Chorionic Gonadotropin Stimulation of Angiogenesis and Pericyte Recruitment

Sarah Berndt, Silvia Blacher, Sophie Perrier d'Hauterive, Marc Thiry, Marie Tsampalas, André Cruz, Christel Péqueux, Sophie Lorquet, Carine Munaut, Agnès Noël,* and Jean-Michel Foidart*

Laboratory of Tumor and Development Biology (S.Be., S.Bl., A.C., C.P., S.L., C.M., A.N., J.-M.F.), Groupe Interdisciplinaire de Génoprotéomique Appliqué-Cancer, Center of Immunology (S.P.H., M.Ts.), Laboratory of Cellular Biology (M.Th.), University of Liège, and Department of Obstetrics and Gynecology (S.P.H., S.L., J.-M.F.), Centre Hospitalier Regional, B-4000 Liège, Belgium

Context: During the periimplantation period, human chorionic gonadotropin (hCG) plays a key role by increasing the uterine blood flow through uterine vessel vasodilatation but also through angiogenesis. Indeed, we previously demonstrated that hCG contributes to endothelial cell recruitment and vessel formation.

Objective: In this study, hCG was proposed as an arteriogenic factor that could promote perivascular cell recruitment and vessel stabilization.

Design: The aortic ring assay, a three-dimensional *ex vivo* angiogenesis system mimicking all the steps of the angiogenesis process was used to study the impact of hCG on pericyte recruitment and vessel maturation.

Setting: The study was conducted at a university hospital laboratory.

Main Outcome Measures: Perivascular cell proliferation, migration, and apposition were quantified by computerized image analysis.

Results: Physiological concentrations of hCG (10–400 IU/ml) significantly enhanced pericyte sprouting and migration and gave rise to the maturation and coverage of endothelial capillaries. In a three-dimensional coculture model of endothelial and perivascular cells, hCG enhanced vessel tube formation and endothelial/mural cell adhesion. In addition, hCG stimulated the proliferation of human umbilical vein endothelial cells and smooth muscle cells. The specificity of these effects was determined by using an anti-hCG blocking antibody. Signaling pathways implicated on this hCG effect is protein kinase A and phospholipase C/protein kinase C dependent for the proliferative effect but only phospholipase C/protein kinase C for the migrative process.

Conclusions: Our findings highlight a novel paracrine role of this early embryonic signal in vessel maturation by stimulating perivascular cell recruitment, migration, and proliferation. **(*J Clin Endocrinol Metab* 94: 4567–4574, 2009)**

Peritrophoblastic angiogenesis at the implantation site is an important step for successful pregnancy. Among the wide range of contributing hormones, cytokines, and growth factors, human chorionic gonadotropin (hCG) plays a key role and modulates: 1) the implantation process by controlling leukemia inhibitory factor and macrophage colony stim-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2009-0443 Received March 2, 2009. Accepted August 17, 2009.
First Published Online October 16, 2009
* A.N. and J.-M.F. are both senior authors.

Abbreviations: AoSMC, Aortic SMC; BIM, bisindolylmaleimide I; CMFDA, 5-chloromethylfluorescein diacetate; EC, endothelial cell; FBS, fetal bovine serum; Fmax, perivascular cells maximal migration distance from the explant; hCG, human chorionic gonadotropin; hCGR, hCG receptor; HUVEC, human umbilical vein endothelial cell; Nf, number of perivascular cells; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor.

ulating factor release, 2) tissue remodeling by increasing matrix metalloproteinase-9 production, and 3) angiogenesis by inducing vascular endothelial growth factor (VEGF) up-regulation (1, 2). LH/hCG receptor (LH/hCGR) was first described on endometrial and myometrial vascular smooth muscle cells and thereafter in vascular endothelial cells (ECs) of the uterine vessels (3, 4). *In vivo* administration of hCG reduced vascular resistance in human uterus and decreased *in vitro* the formation of vasoconstrictor eicosanoids of the vascular wall (4, 5). Zygmunt *et al* (6). showed that hCG was able to promote angiogenesis by stimulating EC migration and capillary sprout formation. We previously showed that the angiogenic activity of hCG is mediated through a direct effect on ECs and through a paracrine dialogue between ECs and endometrial epithelial cells (1). Upon hCG binding to its LH/hCGR at the surface of endometrial ECs, VEGF synthesis and secretion is up-regulated, thereby activating the angiogenic switch in ECs. The endometrial epithelium is thus sensitive to specific embryonic signals, leading to the production of VEGF, the main angiogenic factor (7). As classically observed in pathological conditions (8), VEGF-driven angiogenesis results in the formation of highly abnormal vessels deficient in mural/pericyte coverage. The putative effect of hCG on perivascular cell recruitment and hence on vessel maturation is currently unknown. To address this issue, we used in this study the aortic ring assay, which mimics the different steps of the angiogenic process including EC proliferation, migration, and differentiation into capillaries (9). In addition, we evaluated the effect of hCG on perivascular cell outgrowth and EC coverage by mural cells. The effects of hCG was also investigated in monocultures and cocultures of ECs and smooth muscle cells (SMCs). In this study, we provide evidence for a promoting effect of hCG on vessel maturation through the recruitment of perivascular cells. These novel findings underline a much broader spectrum of activities for hCG than initially anticipated.

