of caspases, final executioners of apoptosis, as well as DNA fragmentation, a late biomarker of apoptosis in somatic cells (23); and to examine whether hCG regulates Bax and Bcl-*2* equilibrium as well as MFG-E8 at the level of messenger RNA (mRNA) expression.

MATERIALS AND METHODS

Human Subjects and Endometrial Biopsies

Endometrial biopsies were obtained from healthy volunteers (21–30 years of age). Study participants were ovulatory women with normal menstrual cycles not using hormonal contraception. The Institutional Review Board of Eastern Virginia Medical School approved this study, and written informed consent was obtained before the recruitment and collection of biopsies. Dating of the secretory-phase biopsies (days 21 through 24) was assessed by urinary ovulation prediction kits and using the criteria of Noyes et al. (2). Biopsies were performed on natural cycles, using one or two passes of an endometrial suction pipelle (Unimar Pipelle; CooperSurgical) (6). The endometrial biopsies were immediately divided into two fragments: one fragment was fixed in formalin and submitted for histologic evaluation by an independent pathologist, and the second and largest fragment was used for primary cultures.

Isolation of Human Endometrial Epithelial and Stromal Cells and Primary Cell Cultures

Endometrial biopsies ($n = 3$–5 per cycle day per experiment) were separated into stromal (ESC) and epithelial cells (EEC), as previously described by others (24, 25) and slightly modified by us (13) (see Supplemental Materials and Methods, available online). The purity of the EEC and ESC preparations was assessed by immunocytochemical analysis of vimentin and cytokeratin, specific markers for stromal and epithelial cells, respectively (13).

Immunocytochemistry and Immunofluorescence

Immunocytochemistry was performed to characterize the phenotype of EEC and ESC by probing for cytokeratin 18 and vimentin, In summary, cells were washed with phosphate-buffered saline (PBS) and fixed with prechilled

methanol (at −20°C) on culture slides; endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes, and nonspecific binding sites were blocked with 2% normal goat serum for 60 minutes at room temperature. The primary antibodies (mouse monoclonal) were serially diluted in a solution of PBS–2% normal goat serum to optimize sensitivity and specificity. The primary antibodies were used at 1:50 dilution and obtained from Abcam. The Envision Kit (Dako North America) was used to visualize localization of the antibody by a brownish precipitate. After primary antibody incubation, slides were washed three times with PBS and incubated with biotinylated goat anti-mouse secondary antibody at a dilution of 1:120 for 30 minutes at room temperature. After rinsing with PBS, the immunoreactive antigen was visualized by incubating with avidin biotinylated horseradish peroxidase (1:100) complex for 30 minutes and 3,3′-diaminobenzidine (0.5 mg/mL) as chromagen for 3 minutes to complete the reaction. Negative controls included sections that were treated with a nonimmune IgG irrelevant control antibody (normal mouse ascites, clone NS-1; Sigma) at similar concentration as primary antibodies. Slides were counterstained with Mayer's hematoxylin (Sigma), then dehydrated in a graded series of ethanol, cleared in xylene, and mounted with mounting media. Representative fields were photographed at ×200 and ×400 magnification with an Olympus microscope using an Olympus Q-color 3 camera.

In addition, immunofluorescence was performed for MFG-E8 and hCG-R (Supplemental Materials and Methods).

In Vitro Decidualization and Hormonal Treatments

In all experiments described below, and to minimize endogenous steroid hormones, after isolation and confluence cells were grown in dishes or slides, washed, and cultured in media supplemented with 10% charcoal-stripped fetal bovine serum (FBS). In vitro decidualization of ESC was accomplished as described previously by treating cultured stromal cells with 1 mM 8-bromo-cyclic adenosine monophosphate (Sigma-Aldrich) for 24 hours (26, 27). Decidualization was confirmed by PRL secretion into the culture medium, both in monolayers as well as in three-dimensional cocultures of endometrial epithelial and stromal cells (data not shown) (28, 29). The EEC and decidualized ESC cells were further treated with MFG-E8 and hCG as described below.

MFG-E8 Effect on Epithelial and Stromal Cells Apoptosis: Caspase Activation and DNA Fragmentation

To study a possible regulatory role of MFG-E8 on apoptosis in the human endometrium, isolated and purified EEC and ESC were treated with 2 μg/mL human recombinant MFG-E8 (R&D Systems) or PBS for vehicle control for 24 hours (30).

