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ORIGINAL PAPER

Estrogen stimulates the human endometrium to express a factor(s) that promotes vascular smooth muscle cell migration as an early step in microvessel remodeling

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Abstract Vascular smooth muscle cell (VSMC) migration is a pivotal early step in blood vessel remodeling; however, very little is known about the regulation of this process in the human endometrium during the menstrual cycle. In this study, explants of human endometrium were incubated with estradiol and/or progesterone and the conditioned medium (CM) applied to cultures of VSMC to test the hypothesis that estrogen and progesterone stimulate endometrial cells to secrete a factor(s) that promotes VSMC migration. Endometrial explants were composed of highly organized glands and stroma. VSMC migration (cells migrated in 21 h/mm^2 fibronectin-coated semipermeable membrane) in the presence of CM from human endometrial explants obtained in the proliferative phase of the menstrual cycle and incubated for 24 h with estradiol was approximately threefold greater ($P < 0.001$) than with medium alone and greater ($P < 0.05$) than with CM from explants treated with estradiol plus progesterone or progesterone. It is concluded, therefore, that estrogen stimulates endometrial secretion of a factor(s) that promotes VSMC migration as an early step in vessel remodeling within the endometrium.

Keywords Vessel remodeling · Endometrium · Human · Estrogen · Progesterone

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Introduction

New capillaries develop, via angiogenesis, and then undergo remodeling and maturation, via investment of vascular smooth muscle cells (VSMC) with endothelial cells, to form a new vascular network during each menstrual cycle to support growth and differentiation of the endometrium for implantation. Improper vascularization of the endometrium may lead to dysfunctional menstrual bleeding [1], implantation failure [2], and infertility [3]. Although ovarian estrogen and progesterone have a pivotal role in establishing the endometrial vascular bed during each menstrual cycle ([4–6]; for reviews), relatively little is known about the mechanisms by which these steroid hormones regulate this fundamentally important process. It is well established that vascular endothelial growth factor (VEGF) regulates angiogenesis by stimulating microvascular endothelial cell permeability [7], survivability [8], proliferation, migration, and assembly into capillary tubes [9]. Angiopoietin-1 (Ang-1), acting via the Tie-2 receptor, promotes the recruitment of VSMC to endothelial cells. Thus, association of VSMC with endothelial cells plays a significant role in the remodeling, maturing, and stabilizing of newly formed blood vessels [10–14].

The endometrium has the capacity to produce many peptide growth factors, including Ang-1, which is

expressed by glandular epithelial and stromal cells of the human [15, 16] and rhesus monkey [17] endometrium. The Tie-2 receptor is expressed by endothelial cells [16–18] and VSMC [19]. Moreover, estradiol and/or progesterone increased glandular epithelial Ang-1 expression and microvessel VSMC proliferation in the rhesus monkey endometrium [17] and vessel maturation as a function of increased mural coverage of vessels in the mouse endometrium [20].

Recruitment of VSMC to endothelial cells is therefore a pivotal early step in vessel remodeling; however, very little is known about the regulation of this process in the human endometrium, one of the few sites in the adult where angiogenesis and vascular remodeling normally occur for reconstruction of the endometrium during each menstrual cycle. In this study, therefore, explants of human endometrium were incubated with estradiol and/or progesterone and the conditioned medium (CM) from the explants applied to cultures of VSMC to test the hypothesis that estrogen and/or progesterone stimulate endometrial cells to secrete a factor(s) that promotes VSMC migration.

Results

Endometrial explant morphology

At the onset of culture, human endometrial explants were composed of highly organized well-defined glands and stroma (Fig. 1a). The integrity of the glands and stroma was in large part maintained in the endometrial explants cultured for 24 h (Fig. 1b) or 96 h (Fig. 1c) with estradiol plus progesterone. However, morphological changes, including disruption of the basement membrane, were observed in the glands and stroma in endometrial explants incubated for 72–96 h without estradiol and progesterone (not shown).