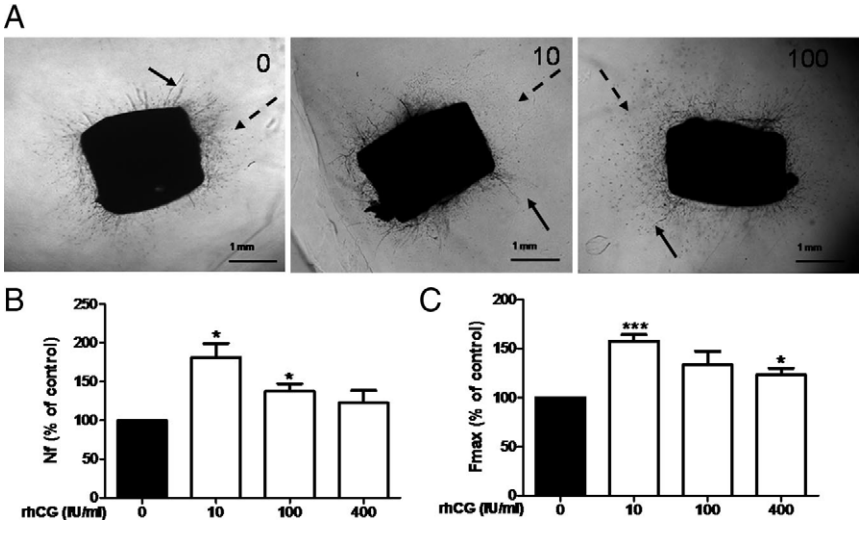

FIG. 1. Effect of hCG on pericyte recruitment in the aortic ring assay. A, Photomicrographs of aortic rings cultured in the absence (0) or presence of increasing concentrations of recombinant hCG (rhCG; 0–100 IU/ml). Endothelial capillaries are depicted by the *arrow*, and fibroblast like-cells are indicated by the *dotted arrow*. B, Quantification of the Nf and Fmax (C) in response to increasing rhCG concentrations (10–400 IU/ml) after 9 d of culture. Data are mean ± SD of one representative experiment, each condition running at least four rings. *, $P < 0.05$; ***, $P < 0.001$.

Materials and Methods

Materials

Reagents tested were purified urinary hCG (Pregnyl, Organon, Roseland, NJ), recombinant hCG (Ovitrelle; Serono, Geneva, Switzerland), protein kinase A (PKA) inhibitor H89 dihydrochloride (Calbiochem, La Jolla, CA), phospholipase C (PLC) inhibitor U73122 (Calbiochem), protein kinase C (PKC) inhibitor bisindolylmaleimide I (BIM; Calbiochem), anti-hCG neutralizing antibody (Sc-7821; Santa Cruz Biotechnology, Santa Cruz, CA), cell tracker Green 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Invitrogen, Carlsbad, CA), and CellTracker red CMRA (Molecular Probes, Invitrogen).

Animals

Wistar rats (±250 g) and C57/Bl 6 mice (10–12 wk old) were provided by the Animal Center of the University of Liège. Experiments were conducted with the approval of the local ethical committee for the care of experimental animals. Mice were maintained with a 12-h light, 12-h dark cycle, with food and water available *ad libitum*.

Aortic ring assay

Rat and mice aortic rings were cultured in three-dimensional type I collagen gels as previously described (10, 11). Effects of different compounds were tested by addition to culture medium at d 0. For quantification, computerized image analysis was performed on a Sun SPARC30 workstation with the software Visilog 5.0 (Noesis, St. Aubin, France) according to Blacher *et al.* (10). The following parameters were determined: number of microvessels; maximal microvessel length, total number of branching in microvessels, number of perivascular cells (Nf), and perivascular cells maximal migration distance from the explant (Fmax).

Whole-mount immunostaining of aortic rings

At d 9 of culture, aortic fragments embedded in collagen gels were washed three times in PBS for 10 min followed by a fixation in 4% paraformaldehyde for 10 min. Samples were washed again in PBS three times for 10 min, and then nonspecific antibody binding was blocked with milk 1.5% for 20 min. Samples were incubated overnight at room temperature with a mix of a lectin and a primary antibody: Griffonia Simplifolia isolectin-B4/Alexa Fluor 488 at 5 μg/ml (Molecular Probes; I21411) and rabbit anti-NG2 chondroitin sulfate pro-

teoglycan antibody (Chemicon, Temecula, CA; AB5320) at 10 μg/ml. After three washes in PBS for 10 min each, the tissues were incubated at room temperature with a secondary goat anti-rabbit biotin antibody (Dako, Glostrup, Denmark; E432) at 1.9 μg/ml. Finally, an incubation with streptavidin Cy3 (Sigma, St. Louis, MO; S6402) was performed for 1 h at room temperature at 1 μg/ml. Samples were mounted with Vectashield-Dapi mounting medium (Vector Laboratories, Burlingame, CA; H-1200), after three washes in PBS for 10 min at room temperature.

Electron transmission microscopy

Aortic three-dimensional gels culture were stopped after 10 d, and gels were fixed 1 h at 4 C with 2.5% glutaraldehyde in a Sörensen 0.1 M phosphate buffer (pH 7.4). Gels were postfixed for 30 min with 1% osmium tetroxide. After deshydratation, samples were embedded into Epon. Ultrathin sections, obtained with a Reichert Ultracut S ultramicrotome (Leica, Germany), were contrasted with uranyl acetate and lead citrate. Observations were done with a Jeol 100 CX II transmission electron microscope at 60 kV (Peabody, MA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were grown on 0.2% gelatin-coated dishes in MCDB 131 medium (Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), HEPES (25 mM), glutamine (2 mM), endothelial cell growth supplement (12 ng/ml; BD Biosciences, Bedford, MA), heparin (2.5 mg/ml), and antibiotics (penicillin-streptomycin, 100 U/ml); for experiments, cells at passages 2–10 were used. Human aortic SMCs (AoSMC) purchased from Lonza (Basel, Switzerland; CC-2671) were grown in complete smooth muscle medium containing 10% FBS (SmBm; Lonza; CC-3181). Cells were at passages 6–10 used for experiments. Mouse SMCs $C^3H/10T1/2$, clone 8 cell line (American Type Culture Collection, Manassas, VA; CCL-226) were grown in basal medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin/streptomycin (100 μg/ml), HEPES (15 mM), and bicarbonate. All cell types were cultured in 5% CO_2 at 37 C, and media were replaced every 2 d.