Apoptosis was evaluated by measuring caspase activity using a Promega Caspase-Glo 3/7 Assay (Supplemental Materials and Methods). Staurosporine (1.5 mM; Sigma-Aldrich) was used as a positive control (31).

Deoxyribonucleic acid fragmentation was detected in the primary stromal cells by TUNEL assay. Briefly, we performed the TUNEL assay with an in situ cell detection kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. The cells were seeded onto a chamber slide and grown for 48 hours. The MFG-E8–treated cells were exposed to 2 μg/mL of the recombinant protein, and untreated cells served as controls. In addition, dose-dependent experiments were performed for MFG-E8 concentrations of 0.5 μg/mL, 1 μg/mL, and 2 μg/mL for 24-hour treatment. Negative controls were established with omission of the terminal transferase, and positive controls consisted of cells treated with DNAseI at 3 U/mL (Sigma) in 50 mM Tris-HCl (pH 7.5) and 1 mg/mL bovine serum albumin, for 10 minutes at room temperature. Thereafter, the cells were washed with PBS/1% bovine serum albumin and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Cells were then incubated with 50 μL of TUNEL reaction mixture (terminal deoxynucleotidyl transferase [TdT] and fluorescein–eoxynucleotide triphosphate [dUTP]) at 37°C for 60 minutes in a humid atmosphere. Cells were labeled with propidium iodide (PI) by the addition of mounting medium containing PI (Vector Laboratories). Results were evaluated under a fluorescence microscope, Olympus BX50, with a DP-70 Olympus camera. Two hundred cells (PI^+) were counted in each of a total of three to five experiments, and results were analyzed as number of apoptotic cells ($TUNEL^+$)/total cells for each treatment dose.

MFG-E8 and Bax/Bcl-2 mRNA Expression in Primary Endometrial Cultures Examined by Quantitative Reverse Transcription–Polymerase Chain Reaction

At doses near those concentrations measured in serum during early pregnancy, lyophilized, highly purified hCG (Sigma-Aldrich) was diluted to 0.5 mIU/mL, 5 mIU/mL, 50 mIU/mL, and 500 mIU/mL with PBS as vehicle control and incubated with each confluent cell line for 6 and 24 hours (32).

RNA Isolation and Reverse Transcription

After incubation and treatment, cells grown in culture dishes were washed and scraped, and total RNA was extracted from the primary EEC and ESC cultures using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Ribonucleic acid isolation and reverse transcriptions were performed according to our previously published protocols (13, 33) (Supplemental Materials and Methods).

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed using a Lightcycler Fastart DNA Master Plus SYBR green I and a Lightcycler 2.0 instrument (Roche Applied Science) in a 20-μL reaction volume composed of 2 μL of each complementary DNA and 0.5 μM of each sense and antisense primer (glyceraldehyde 3-phosphate dehydrogenase used with 0.3 μM) (details in Supplemental Materials and Methods).

The primer source/sequence and the expected lengths of the resulting polymerase chain reaction products for MFG-E8, Bax, Bcl-2, and glyceraldehyde 3-phosphate dehydrogenase are shown in Supplemental Table 1. Primers were individually designed using Oligo Explorer or Primer 3 software and purchased from Invitrogen.

Statistical Analysis

Three to five independent experiments were performed for each cell type, treatment, and treatment concentration condition. Means from the individual experiments were calculated and evaluated for normality. Results of caspase activation and quantitative real-time polymerase chain reaction were analyzed by one-way analysis of variance or the nonparametric equivalent (Kruskal–Wallis test), to compare experiments with multiple treatments. Dunn's post hoc test for pairwise multiple comparisons was used when appropriate. The proportions of cells with DNA fragmentation (TUNEL) were compared by logistic regression. All data are expressed as mean $\pm$ SD. Significance was defined as $P<.05$. Sigma Plot (version 11.0; Systat Software) statistical software was used for data analysis and graph development.