VSMC morphology

VSMC applied to fibronectin-coated filters maintained their well-defined stellate appearance after 21 h in the presence of DMEM/F12-vehicle alone (Fig. 2a) or CM from human endometrial explants incubated for 24 h with estradiol (Fig. 2b) and/or progesterone (not shown). However, VSMC incubated with CM from explants cultured for 96 h with medium alone exhibited a multicellular aggregate appearance (Fig. 2c), an effect prevented by CM from estradiol/progesterone-treated explants (Fig. 2d).

VSMC migration

The 24-h explant cultures

VSMC migration (means ± SE, number of cells migrated over a 21 h/mm^2 membrane area, Fig. 3) in the presence of CM from human endometrial explants obtained in the proliferative phase of the menstrual cycle and incubated for 24 h with estradiol (169 ± 7, E_2 CM) was almost threefold greater ($P < 0.001$) than that with DMEM/F12 containing estradiol but no CM (56 ± 6, E_2 no CM) and greater ($P < 0.01$) than that with CM from explants incubated without steroids (128 ± 10, VEH CM), or with estradiol plus progesterone (125 ± 6, E_2/P_4 CM), or progesterone (131 ± 7, P_4 CM). Thus, progesterone attenuated the increase in VSMC migration observed with CM from explants incubated with estradiol alone. However, VSMC migration in the presence of CM from endometrial explants cultured with medium-vehicle alone or with estradiol/progesterone or progesterone was greater ($P < 0.05$) than that in the absence of CM, while estradiol and/or progesterone added directly to VSMC in the absence of CM had no effect.

In contrast to the results obtained with endometrial tissue obtained in the proliferative phase, VSMC migration was similar with CM from explants of secretory

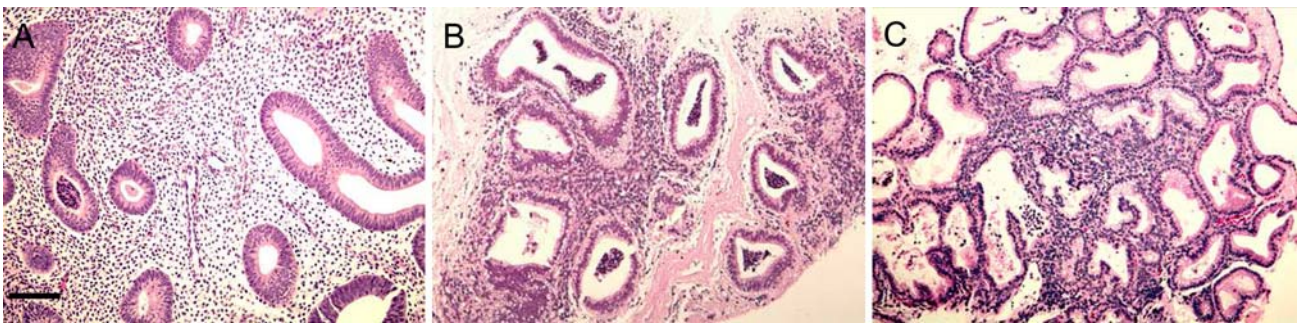

Fig. 1 Histology of human endometrial explants at the onset of culture with DMEM/F12 containing ethanol vehicle (**a**), and 24 h (**b**), and 96 h (**c**) after incubation with DMEM/F12 containing estradiol plus progesterone. Magnification bar = 100 μm