10 T1/2 cell migration

To evaluate SMC migration, the wound assay model was used as described by Savani *et al.* (12). 10 T1/2 cells (10^5) were seeded on 24-well microplates and grown overnight in 10% FBS basal medium. A cross-shape scratch was made with a tip in cell monolayers. After 4 and 9 h, pictures were acquired at the same magnification and location in the bottom of the dish. Quantification was done using ImageJ software (National Institutes of Health, Bethesda, MD) by measurements of wounded areas at time 0, 4, and 9 h.

Proliferation assays

Proliferation tests were performed on AoSMCs, 10 T1/2 cells, and HUVECs cultured for 24 h. Cells (5×10^3) were seeded and allowed to adhere in 96-well microplates. For HUVEC proliferation, 96-well microplates were coated with gelatin at 2 mg/ml. Cells were treated with or without hCG (10–1000 U/ml), H89 (10 μM), U73122 (10 μM), or BIM (1 μg/ml) alone or combined with hCG (100 IU/ml) in their respective culture media containing 2% FBS. Cell proliferation was evaluated using 5-bromo-2′-deoxyuridine incorporation into cells followed by a cell proliferation colorimetric assay (Roche Applied Science, Penzberg, Germany) as previously described (13).

Boyden chamber migration assay

AoSMC cell migration was assessed using Boyden chamber assay. Cells were seeded on top of polycarbonate filters (8 μm pore) of Transwell Permeable Support (Costar, Corning Inc., Lowell, MA). Cells in stock culture were trypsinized, suspended in serum-free medium containing 0.1% BSA, and placed on the upper compartment of the chamber (7.10^4 cells/filter). The lower compartment of the chamber was filled with serum-free medium containing 1% BSA, supplemented with or without hCG, anti-hCG, H89, U73122, and BIM. After an incubation period of 24 h at 37 C, filters were fixed in methanol. Cells on filters were stained with crystal violet solution 0.1% (Sigma). Nonmigrated

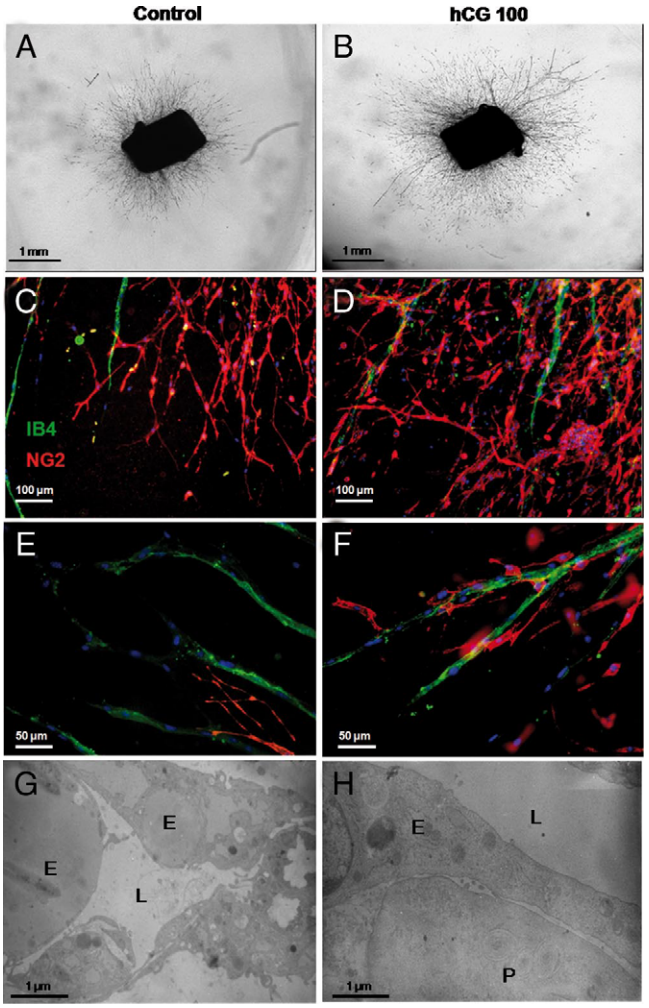

FIG. 2. Effect of hCG on pericyte outgrowth from the aortic rings. A and B, Photomicrographs of aortic rings cultured in the absence (control) (A, C, E, and G) or presence of uhCG 100 IU/ml (B, D, F, and H) (n = 5 rings per condition). Whole-mount double immunostainings of the collagen gels at d 9 of culture with an anti-EC-specific isolectin (IB4) in *green* and a pericyte-specific antibody (NG2) in *red*; nuclei were counterstained with 4′,6′-diamino-2-phenylindole (*blue*) (C–F). Representative electron micrographs (G) showing a section of immature nude endothelial tubes composed of ECs (E) delimiting a lumen (L) and a mature endothelial tube (H) composed of endothelial cells (E) delimiting a larger lumen (L) and surrounded by a perivascular cell (P).