RESULTS

Characterization of Human EEC and ESC: Markers of Purity

The purity of the human endometrial epithelial and stromal separation procedures and cultures was evaluated by staining for cytokeratin and vimentin. As expected, the primary epithelial cell cultures stained positive for cytokeratin but not for vimentin, whereas stromal cells cultures stained for vimentin but not for cytokeratin (Fig. 1A and B). Analysis confirmed >99% purity of EEC and ESC preparations in all experiments.

Expression of MFGE-8 and hCG-R Proteins in EEC and ESC

Milk fat globule–E8 protein was immunolocalized in primary EEC with strong staining, whereas ESC were negative (Fig. 1C). The presence of hCG-R was demonstrated in both EEC (not shown) and ESC (Fig. 1D).

MFG-E8, an Epithelial Product, Has Proapoptotic Activity in Human ESC

At 24 hours of culture, treatment of human ESC with human recombinant MFG-E8 (2 μg/mL) resulted in a statistically significant 13-fold increase in caspase activity ($P<.04$) (Fig. 2). In contrast, treatment of human EEC did not result in significant change in caspase activity (not shown).

Similarly, treatment of human ESC with MFG-E8 (2 μg/mL) resulted in a statistically significant increase in DNA fragmentation as detected by TUNEL assay (15% in treated cells vs. 0.7% in controls, $P<.0001$). Figure 3 shows merged photographs of PI (red fluorescence) and TUNEL (green fluorescence at $\times$200) comparing positive control (DNAase) (Fig. 3A), negative control (absence of enzyme) (Fig. 3B), untreated cells, and cells treated with MFG-E8 (2 μg/mL). Figure 3D shows results of dose-dependent experiments that demonstrated a 30-fold increase in TUNEL$^+$ cells upon MFG-E8 treatment ($P=.0001$).

hCG Modulation of MFG-E8, Bax, and Bcl-2 mRNA Expression

Functional studies were performed by treating the EEC and ESC with increasing hCG concentrations for 6 and 24 hours. The hCG treatment did not result in changes in MFG-E8 mRNA levels at any investigated time (not shown). Whereas hCG did not affect Bax or Bcl-2 gene expression in EEC, Bax mRNA levels significantly decreased in ESC cells upon hCG treatment ($P<.05$) at the higher doses (50 and 500 mIU/mL) and for the longest treatment duration (24 hours) (Fig. 4).

DISCUSSION

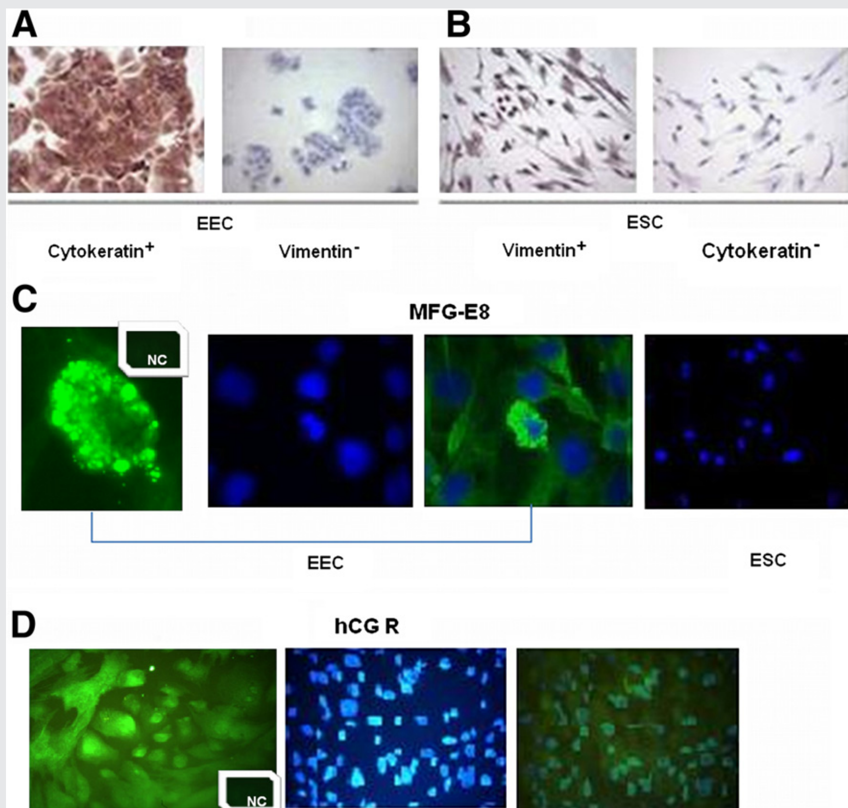
The present studies confirmed and extended our original findings that MFG-E8 is almost exclusively an epithelial protein, with practically undetectable expression in the stromal cells of the human endometrium, as previously evaluated in fixed tissue from endometrial biopsies and in endometrial cell primary cultures (13). Peak epithelial cell expression of MFG-E8 mRNA and protein occurred during the window of implantation; and this was temporally coincident with the highest epithelial cell expression of one of its specific receptors, $\alpha v\beta 3$ integrin (13). These findings were consistent with previous work that found a similar distribution of the heterodimeric $\alpha v\beta 3$ integrin receptor with a diminution of staining intensity in the stromal compartment (34). These two molecules colocalized in the endometrial epithelium, but more data are needed to establish functional interaction among the proteins in these cells. It should be noted that stromal cells had positive and highest immunostaining in endometrial tissue also during the receptive phase (13).