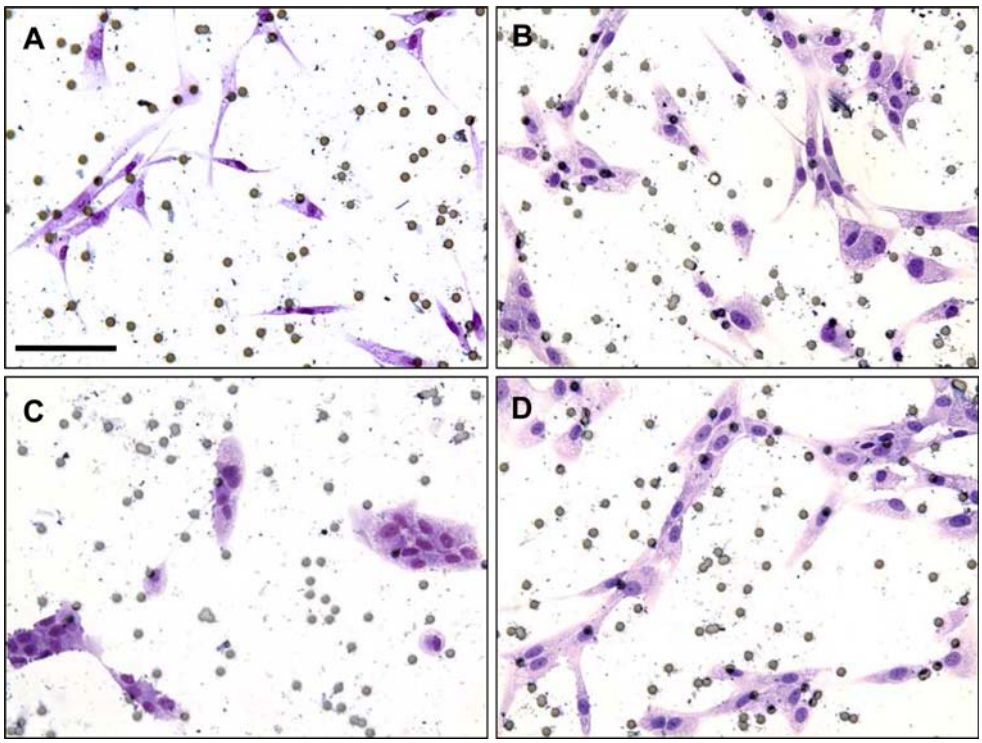

Fig. 2 Histology of human VSMC on top of fibronectin-coated filters 21 h after incubation with DMEM/F12 and ethanol vehicle alone (**a**), or CM (20% volume/volume) from human endometrial explants incubated for 24 h with estradiol (**b**), or 96 h with DMEM/F12-ethanol vehicle alone, (**c**) or with estradiol plus progesterone (**d**). Magnification bar = 100 μm

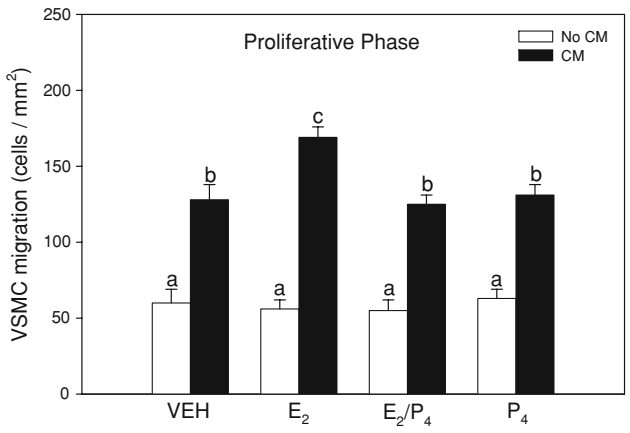

Fig. 3 VSMC migration (cells migrated in 21 h/mm^2 membrane area) in the presence of either CM (*black bars*) from explants of human endometrium obtained during the proliferative phase of the menstrual cycle and that had been cultured for 24 h with DMEM/F12 and ethanol vehicle alone (VEH), 10^{-8} M estradiol (E_2), 10^{-8} M estradiol plus 10^{-7} M progesterone (E_2/P_4), or 10^{-7} M progesterone (P_4), or DMEM/F12 without CM (*white bars*), but containing estradiol and/or progesterone. Values represent the means (±SE) of six separate endometrial explants from six subjects and the respective VSMC cultures. Values with different letter superscripts are different ($P < 0.05$) from one another