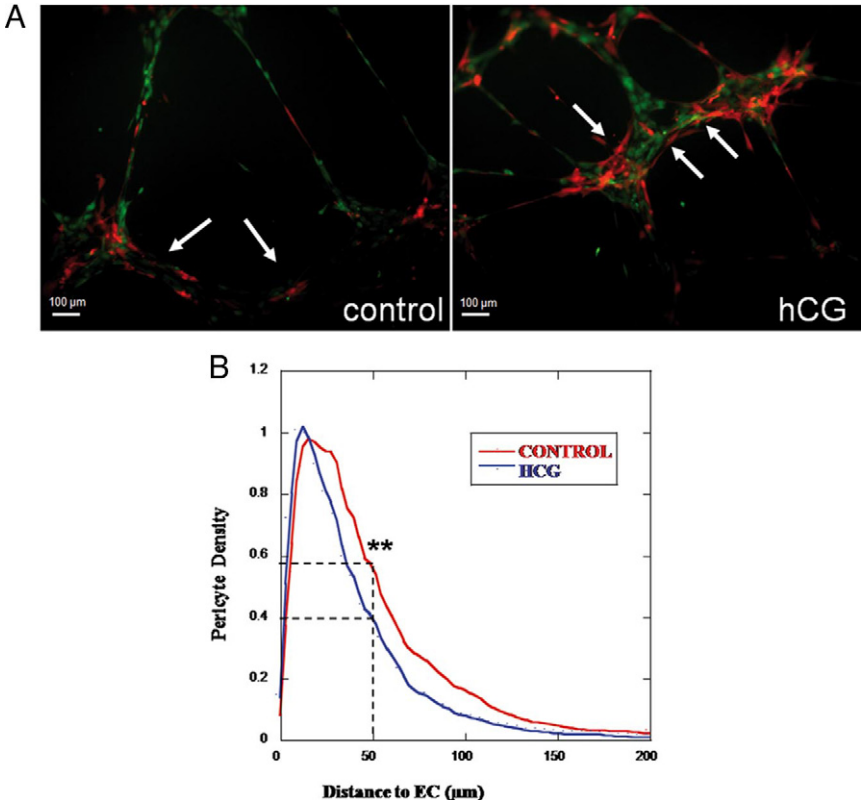

FIG. 3. Effect of hCG on EC-pericyte interactions in a three-dimensional matrigel coculture model. A, Photomicrographs (*scale bars,* 100 μm) of HUVECs (*green* labeled) and AoSMCs (*red*-labeled) after 12 h of coculture without (ctrl) or with uhCG (100 IU/ml hCG). *Arrows* point area in which AoSMCs are close to endothelial tube-like structures. B, Computer-assisted quantification of AoSMC distribution around HUVEC tube network. Upon hCG treatment, pericytes were closer to ECs (*blue curve*). The Student *t* test was used for statistical analysis (n = 20). **, $P < 0.05$.

cells at the upper surface of the filters were wiped away with a cotton swab. Quantification of the migration assay was done by colorimetric measurement (λ = 560 nm) of cells at the lower surface of the filter.

HUVEC and AoSMC coculture assays

AoSMC and HUVEC coculture assays were performed in a modified matrigel overlay assay as previously reported (14). Briefly, HUVEC cells (15×10^3 CMFDA green labeled cells) were seeded on top of a 300 μl matrigel layer (15) in 24-well microplates and allowed to form tubular structures. Six hours later, AoSMCs (15×10^3 CMRA red labeled) were overseeded on HUVECs, and 100 IU/ml hCG were added or not. After 24 h, cultures were stopped, rinsed with PBS, and fixed with 4% paraformaldehyde until microscopic examination. To analyze the AoSMC distribution around the EC tube network, an original computer-assisted quantification was developed by implementing an algorithm using the image analysis tool box of MATLAB7.1 software (The Mathworks, Natick, MA). This method calculated the smallest distance separating green pixel corresponding to EC and red pixel corresponding to AoSMCs identified by cell tracker green CMFDA and CellTracker red CMRA, respectively.

Statistical analyses

Statistical analysis were conducted with GraphPad Prism software (GraphPad Inc., La Jolla, CA). Aortic rings experiments including four explants per condition were repeated at least twice. Student's *t* test was used to evaluate whether differences among groups were significant. For computerized image analysis, statistical analysis were performed with the statistics toolbox of MATLAB 7.1 (MathWorks) using Student's *t* test or using Wilcoxon test, with regard to heteroscedasticity. In the other experiments, Mann-Whitney tests were used for statistical analysis. $P < 0.05$ was considered as statistically significant.

Results

hCG promotes perivascular cell recruitment in the aortic ring assay

According to our previous results (1), the incubation of rat aortic fragments embedded in a collagen gel with recombinant hCG induced an angiogenic response characterized by enhanced capillary formation (Fig. 1. A). Interestingly, dispersed cells spread out, suggesting a possible mobilization of perivascular cells in addition to that of EC. These cells have been quantified through a computerized quantification (10) and were identified as mural cells by immunostaining. The addition of hCG (10–400 IU/ml) to the aortic ring culture media resulted in an increase of Nf (Fig. 1B) and distance of cell migration from the aortic explant (Fmax) (Fig. 1C). Similar results were obtained with urinary hCG. For next experiments, urinary hCG (uhCG) was used at its optimal concentration (100 IU/ml).

Whole-mount immunostainings of mice aortic ring cultures (d 9 of culture) were performed to identify the ECs through Isolectin B4 staining and pericytes through NG2 detection. They revealed the pericyte phenotype of perivascular cells that were growing out in the presence of stimulatory dose of hCG (100 IU/ml) (Fig. 2). In control conditions, in the absence of hCG, two types of capillary-like structures were detected, one composed of naked endothelial cells and the other one composed of pericytes (Fig. 2, C and E). In sharp contrast, on hCG treatment, pericyte proliferation and migration were drastically stimulated (Fig. 2D), and endothelial tubes displayed a mature phenotype with a pericyte coating (Fig. 2, F and H). Observations by electron transmission microscopy confirmed the presence of perivascular cells upon treatment with hCG (Fig. 2H). In sharp contrast, in the absence of hCG,