Little is known about MFG-E8 function and regulation in the human endometrium. We have presented initial data from in vitro studies showing that the intracellular protein production of MFG-E8 in EEC seems not to be regulated by the ovarian steroids estrogen and P, but that it is up-regulated by PRL, a well-known product of decidualized stroma (13). This is consistent with the fact that in peripheral macrophages, PRL is known to up-regulate MFG-E8 production (35). Such an interaction might represent a paracrine effect of differentiated–decidualized stromal cells on epithelial cell function.

We also examined whether hCG, an early embryonic product, could modulate endometrial MFG-E8 expression. Although the luteotropic role of hCG in human reproduction has been extensively studied, the possible functions of hCG in the human endometrium, given the presence of hCG-R, need to be unveiled. Earlier investigations have suggested roles in immunomodulation and decrease of endometrial apoptosis in humans (21, 22). Confirming previous studies, the presence of hCG-R was documented in the present work in both the epithelial and stromal primary cultures.

We found that hCG did not modulate MFG-E8 expression at the gene level, but it did enhance MFG-E8 secretion in the culture medium in the form of vesicles (36). Oshima et al. (37) have demonstrated by scanning electron microscopy that

FIGURE 1



Characterization of cultured EEC and ESC. Immunocytochemical analysis of human EEC (A) and ESC (B) using markers of epithelial (cytokeratin) and stromal (vimentin) phenotypes, confirming purity of cultures (original magnification, ×200). (C) Milk fat globule–E8 protein immunolocation (fluorescein isothiocyanate conjugate) in primary EEC and ESC in culture by immunofluorescence (MFG-E8 green, 6-diamino-2-phenylindole counterstain *blue*, and *green* and *blue* merged; original magnification, ×400; NC = negative control). The EEC showed strong immunoreactivity, whereas ESC (merged) were negative. (D) Immunolocation of hCG-R in ESC cells by immunofluorescence (MFG-E8 *green*, 6-diamino-2-phenylindole counterstain *blue*, and *green* and *blue* merged; original magnification, ×250; NC = negative control).

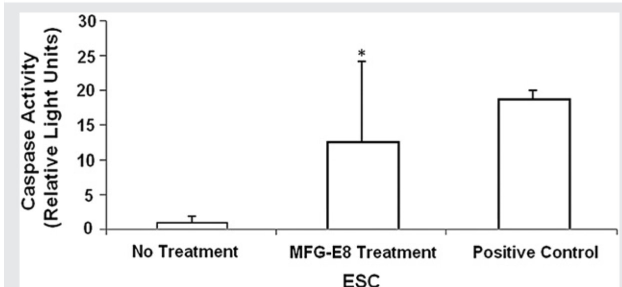
Riggs. MFG-E8 control of endometrial apoptosis. Fertil Steril 2012.

MFG-E8 is secreted by mammary epithelium into the culture medium in association with small membrane vesicles with a size from 100–200 nm in diameter. These results suggest a possible role of MFG-E8 in the membrane vesicle secretion, such as budding or shedding of plasma membrane (microvesicles) and exocytosis of endocytic multivesicular bodies (exosomes). Further studies with confocal microscopy are needed to elucidate whether epithelial cell MFG-E8 vesicular secretion occurs from the apical (exocrine) and/or basolateral membranes (paracrine), or both.