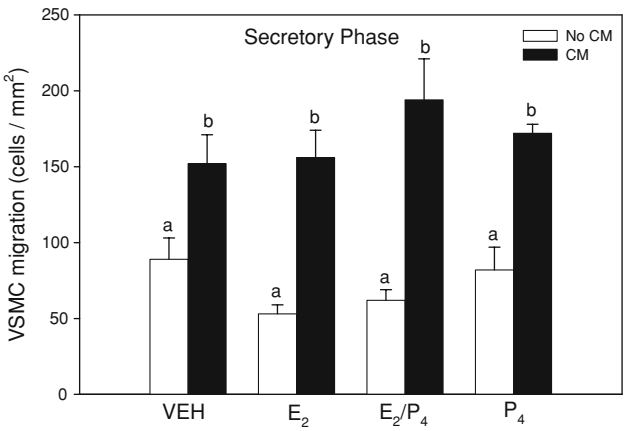

Fig. 4 VSMC migration in the presence of either CM (*black bars*) from explants of human endometrium obtained during the secretory phase and cultured for 24 h with vehicle, estradiol, and/or progesterone or DMEM/F12 without CM (*white bars*), but containing estradiol and/or progesterone. Values are the means (±SE) of three to eight separate explants from three to eight subjects and the respective VSMC cultures. Values with different letter superscripts are different ($P < 0.05$) from one another

endometrium cultured for 24 h without steroids (152 ± 19) or with estradiol (156 ± 18), estradiol plus progesterone (194 ± 27), or progesterone (172 ± 6, Fig. 4). However, VSMC migration in the presence of CM from secretory explants cultured with or without estradiol and/or progesterone remained greater ($P < 0.05$–0.001) than their respective no CM controls, while estradiol and/or progesterone added directly to VSMC again had no effect upon migration (Fig. 4).

The 72–96-h explant cultures

In contrast to the efficacy of CM from endometrial explants incubated for 24 h with DMEM/F12 alone to induce

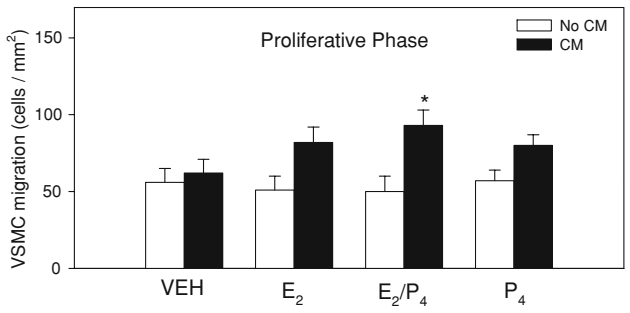

Fig. 5 VSMC migration in the presence of either CM (*black bars*) from explants of human endometrium obtained during the proliferative phase and cultured for 72–96 h with vehicle, estradiol, and/or progesterone or DMEM/F12 without CM (*white bars*), but containing estradiol and/or progesterone. Values are the means (±SE) of four separate explants from four subjects. *Different ($P < 0.05$) from VEH CM and E_2/P_4 no CM

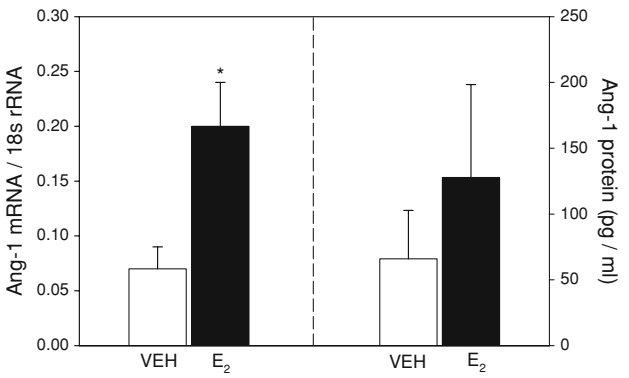

Fig. 6 **a** Ang-1 mRNA levels (corrected for 18S rRNA) and **b** Ang-1 protein (pg/ml) levels in CM of endometrial explants obtained in the proliferative phase and incubated for 24 h with DMEM/F12-vehicle (VEH, $n = 7$) or estradiol (E_2, $n = 7$). *Different ($P < 0.05$) versus VEH