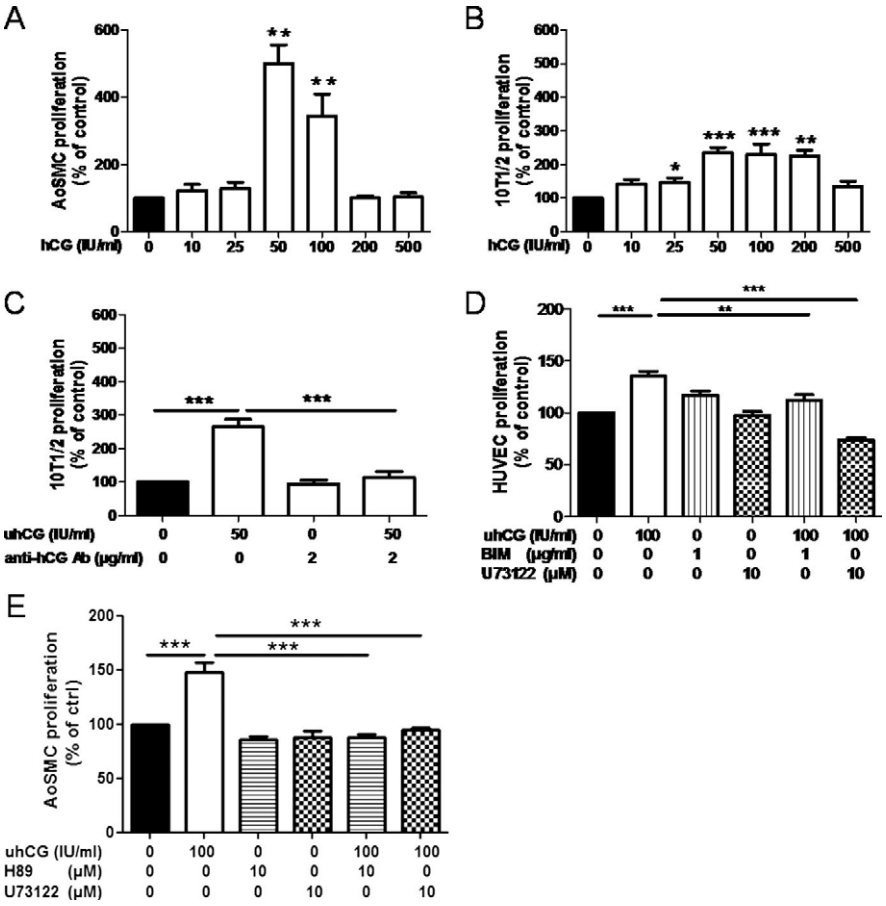

FIG. 4. Effect of hCG on human and murine SMC proliferation. Proliferation of AoSMCs treated for 24 h with 10–500 IU/ml uhCG (A) and 10 T1/2 cell line (B) were evaluated by 5-bromo-2′-deoxyuridine colorimetric immunoassay. Data are expressed as means ± SD (n = 10). **, $P < 0.01$. C, Proliferation of 10 T1/2 cells treated or not with uhCG (50 IU/ml) or anti-hCG antibody (micrograms per milliliter) for 48 h. Data are expressed as means ± SD (n = 10). ***, $P < 0.001$. D, Proliferation of HUVECs after 24 h of culture with or without uhCG 100 IU/ml, combined or not with BIM (1 µg/ml) or U73122 (10 µM). E, Effect of hCG (100 IU/ml), H89 (10 µM), or U73122 (10 µM) alone or in combination on AoSMC cell proliferation rate. Data are expressed as means ± SD (n = 4). *, $P < 0.05$; **, $P < 0.05$; ***, $P < 0.001$. Results are expressed as percentage of control.

capillaries were formed of a single layer of naked ECs delimiting a lumen (Fig. 2G).

hCG promotes EC and SMC interactions in a three-dimensional *in vitro* coculture model of HUVECs and human AoSMCs

The interactions between EC and human AoSMCs were then investigated *in vitro* in a coculture system on matrigel. When HUVECs were seeded on matrigel, they organized into tube-like structures. AoSMCs were then seeded on top of the HUVEC culture to study pericyte-endothelial cell interactions (Fig. 3). Under hCG treatment, pericytes became more closely apposed to the ECs (Fig. 3A). An original computer-assisted method of quantification was used to measure the distance separating pericytes from ECs (Fig. 3B). The density of mural cells apposed to ECs (distance ≤5 µM) was 20% higher under hCG treatment (**, $P < 0.05$) (Fig. 3B). In contrast, the density of mural cells present at a distance higher than 50 µm from ECs was 1.5-fold lower on hCG treatment (**, $P < 0.05$).

hCG promotes SMC and EC proliferation through PKA/PKC signaling pathways

We previously reported that hCG can directly modulate the proliferation of ECs (1). To evaluate the putative direct effect of hCG on perivascular cells, we next determined the impact of hCG on the proliferation of human AoSMCs. Increasing concentrations of hCG (10–500 IU/ml) stimulated AoSMC proliferation in a dose-dependent manner (Fig. 4A). This dose-response curve displayed a bell shape with maximal effect of hCG between 50 and 100 IU/ml. Similar results were obtained by using murine smooth muscle cells (10 T1/2) (Fig. 4B). The specificity of this effect was assessed by the addition of an anti-hCG-blocking antibody together with hCG (Fig. 4C).

Cell signaling pathways involved in the proliferative effects exerted by hCG on ECs and SMCs have next been investigated by inhibiting different pathways putatively involved in LH/hCGR signaling (Fig. 4, D and E). Addition of a PKC inhibitor (1 µg/ml BIM) and a PLC inhibitor (10 µM U73122) abolished the effect of hCG on HUVEC proliferation (Fig. 4D). Addition of a PKA inhibitor (10 µM H89) and a PLC inhibitor (10 µM U73122) abolished the effect of hCG on AoSMCs (Fig. 4E).

hCG induces SMC migration

A wound healing *in vitro* assay was first applied to determine the effect of hCG on SMCs (10T1/2). Recolonization of wounded areas was evaluated after 4 and 9 h by measuring the width of the gap in the same areas. At each time point, the size of the gap was measured, normalized to each native wound surface (time 0) and data were averaged (n = 24). Already after 4 h, hCG induced cell migration at a higher extent than in control condition (Fig. 5, A and B). After 9 h the recolonization was increased 1.4-fold (Fig. 5, A and B). A Boyden migration chamber assay was next used to further determine the promigratory effect of hCG on both smooth muscle cells