Apoptosis has been suggested to play a pivotal role in the reproductive physiology of the endometrium (38–41). Several studies in humans have reported the presence of endometrial apoptotic cells, appearing mainly at the beginning of the secretory phase and finally peaking during the menstrual phase (38–41). Von Rango et al. (42) previously noted that the occurrence of apoptosis during the midsecretory phase coincided with the window of endometrial receptivity for implantation of the embryo. They postulated that endometrial apoptosis was probably involved in the process of endometrial preparation for implantation and pregnancy.

Regarding MFG-E8 function in the endometrium, we demonstrated that recombinant MFG-E8 increased ESC caspase activity, with signs of late stages of apoptosis as

FIGURE 2



Effect of recombinant MFG-E8 (2 μg/mL) on caspase activation in ESC (*P<.04 vs. controls without treatment). Positive controls: cells treated with staurosporin. Milk fat globule–E8 significantly increased caspase-3 activity in endometrial stromal cells compared with controls.

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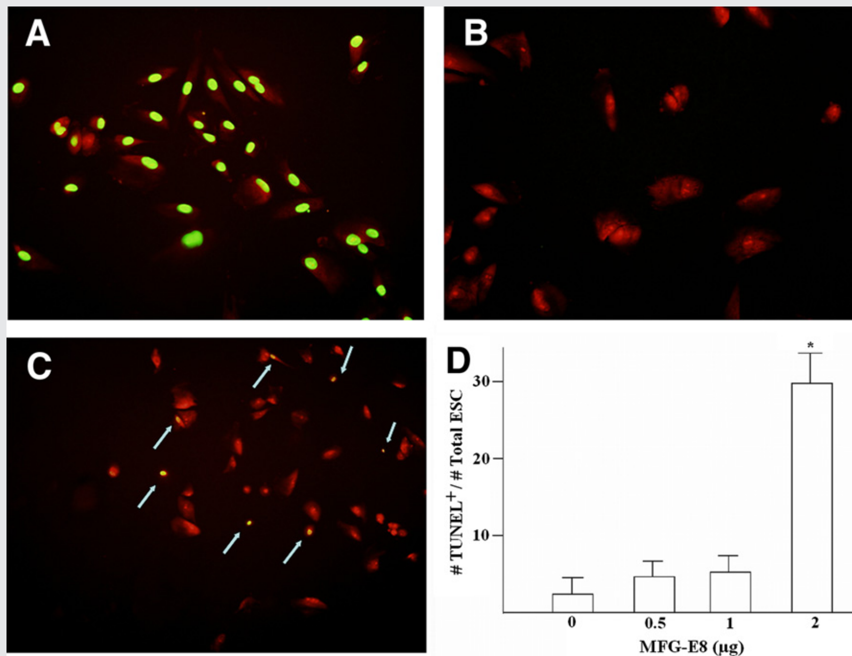
FIGURE 3



Effect of recombinant MFG-E8 (2 μg/mL) on DNA fragmentation (TUNEL) in ESC (*$P<.0001$ vs. controls without treatment). Milk fat globule–E8 significantly increased ESC DNA fragmentation in a dose-dependent manner. Merged photographs of PI (*red* fluorescence) and TUNEL (*green* fluorescence at $\times$200). (A) Positive control (DNAase). (B) Negative control (absence of enzyme). (C) Untreated cells. (D) Cells treated with MFG-E8; dose-dependent effect of MFG-E8 ($P=.0001$).