VSMC migration, VSMC migration in the presence of CM from explants obtained in the proliferative phase and incubated for 72–96 h with DMEM/F12 alone (i.e., without estradiol and progesterone, 62 ± 9, Fig. 5) was not increased and thus similar to that exhibited in absence of explant CM (56 ± 9). However, VSMC migration after the addition of CM from endometrial explants incubated for 72–96 h with estradiol plus progesterone (93 ± 10) was twofold greater ($P < 0.05$) than with CM from explants cultured with medium alone or after addition of estradiol plus progesterone directly to VSMC (50 ± 10, Fig. 5).

Endometrial explant Ang-1 mRNA and CM Ang-1 protein levels

Ang-1 mRNA levels (expressed as a ratio of 18S rRNA) in the proliferative phase explants incubated 24 h with estradiol (0.20 ± 0.04, Fig. 6a) were approximately threefold greater ($P < 0.02$) than with DMEM/F12 alone (0.07 ± 0.02).

Ang-1 protein levels in CM of endometrial explants incubated for 24 h with estradiol (128 ± 71, Fig. 6b) were twofold but not significantly greater than with DMEM/F12 alone (66 ± 37).

Discussion

This study shows that CM from explants of endometrium obtained from women in the proliferative phase of the menstrual cycle and incubated for 24 h with estradiol stimulated the migration of VSMC, while estradiol added directly to VSMC had no effect. We propose, therefore, that estradiol stimulates the endometrium to express a factor(s) that promotes in a paracrine manner VSMC migration. Following their migration and recruitment, VSMC associate morphologically and functionally with endothelial cells resulting in remodeling, maturation, and stabilization of the newly formed blood vessels. Therefore, estrogen appears to have an important role in promoting VSMC migration as an early step in the sequence of events involved in vessel remodeling in the endometrium.

This study further showed that progesterone attenuated the increase in VSMC migration exhibited by CM from proliferative phase endometrial explants incubated for 24 h with estradiol, while CM from endometrial tissue obtained in the progesterone-dominated secretory phase and treated with estradiol was no more effective in increasing VSMC migration than with CM from vehicle-treated explants. The apparent suppressive effect of progesterone on estrogen-induced VSMC migration in this study is reminiscent of the inhibitory effect that progesterone has on other fundamentally important actions of estrogen in the endometrium, including the generation of the receptors for estrogen and progesterone [4, 21]. However, progesterone promoted VSMC proliferation in the rhesus monkey endometrium [17] and the association of VSMC with endothelial cells in the mouse endometrium [20]. Therefore, the roles of estrogen and progesterone on VSMC migration and recruitment must be complex, interactive, and temporally ordered to ensure the normal progression of vessel remodeling and maturation with advancing stages of the menstrual cycle.

In this study, CM from explants of endometrium collected in either the proliferative or secretory phase and incubated for 24 h with DMEM/F12 medium-vehicle alone significantly increased VSMC migration. However, this effect was lost when the interval for endometrial explant incubation was extended to 72–96 h. It appears that endometrial cells retain an upregulated angioregulatory capacity, i.e., expression of a factor(s) that promotes VSMC migration, for at least 24 h after removal from the

in vivo environment of elevated endogenous estradiol levels typical of the advancing human menstrual cycle. We have shown a similar short-term retention in vitro of estrogen-induced 11β-hydroxysteroid dehydrogenase expression important for corticosteroid metabolism in placental trophoblast cells isolated from baboons [22].