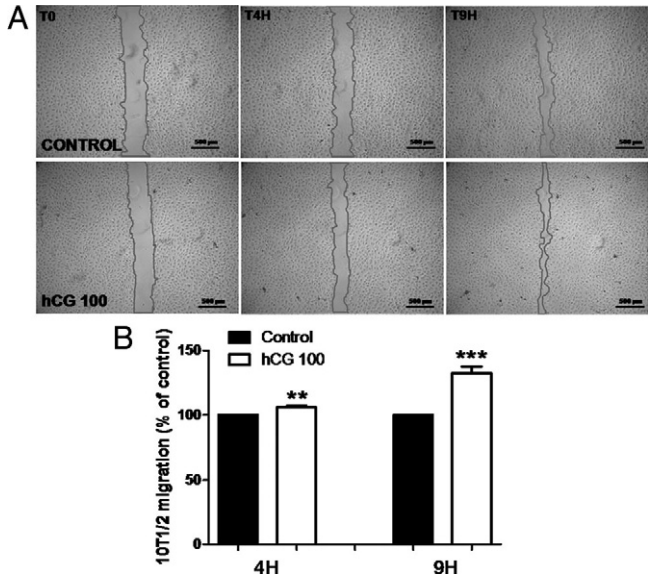

FIG. 5. Effect of hCG on SMC migration in a wound-healing assay. A, Photomicrographs of the wound area in 10 T1/2 cell monolayer in a kinetic assay (at time 4 and 9 h) without (CTRL) or with addition of uhCG 100 IU/ml to the culture medium. B, Semiautomatic quantification of recolonized areas defined as the primitive wound surface divided by wound surfaces persisting after 4 and 9 h. Control condition was set to 100%. Data are expressed as means ± SD (n = 24). **, $P < 0.05$; ***, $P < 0.001$.

(AoSMCs) and ECs (HUVECs). Incubations of a stimulatory dose of hCG (100 IU/ml) resulted in a significant enhancement of both HUVEC (Fig. 6 A) and AoSMC migration (Fig. 6B). HUVECs and AoSMCs seeded on Boyden chambers filters were incubated for 24 h with hCG (100 IU/ml) alone or in combination with signaling pathway inhibitors. Whereas hCG-induced migration of HUVECs was abolished by PKA (H89) or PLC (U73122) inhibitor (Fig. 6C), only the PLC and PKC inhibitor (BIM) was able to block the hCG-stimulated migration of AoSMCs (Fig. 6, D and E). All biological effects described above were observed in the presence of urinary and recombinant hCG.

Discussion

hCG is a key actor of placental vascularization (16–18), which is one of the crucial local adaptations to successful pregnancy. hCG stimulates directly angiogenesis through EC recruitment, enhanced proliferation, and migration (19). It also stimulates the release of VEGF by human epithelial endometrial cells that causes a paracrine stimulation of ECs. VEGF-mediated angiogenesis results in the formation of naked, dilated, and leaky capillaries resulting in tissue ischemia. Indeed, normal efficient blood circulation requires the coverage of newly formed endothelial tubes by vascular SMCs. In this study, through the use of five different models (aortic ring, cell proliferation assay, wound healing assay, Boyden chamber assay, and tube formation on matrigel), we report a potent effect of recombinant and urinary hCG on various SMC activities including proliferation, migration, and capacity to cover EC capillaries.

Because of the complexity of the mechanism underlying the angiogenic reaction and vessel maturation through pericyte recruitment, a panel of complementary models (9) has been used to investigate the mechanisms of action of hCG. The aortic ring assay comprises three different cell types (ECs, fibroblastic cells, and SMCs) and mimics four steps of the angiogenic process: 1) EC proliferation, 2) EC migration, 3) EC differentiation into capillaries, and 4) maturation by pericyte coverage (20). hCG was able to enhance the sprouting of both IB4-positive EC and NG2-positive pericytes. Interestingly, both their number and distance of migration were stimulated on hCG treatment. Of great interest is the finding that hCG promoted the coverage of endothelial capillaries by pericytes, whereas in its absence, EC and pericytes spread out in an independent manner. The impact of hCG on EC/mural cell interactions was then confirmed in a three-dimensional coculture model leading to tube-like structure formation. When EC and pericytes were cocultured on matrigel, hCG stimulated an affixing of close pericytes to ECs, which mimics the vascular maturation process. Our data are in accordance with clinical observations performed on hCG-rescued human corpus luteum. Indeed, hCG treatment of women increased endothelial cell proliferation and pericyte recruitment indicating an increase rate of angiogenesis together with vessel stabilization (21).

The presence of LH/hCGRs has been evidenced *in vivo* in the endothelium and smooth muscle of uterine blood vessels. Their expression levels are significantly increased in the intramyometrial segment (3, 4). In line with these findings, immunostaining of LH/hCGR revealed positivity on AoSMCs (data not shown). Our previous work reported that hCG has a direct stimulating effect on ECs (1). Accordingly, vascular uterine endothelial cells expressing LH/hCGR responded to physiological doses of hCG with increased capillary formation *in vitro* (6). The LH/hCGR-dependent effect of hCG is further supported by the application of the aortic ring assay to LH/hCG-deficient mice (22). This genetic approach clearly demonstrated an abrogation of hCG angiostimulation in the absence of LH/hCGR.

To further dissect the mechanism of hCG action, more simple models consisting of monocultures of EC or pericytes/SMCs were used to determine whether hCG can exert an effect on cell proliferation and/or cell migration. hCG induced a strong proliferative effect on both ECs and