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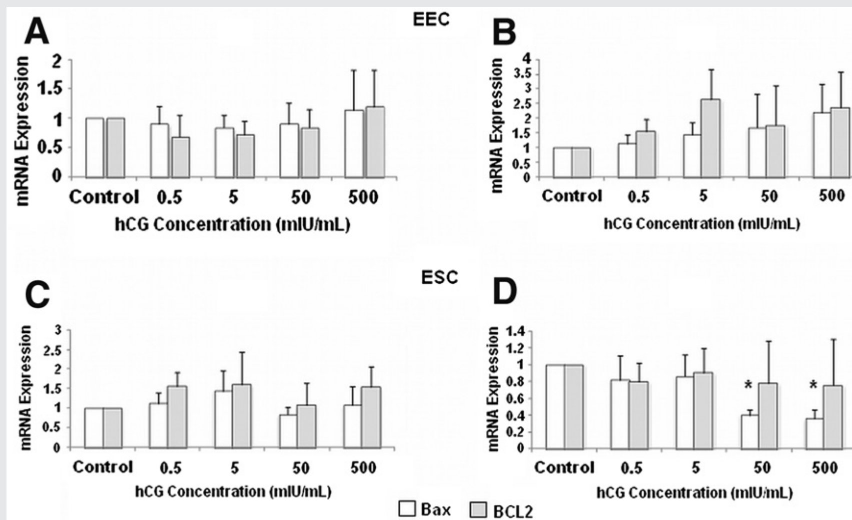
evidenced by DNA fragmentation, clearly indicating a proapoptotic effect of the molecule. The MFG-E8 proapoptotic activity in stromal cells around the window of implantation was dose-dependent, and the extent of this activity (approximately 15% of apoptotic cells) was moderate and consistent with what is observed in the midsecretory endometrium (22% apoptosis by TUNEL by Castro et al. [43]), later increasing to 80% apoptotic cells in the late secretory, premenstrual phase

FIGURE 4



Effect of hCG on gene expression of Bax and Bcl-2 in cultured cells. In EEC, hCG did not have any effect at the doses and time points examined (A: 6 hours of culture; B: 24 hours of culture). In ESC, hCG had no effect on Bcl-2 and did not affect Bax expression at 6 hours (C); on the other hand, Bax mRNA levels significantly decreased (*$P<0.05$) in ESC upon hCG treatment at the higher doses (50 and 500 mIU/mL) for the longest treatment duration (24 hours) (D).

Riggs. MFG-E8 control of endometrial apoptosis. Fertil Steril 2012.

(38). Milk fat globule–E8 involvement in elimination of apoptotic cells has been reported in the mouse intestinal tract, retinal epithelium, and in patients with autoimmune diseases such as systemic lupus erythematosus (44–48). Additionally, breast epithelial MFG-E8 has been proposed to act as a paracrine stromal factor for mammary gland morphogenesis (49). In the mouse mammary gland model, SED1 (the mouse homologue of MFG-E8), secreted by either myoepithelial or luminal epithelial cells, participates in branching morphogenesis by virtue of its N-terminal RGD motif binding to αv-containing integrins on myoepithelial cells. Additionally, the angiogenic role of MFG-E8 has been demonstrated in vivo in a mouse model of acute hindlimb ischemia (50) as well as in vitro, stimulating adhesion and proliferation of both endothelial and vascular smooth muscle arterial cells (51). We propose that, given the known functions of MFG-E8 in tissue remodeling, the high tissue and vascular turnover within the human endometrium, and the present findings, MFG-E8 is an active participant in endometrial stromal remodeling during the secretory phase.

Under the conditions studied herein, hCG decreased Bax but not Bcl-2 mRNA expression. These findings are consistent with prior work that demonstrated diminished apoptosis and enhanced stromal differentiation during the window of implantation in the baboon (52) and in the human endometrium (22, 38, 39). The antiapoptotic role of hCG in the endometrium has been reported, with hCG increasing protein levels of Bcl-2 (53) with concomitant down-regulation of Bax (53). Li et al. (54) demonstrated that lactadherin/MFG-E8 increases human endothelial cell apoptosis with up-regulation of bax/bcl protein ratio.

Evidence has been presented that the generation of an hCG gradient created by secretion of hCG by the syncytiotrophoblasts stimulates a differentiation pathway toward a hormonally active phenotype, whereas other decidual factors stimulate differentiation toward a junctional and anchoring trophoblast at the maternal decidual level, thereby stabilizing stromal cells at that site (55).