The CM from endometrium cultured with medium alone for 72–96 h resulted in a level of VSMC migration that was considerably lower than that observed with CM from explants incubated with medium alone for 24 h. However, concomitant addition of estradiol and progesterone to endometrial explants cultured for 72–96 h significantly increased, albeit moderately, VSMC migration. The integrity of the endometrial explants, as well as the VSMC to which the explant CM was applied, however, was disrupted by long-term culture of the endometrium in medium alone, an effect prevented by the addition of both estradiol and progesterone. Tissue breakdown [23] and the expression of matrix metalloproteinases that initiate extracellular matrix remodeling [24] have also been reported in human endometrial explants cultured for 48–96 h in the presence or absence of serum, effects prevented by the addition of estradiol and progesterone. We speculate, therefore, that long-term incubation of the endometrium in the absence of estradiol/progesterone results in tissue breakdown and instability which hinders the production of the angiostimulatory factor(s) that upregulate VSMC migration and estrogen and progesterone prevent these deleterious effects.

Although further study is needed to definitively establish the angiomodulatory factor(s) present in CM from estradiol-treated endometrial explants, transgenic mouse studies have shown that Ang-1 has an essential role in recruitment of VSMC to and their coupling with endothelial cells [11]. We have recently shown that Ang-1 stimulated the migration of human VSMC cytoplasmic processes to and contact with endothelial cells cocultured on opposite sides of semipermeable membrane [25]. Moreover, estradiol increased Ang-1 mRNA expression in vivo in the sheep [26] and rhesus monkey [17] endometrium, and in vitro in human endometrial glandular epithelial cells [27] and endometrial explants of this study. Ang-1 protein levels assessed by ELISA also were increased twofold, but not significantly because of variability, in CM from estradiol-treated endometrial explants of this study. However, because Ang-1 exists as a quaternary multimer [28] which determines receptor interaction [29] and the ELISA kit only detected the monomeric form of Ang-1, further studies, e.g., using Western immunoblot, are required to assess the potential effect of estrogen on the expression of the fully active multimeric forms of Ang-1 by the endometrium and impact on VSMC migration. Collectively, based on the results of this and previously published studies, we propose that estradiol stimulates human endometrial glandular epithelial and/or stromal cells to express Ang-1 and/or other angioregulatory factors, such as platelet-derived growth factor [30, 31], basic fibroblast growth factor [32], or hepatocyte growth factor [33], which promote in a paracrine manner VSMC migration as an early step in vessel remodeling in the endometrium.

VSMC and endothelial cells express estrogen and progesterone receptors [4, 34, 35] and estradiol has been reported to directly regulate endothelial cell [36] and VSMC [17] function. Moreover, because blood vessel tissue, in addition to glandular epithelial and stromal cells, was likely present in the human endometrial explants employed in this study, it is possible that angioregulatory factors expressed by the vascular tissue and present in CM stimulated VSMC migration. However, in this study, the addition of estradiol directly to human VSMC clearly had no effect on their migratory capacity. Therefore, we propose that glandular epithelial and/or stromal cells, via their expression of Ang-1 and/or other factors, have an essential role in mediating the stimulatory effect of estrogen on VSMC migration and vessel remodeling within the uterus.

In summary, the results of this study show that CM from explants of human endometrium obtained in the proliferative phase and incubated for 24 h with estradiol stimulated VSMC migration across a semi-permeable membrane, while estradiol added directly to VSMC had no effect. Moreover, progesterone attenuated the increase in VSMC exhibited by CM from proliferative phase endometrial explants incubated for 24 h with estradiol, while CM from endometrial tissue obtained in the progesterone-dominated secretory phase and treated with estradiol was ineffective. Finally, long-term culture of endometrial explants resulted in disruption of tissue integrity and a decline in VSMC migration, effects prevented by the addition of estradiol and progesterone. Therefore, we propose that estrogen stimulates the endometrium to express a factor(s) that promotes VSMC migration as an early step in blood vessel remodeling

Materials and methods

Human endometrial explant culture

Endometrial tissue was collected in ice-cold saline from women in the proliferative ($n = 9$) or secretory ($n = 8$) phases of the menstrual cycle after hysterectomy performed for benign gynecological conditions. All patients were of reproductive age (21–49 years old) and not treated with steroids, GnRH, or other hormonal regimens. Endometrial tissue was dated by histological examination [37] and menstrual cycle records. The study was approved by the Institutional Review Boards of the University of

Maryland School of Medicine and Mercy Medical Center, Baltimore, MD.