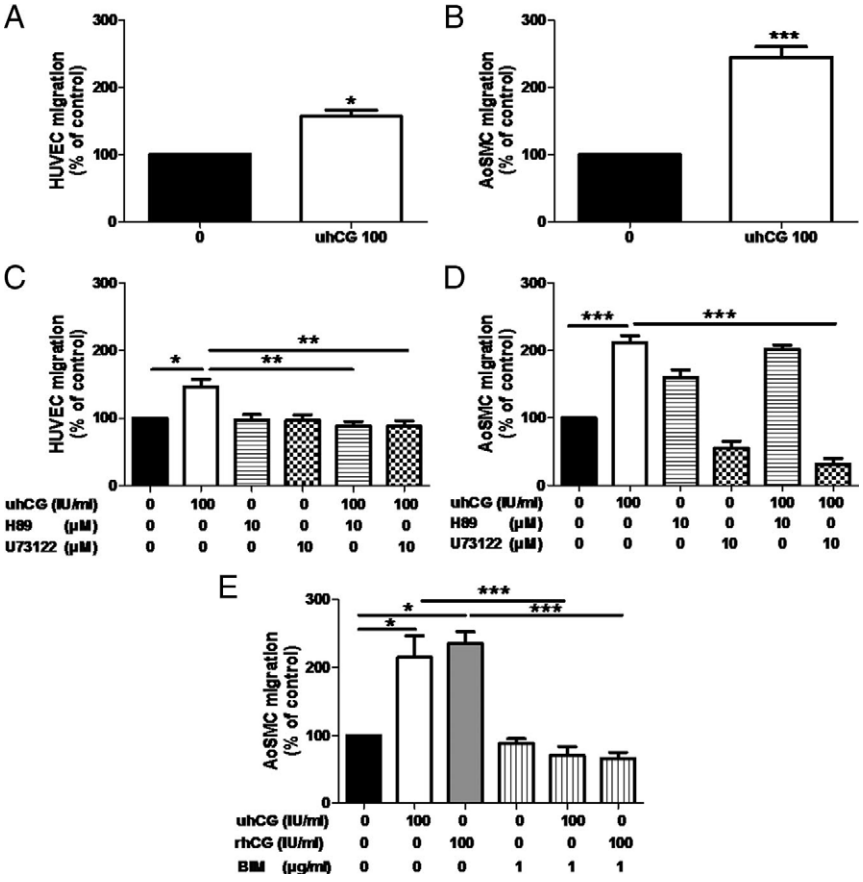

FIG. 6. Effect of hCG on HUVEC and AoSMC migration in a Boyden chamber assay. Migration of HUVECs (A) or AoSMCs (B) in the absence or presence of uhCG 100 IU/ml. HUVECs (C) and AoSMCs (D) were treated or not with uhCG 100 IU/ml, H89 (10 μM), or U73122 (10 μM) alone or in combination. Inhibition of hCG-induced migration by PKC inhibitor (BIM) (E). Data are (means ± SD) expressed as percentage of control condition (n = 4). *, $P < 0.05$; **, $P < 0.05$; ***, $P < 0.001$.

SMCs from murine or human origin, which is consistent with previous reports (23–25). Blocking antibody addition and use of recombinant hCG further confirmed the specificity of this effect. This effect was dose dependent with an optimal effect at 50–100 IU/ml corresponding to the physiological hCG concentration achieved in the first 2 wk of pregnancy. Such hCG concentration has proven a similar optimal angiogenic response in terms of endothelial cell proliferation and sprouting in different *in vitro* models (1, 19) and led to a stimulation of trophoblast migration *in vitro* (26).

We previously reported that hCG-mediated proliferative effect was PKA dependent (1). In the present study, we provide additional data that the stimulation of HUVEC proliferation by hCG is also sensitive to PKC and PLC inhibition. AoSMC proliferation was affected by PKA and PLC pathway inhibitions under hCG treatment. This is in line with previous reports showing that in porcine myometrial cells, LH/hCGR activation is coupled with two different signaling systems: adenylate cyclase and PLC (27).

SMC-induced migration was further studied in a wound healing assay in which hCG enhanced 10T1/2 migration already after 4 h of treatment and further after 9 h. This early promigrative effect signed a direct effect on migration property and was not reflecting an enhancement of cell proliferation in the wounded area. hCG caused a 2.5-fold increase of AoSMC migration in a Boyden chamber assay and a 2-fold increase of HUVEC migration. The effect of hCG on EC migration was clearly more potent when uterine microvascular endothelial cells were used (6). The present study demonstrates for the first time that hCG enhances pericyte/SMC migration. To specify the signaling pathways involved in this process, PKA and PLC inhibitors were tested in combination or not with hCG in the Boyden chambers model. We demonstrated that HUVEC-hCG induced migration signals through PKA and PLC pathways, whereas it involves only the PLC pathway in AoSMCs. Interestingly, this suggests that the signaling pathways involved in hCG's promigrative property are cell type dependent.

In summary, our results support the complex and key role played by hCG in the angiogenic process occurring at the implantation site. They provide evidence for a novel function of hCG in vessel maturation through the stimulation of pericyte recruitment.

Acknowledgments

The authors thank Benoît Brouwers for his skillful and excellent technical assistance.

Address all correspondence and requests for reprints to: Agnès Noël, Laboratory of Tumor and Developmental Biology, University of Liège, Tour de Pathologie, Centre Hospitalier Universitaire (B23), Sart Tilman, B-4000 Liège, Belgium. E-mail: agnes.noel@ulg.ac.be.