It can be speculated that one of many MFG-E8 functions may be to act as a paracrine factor (epithelial to stromal cells) to influence endometrial remodeling through stimulation of apoptosis in the stromal compartment at the critical time of early embryonic invasion. Milk fat globule–EGF 8 presence in chorionic villae as demonstratd by us (14) suggests continuing participation of this molecule in later stages of invasion and placentation. On the other hand, hCG secreted by the trophoblast cells plays an inverse role in the stabilization of the endometrium via an antiapoptotic effect on stromal cells at the implantation site, perhaps with a different temporal–spatial relationship to MFG-E8, and resulting in controlled/limited invasion. We hypothesize that MFG-E8, with peak expression in the endometrial epithelium in the midsecretory phase, can promote stromal/matrix remodeling by inducing apoptosis, and endothelial cell proliferation. In the secretory phase, MFG-E8 may also aid in vascular remodeling by inducing endothelial cell adhesion and proliferation (15). At a later time of implantation, the blastocyst may counterbalance or limit apoptosis via its secretion of hCG.

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SUPPLEMENTAL MATERIALS AND METHODS

Isolation of Human EEC and ESC and Primary Cell Cultures

Endometrial biopsies (n = 3–5 per cycle day per experiment) were separated into stromal (ESC) and epithelial cells (EEC) as previously described by others (1, 2) and slightly modified by us (3). Briefly, the tissue samples were minced and incubated with shaking for 1 hour at 37°C, in DMEM/F-12 1:1 (Dulbecco's modified Eagle's medium/Ham's F-12; GIBCO) containing 0.1% collagenase type I and 0.005% deoxyribonuclease type I (Sigma). After digestion, stromal cells and epithelial clumps were then separated on a size basis using gravity sedimentation and membrane filtration. The pellet (epithelial-rich fraction) was further rinsed with DMEM two or three times. The epithelial pellet was purified of residual stromal cells and undigested clumps as follows: partially purified epithelium was placed in 75-cm^2 Falcon flasks (BD Biosciences) in DMEM containing 2% fetal bovine serum (FBS; GIBCO) and then incubated at 37°C in a 95% air–5% CO_2 atmosphere; under these conditions the residual stromal cells and macrophages selectively attached to the plastic within 20–30 minutes. The media containing unattached epithelial cells were transferred to a new flask. After repetition of this procedure twice the purified epithelial cells were collected, and their structural integrity was assessed by inverted microscopy. The EEC and ESC were then cultured in EEC medium supplemented with 10% FBS. The EEC medium was composed of 75% DMEM (GIBCO) and 25% MCDB-105 (Sigma) containing antibiotics (penicillin/streptomycin 1% vol/vol; GIBCO), supplemented with 10% charcoal and dextran-treated FBS (Hyclone) and 5 pg/mL insulin (Sigma). The cells were cultured in 6- or 12-well dishes (Corning) or culture slides (BD Biosciences) until reaching 80%–90% confluence and were used in the subsequent experiments.

RNA Isolation and Reverse Transcription

After incubation and treatment, cells grown in culture dishes were washed and scraped, and total RNA was extracted from the primary EEC and ESC cultures using an RNeasy kit (Qiagen) according to the manufacturer's instructions. The RNA isolation and reverse transcriptions were performed according to our previously published protocols (3, 4).

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (PCR) was performed using a Lightcycler Fastart DNA Master Plus SYBR green I and a Lightcycler 2.0 instrument (Roche Applied Science) in a 20-μL reaction volume composed of 2 μL of each complementary DNA (cDNA) and 0.5 μM of each sense and antisense primer (glyceraldehyde 3-phosphate dehydrogenase [GADPH] used with 0.3 μM). Before amplification, samples were denatured at 95°C for 10 minutes, followed by template amplification by 45 cycles of denaturation 95°C for 10 seconds, annealing of primer at the specific temperature given in Supplemental Table 1 for 5 seconds, and extension at 72°C for 10 seconds followed by final extension at 72°C for 10 minutes. The melting protocol consisted of heating the samples to 95°C, followed by cooling to 65°C for 15 seconds and slowly heating at 0.1°C per second to 95°C while monitoring fluorescence. Melting curve analysis was performed after each run to verify specific amplification. Negative control(s) consisted of water replacing the cDNA solution (no template control). All PCR products exhibited a single melting curve peak and were identified as single bands of the appropriate size on ethidium bromide–stained 3% agarose gel electrophoresis. The cDNA levels were obtained using a standard curve, and the values obtained were normalized to those found for GAPDH (a housekeeping gene that catalyzes the sixth step of glycolysis) for differing amounts of starting material (5, 33). The primer source/sequence and the expected lengths of the resulting PCR products for milk fat globule–epidermal growth factor 8 (MFG-E8), Bax, Bcl-2, and GADPH are shown in Supplemental Table 1. Primers were individually designed using Oligo Explorer or Primer 3 software and purchased from Invitrogen.