Explant cultures were employed, rather than monolayer cultures of dispersed cells, to maintain an intact epithelial–stromal tissue architecture. Endometrial tissue was prepared for explant culture using previously established conditions [38] with modifications. Endometrial tissue was washed in saline, cut into 2 mm^3 pieces in ice-cold 1× calcium/magnesium-free Hanks Balanced Salt Solution (Gibco-Invitrogen Corp., Grand Island, NY) and a wet weight obtained. Explants (25–30 mg) were placed on 30-mm Millicell-CM culture inserts (0.4-μm pore size; Millipore, Billerica, MA) in six-well plates containing 1.2 ml of Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12; Sigma Chemical Co., St. Louis, MO) without phenol red, insulin, or serum, but supplemented with 2.5-mM L-glutamine and 1× penicillin, streptomycin, and amphotericin B in the bottom chamber. Each individual tissue culture insert containing explants from a separate patient in the proliferative or secretory phase were incubated at 37°C in a humidified atmosphere of 95% air: 5% CO_2 in the presence/absence of 0.003% ethanol vehicle, 10^{-8} M 17β-estradiol (Sigma), 10^{-7} M progesterone (Sigma), or estradiol plus progesterone for 24 h. The concentrations of steroids were based on previous reports [23, 24] showing that endometrial explants were maintained in good condition for 3–4 days in the presence of 10^{-9}–10^{-8} M estradiol and 10^{-7} M progesterone. To eliminate potential confounding effects of endogenous estradiol and/or progesterone exposure in vivo, additional experiments were performed in which the explants were incubated in the presence/absence of vehicle control, estradiol, and/or progesterone for 72–96 h with media change every 24 h. After incubation, explant CM was removed, centrifuged at 16,000*g* for 10 min and stored at −80°C. Representative explants were fixed in 10% neutral-buffered formalin for histology or stored in liquid nitrogen for RNA analysis.

VSMC culture and migration

Cryopreserved human female aortic VSMC (Lonza, Walkersville, MD) were obtained at passage 3 and plated in T75 flasks at 2.5×10^3 cells/cm^2 in a specialized smooth muscle cell growth media (SmGM-2 bulletkit, Lonza) containing 5% FBS, human epidermal growth factor, insulin, human fibroblast growth factor-B, gentamicin, and amphotericin B. The attached cells were grown for a preconfluent period of 5–7 days at 37°C in a humidified atmosphere of 95% air: 5% CO_2 with media change every 2 days, trypsinized and either cryopreserved or subcultured on T75 flasks. Cells from passages 4–8 were used in this study.

VSMC migration was performed based on the Boyden chamber principle [39] using modifications of previously established conditions [40]. Migration was evaluated in 24-well cell culture chambers containing 8-μm pore polyethylene terephthalate (PET) membrane inserts (BD Biosciences, Franklin Lakes, NJ) coated on both sides with 10 μg/ml human fibronectin (Chemicon-Millipore, Temecula, CA). VSMC (2.5×10^4) cells were applied to the upper side of the inserts and incubated in 1.0 ml DMEM/F12 supplemented with 0.1% BSA (Sigma) added to the upper chamber for 21 h at 37°C in a humidified atmosphere of 95% air: 5% CO_2 in the presence/absence of CM (20% volume/volume) from human endometrial explants cultured with vehicle (VEH CM), estradiol (E_2 CM), estradiol plus progesterone (E_2/P_4 CM) or progesterone alone (P_4 CM), or DMEM/F12 media from wells without explants (previously stored at −80°C, 20% volume/volume) containing ethanol vehicle (VEH no CM), estradiol (E_2 no CM), estradiol plus progesterone (E_2/P_4 no CM), or progesterone alone (P_4 no CM) added beneath the inserts to the lower chamber. The 20% volume/volume dilution of the endometrial explant CM was chosen because it stimulated VSMC migration without inducing observable signs of cellular stress (data not shown). After 21 h, VSMC were removed from the upper side of the membrane insert by a cotton swab and the inserts fixed at room temperature with 0.75 ml of HEMA 3 fixative solution for 7 min and stained in HEMA 3 solutions I and II (Fisher Scientific, Kalamazoo, MI) for 2 min. Membranes were washed three times in deionized water, removed from the inserts, and the lower side mounted facing up on a slide with immersion oil and cover slipped. Migration was quantified by counting at the light microscopic level the number of VSMC in 20 random high-powered fields (final magnification 400 and 1,000×) of the membrane. The areas of the fields were determined and the results are expressed as the number of migrating cells per mm^2 membrane area.