This work was supported by grants from the Fonds National de la Recherche Scientifique (Belgium), European FP7 program (MICROENVIMET Grant 201279), IUAP (interuniversity attraction pole) program, Grant Télévie, European Network of Excellence EMBryo Implantation Control, and the Fondation Léon Fredericq (University of Liège). S. B. is a recipient of a Télévie grant.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Berndt S, Perrier d'Hauterive S, Blacher S, Péqueux C, Lorquet S, Munaut C, Applanat M, Hervé MA, Lamandé N, Corvol P, van den Brûle F, Frankenne F, Poutanen M, Huhtaniemi I, Geenen V, Noël A, Foidart JM** 2006 Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium. FASEB J 20:2630–2632
2. **Licht P, Russu V, Lehmeyer S, Möll J, Siebzehnrübl E, Wildt L** 2002 Intrauterine microdialysis reveals cycle-dependent regulation of endometrial insulin-like growth factor binding protein-1 secretion by human chorionic gonadotropin. Fertil Steril 78:252–258
3. **Lei ZM, Reshef E, Rao V** 1992 The expression of human chorionic gonadotropin/luteinizing hormone receptors in human endometrial and myometrial blood vessels. J Clin Endocrinol Metab 75:651–659
4. **Toth P, Li X, Rao CV, Lincoln SR, Sanfilippo JS, Spinnato 2nd JA, Yussman MA** 1994 Expression of functional human chorionic gonadotropin/human luteinizing hormone receptor gene in human uterine arteries. J Clin Endocrinol Metab 79:307–315
5. **Toth P, Lukacs H, Gimes G, Sebestyen A, Pasztor N, Paulin F, Rao CV** 2001 Clinical importance of vascular LH/hCG receptors—a review. Reprod Biol 1:5–11
6. **Zygmunt M, Herr F, Keller-Schoenwetter S, Kunzi-Rapp K, Münstedt K, Rao CV, Lang U, Preissner KT** 2002 Characterization of human chorionic gonadotropin as a novel angiogenic factor. J Clin Endocrinol Metab 87:5290–5296
7. **Ferrara N, Gerber HP, LeCouter J** 2003 The biology of VEGF and its receptors. Nat Med 9:669–676
8. **Carmeliet P** 2003 Angiogenesis in health and disease. Nat Med 9:653–660
9. **Berndt S, Bruyère F, Jost M, Noel A** 2008 *In vitro* and *in vivo* models of angiogenesis to dissect MMP functions. Edwards D, Hoyer-Hansen G, Blasi F, Sloane BF, eds. The cancer degradome. Proteases and cancer biology. New York: Springer; 303–323
10. **Blacher S, Devy L, Burbridge MF, Roland G, Tucker G, Noël A, Foidart JM** 2001 Improved quantification of angiogenesis in the rat aortic ring assay. Angiogenesis 4:133–142
11. **Masson V, Devy L, Grignet-Debrus C, Bernt S, Bajou K, Blacher S, Roland G, Chang Y, Fong T, Carmeliet P, Foidart JM, Noël A** 2002 Mouse Aortic Ring Assay: A New Approach of the Molecular Genetics of Angiogenesis. Biol Proced Online 4:24–31
12. **Savani RC, Wang C, Yang B, Zhang S, Kinsella MG, Wight TN, Stern R, Nance DM, Turley EA** 1995 Migration of bovine aortic smooth muscle cells after wounding injury. The role of hyaluronan and RHAMM. J Clin Invest 95:1158–1168
13. **Perrier d'Hauterive S, Charlet-Renard C, Berndt S, Dubois M, Munaut C, Goffin F, Hagelstein MT, Noël A, Hazout A, Foidart JM, Geenen V** 2004 Human chorionic gonadotropin and growth factors at the embryonic-endometrial interface control leukemia inhibitory factor (LIF) and interleukin 6 (IL-6) secretion by human endometrial epithelium. Hum Reprod 19:2633–2643
14. **Saunders WB, Bohnsack BL, Faske JB, Anthis NJ, Bayless KJ, Hirschi KK, Davis GE** 2006 Coregulation of vascular tube stabilization by endothelial cell TIMP-2 and pericyte TIMP-3. J Cell Biol 175:179–191
15. **Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM, Martin GR** 1979 Laminin—a glycoprotein from basement membranes. J Biol Chem 254:9933–9937
16. **Zygmunt M, Herr F, Munstedt K, Lang U, Liang OD** 2003 Angiogenesis and vasculogenesis in pregnancy. Eur J Obstet Gynecol Reprod Biol 110(Suppl 1):S10–S18
17. **Licht P, Russu V, Wildt L** 2001 On the role of human chorionic gonadotropin (hCG) in the embryo-endometrial microenvironment: implications for differentiation and implantation. Semin Reprod Med 19:37–47
18. **Licht P, Fluhr H, Neuwinger J, Wallwiener D, Wildt L** 2007 Is human chorionic gonadotropin directly involved in the regulation of human implantation? Mol Cell Endocrinol 269:85–92
19. **Herr F, Baal N, Reisinger K, Lorenz A, McKinnon T, Preissner KT, Zygmunt M** 2007 HCG in the regulation of placental angiogenesis. Results of an *in vitro* study. Placenta 28(Suppl A):S85–S93
20. **Nicosia RF, Ottinetti A** 1990 Growth of microvessels in serum-free matrix culture of rat aorta. A quantitative assay of angiogenesis *in vitro*. Lab Invest 63:115–122
21. **Wulff C, Dickson SE, Duncan WC, Fraser HM** 2001 Angiogenesis in the human corpus luteum: simulated early pregnancy by HCG treatment is associated with both angiogenesis and vessel stabilization. Hum Reprod 16:2515–2524
22. **Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I** 2001 Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. Mol Endocrinol 15:172–183
23. **Környei JL, Lei ZM, Rao CV** 1993 Human myometrial smooth muscle cells are novel targets of direct regulation by human chorionic gonadotropin. Biol Reprod 49:1149–1157
24. **Lee TK, Kim DI, Song YL, Lee YC, Kim HM, Kim CH** 2004 Differential inhibition of Scutellaria barbata D. Don (Lamiaceae) on HCG-promoted proliferation of cultured uterine leiomyomal and myometrial smooth muscle cells. Immunopharmacol Immunotoxicol 26:329–342
25. **Horiuchi A, Nikaido T, Yoshizawa T, Itoh K, Kobayashi Y, Toki T, Konishi I, Fujii S** 2000 HCG promotes proliferation of uterine leiomyomal cells more strongly than that of myometrial smooth muscle cells *in vitro*. Mol Hum Reprod 6:523–528
26. **Zygmunt M, McKinnon T, Herr F, Lala PK, Han VK** 2005 HCG increases trophoblast migration *in vitro* via the insulin-like growth factor-II/mannose-6 phosphate receptor. Mol Hum Reprod 11:261–267
27. **Kisielewska J, Flint AP, Ziecik AJ** 1996 Phospholipase C and adenylate cyclase signalling systems in the action of hCG on porcine myometrial smooth muscle cells. J Endocrinol 148:175–180