The quality of total RNA extracted was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies), and quantification of total RNA was performed on a NanoDrop spectrophotometer (Thermo Scientific) (3, 4).

Reverse Transcription

Complementary DNA was generated from 300 ng of total RNA in a volume of 20 μL containing the following: 2.5 μM random hexamers, 2.5 U/μL with murine leukemia virus reverse transcriptase, 1 U/μL RNase inhibitor, 1× PCR buffer, 1 mM each decoy nucleoside triphosphate (NTP), and 5 mM $MgCl_2$ (Applied Biosystems). The reverse transcription reaction parameters were as follows: 23°C for 10 minutes, 42°C for 15 minutes (reverse transcription reaction), and 99°C for 5 minutes in an iCycler thermal cycler (BioRad). The cDNA solutions were then stored at −20°C. Preparations without reverse transcriptase were used as negative controls, in which the absence of PCR products indicated the absence of contaminating genomic DNA (3, 4).

Immunocytochemistry and Immunofluorescence

Briefly, isolated EEC and ESC were grown on glass slides (BD Biosciences) and then washed and fixed in situ with cold methanol for 10 minutes. After washing with phosphate-buffered saline, 2% normal serum was used for blocking for 60 minutes, and then samples were incubated with primary antibody anti–MFG-E8 (0.1 μg/mL; Abcam) and anti-hCG-R (0.5 μg/mL; Abcam) overnight at 4°C. A goat anti-mouse antibody conjugated with fluorescein isothiocyanate conjugate was used as second antibody (Santa Cruz Biotechnologies) and incubated for 90 minutes at room temperature. Negative controls included sections that were treated with a nonimmune IgG irrelevant control antibody (normal mouse ascites, clone NS-1; Sigma) at similar concentration as primary antibodies. After washing the second antibody, slides were mounted with anti-fading medium (Vector Laboratories) and analyzed under fluorescence microscopy (Nikon Eclipse E600) equipped with a SPOT-RT Slider digital camera (Diagnostic Instruments).

MFG-E8 Effect on Epithelial and Stromal Cells Apoptosis: Caspase Activation and DNA Fragmentation

Briefly, the assay is a homogenous luminescent assay that measures caspases-3 and -7 activity through luciferase activity. If caspase cleavage occurs, a substrate for luciferase (aminoluciferin) is released, yielding a luciferase reaction and the production of luminescent signal (relative light units) measured on an MLX microplate luminometer (Dynex 4.06; Dynex Technologies). Higher relative light units values are associated with greater caspase activity. Cells were grown in dishes, harvested using a sterile cell scraper, and prepared by mixing the cell substrate with an equal amount of reconstituted reagent and briefly placing the mixture on a plate shaker, incubated at room temperature for 30 minutes, and followed by luminescence measurement. Staurosporine (1.5 mM; Sigma-Aldrich) was used as a positive control (4).

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SUPPLEMENTAL TABLE 1

Oligonucleotide primers used for PCR.

Gene name	Primer sequence	Annealing temperature (°C)	Product size (bp)
GADPH	Fw: 5′-GAGTCAACCGGATTTGGTCGT Rv: 5′-CGTAGCAAGGCACAGATCAG	58	123
MFG-E8	Fw: 5′-GCCCTGGATATCTGTTCCAA Rv: 5′-GCTCGACACATTTCGTCTCA	58	151
Bax	Fw: 5′-CTGGACAGTAACATGGAGCTG Rv: 5′-GGCGTCCCAAAGTAGGAGA	60	296
Bcl-2	Fw: 5′-CCTGTGGATGACTGAGTACC Rv: 5′-GAGACAGCCAGGAGAAATCA	58	128

Note: Fw = forward; Rv = reverse.

Riggs. MFG-E8 control of endometrial apoptosis. Fertil Steril 2012.