Ang-1 mRNA by real-time RT-PCR

Proliferative phase endometrial explants were homogenized in a 4 M guanidine isothiocyanate/0.83% 2β-mercaptoethanol containing buffer and total RNA isolated by silica gel spin column centrifugation as described by the manufacturer (RNeasy, Qiagen, Valencia, CA). The amount of total RNA in each sample was quantified by UV-absorption spectrophotometry at 260 nm.

Ang-1 primers

Oligonucleotide Ang-1 primers were based on the Ang-1 human gene sequence (NCBI database accession #U83508) and supplied by TIB MOLBIOL (Adelphia, NJ):

5′-GGGGGAGGTTGGACTGTAAT-3′ (position 1270–1289) and 5′-AGGGCACATTTGCACATACA-3′ (position 1631–1612).

18S rRNA primers

Oligonucleotide 18S rRNA primers were based on the human gene sequence (NCBI database, accession #M10098): 5′-TCAAGAACGAAAGTCGGAGG-3′ (positions 1126–1145) and 5′-GGACATCTAAGGGCATCAC A-3′ (positions 1614–1595).

RT and real-time PCR

RT of total endometrial RNA was performed according to manufacturer's directions (Invitrogen, Carlsbad, CA). A 13-μl reaction volume containing 1 mM each of deoxy (d)-ATP, dCTP, dGTP and dTTP, 1× RT buffer, 250 ng random primers, and total RNA was incubated at 65°C for 5 min and on ice for 1 min. Reaction buffer, 200 U Superscript III RT (Invitrogen) and 40 U RNAguard (Amersham Pharmacia Biotech, Piscataway, NJ) were incubated at 25°C for 5 min and 50°C for 60 min. The RT reaction was terminated by heat inactivation at 70°C for 15 min and cooled to 4°C.

The mRNA levels were quantified via a LightCycler real-time PCR unit (Roche Diagnostics Corp.) using Fast Start DNA Master SYBR Green I Kit for PCR. A 19-μl reaction mix containing either target mRNA- or 18S rRNA-specific primers was combined with 1-μl aliquot of RT reaction. The reaction profile consisted of denaturation at 95°C for 8 min, 40 cycles of amplification (95°C for 5 s, 52°C for 5 s, and 72°C for 16 s) and product formation measured and displayed in real time. Efficiency-corrected calibrator-normalized relative quantification was performed with analysis software (LightCycler version 4) using pre-made standard curves specific for each product to correct for differences in the efficiencies of target and reference genes, and an in-run calibrator to normalize quantification. Specificity of the products was confirmed by melting curve analysis, agarose gel electrophoresis, and inclusion of negative controls with no template or no RE in the reaction.

Enzyme-linked immunoassay (ELISA) of Ang-1 protein

Levels of the monomeric form of Ang-1 protein in CM of proliferative phase endometrial explants after the 24-h incubation period were determined by ELISA using a commercially available kit (R&D Systems, Inc., Minneapolis, MN), according to the manufacturers instructions. The limit of the sensitivity of the assay was less than 5 pg/ml.

Statistical analysis

Data are expressed as the means ± SE. Statistical analysis of the data was performed either by two-factor ANOVA with post hoc comparisons by Bonferroni test (VSMC migration) or by Student's *t* test (Ang-1 levels). A P value of <0.05 was considered to be statistically significant.

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