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Human Chorionic Gonadotropin Produced by the Invasive Trophoblast But Not the Villous Trophoblast Promotes Cell Invasion and Is Down-Regulated by Peroxisome Proliferator-Activated Receptor-γ

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A critical step in the establishment of human pregnancy is the invasion of the uterus wall by extravillous cytotrophoblasts (EVCTs) during the first trimester. It is well established that human chorionic gonadotropin hormone (hCG) is secreted by the endocrine syncytiotrophoblast (ST) into the maternal compartment. We recently reported that invasive EVCTs also produce hCG, suggesting an autocrine role in the modulation of trophoblast invasion. Here we analyzed the role of hCG secreted *in vitro* by primary cultures of invasive EVCT and noninvasive ST. We first demonstrated that LH/CG receptor was present in EVCTs *in situ* and *in vitro* as well as in an EVCT cell line (HIPEC65). We next showed that hCG secreted by EVCTs stimulated progesterone secretion by MA10 cells in a concentration-dependent manner. Incubation of HIPEC65 with EVCT supernatants induced a 10-fold increase in cell invasion, whereas ST supernatants had no effect. This stimulating effect was strongly decreased when hCG was depleted from EVCT supernatants containing a large amount of the hyperglycosylated form of hCG, which is almost undetectable in ST supernatants. Finally, we investigated the regulation of hCG expression by peroxisome proliferator-activated receptor (PPAR)-γ, a nuclear receptor shown to inhibit trophoblast invasion. Activation of PPARγ decreased α- and β-subunit transcript levels and total hCG secretion in primary EVCTs. Our results offer the first evidence that hCG secreted by the invasive trophoblast, likely the hyperglycosylated form of hCG, but not by the syncytiotrophoblast, promotes trophoblast invasion and may be a PPARγ target gene in trophoblast invasion process. (*Endocrinology* 148: 5011–5019, 2007)

THE HUMAN PLACENTA is characterized by extensive invasion of cytotrophoblasts into the uterus wall, allowing direct contact of cytotrophoblasts with the maternal blood, and by the extent and specificity of its hormonal production (1). After the initial phase of nidation, human cytotrophoblasts differentiate along either the villous or extravillous cytotrophoblast pathway (Fig. 1A) (2).

The mononucleated villous cytotrophoblasts (VCTs) that cover the floating chorionic villi aggregate and fuse to form a multinucleated syncytiotrophoblast (ST), which is involved in the exchange of gases and nutrients between the mother and the fetus. The ST represents the endocrine tissue of the placenta, secreting large amounts of protein hormones including human chorionic gonadotropin hormone (hCG) (3). hCG, specific to humans and great apes, is a glycoprotein composed of an α-subunit (αhCG), which is common to pituitary gonadotropins such as LH, FSH, and TSH and a β-subunit (βhCG), which confers the biological specificity of the hormone (4, 5). αhCG is encoded by a single gene and βhCG by six genes, of which CGβ5 is expressed predominantly in the placenta (6, 7). After implantation, hCG is the first trophoblast signal detected in the maternal blood and is used to diagnose pregnancy. hCG and free βhCG are detected in the maternal blood from the first week of pregnancy, with a peak level at 12 wk followed by a decrease up to term; αhCG levels increase progressively throughout pregnancy (8). Maintenance of pregnancy during the first trimester depends on the synthesis of hCG, which prevents regression of the corpus luteum (9), allowing the maintenance of ovarian progesterone secretion (3). In addition to its well-established endocrine role, hCG increases its own synthesis in an autocrine fashion by inducing the formation of the ST through the LH/CG receptor and a cAMP/protein kinase A-dependent pathway (10, 11).

Extravillous cytotrophoblasts (EVCTs) are located at the tip of the anchoring villi contacting the uterine wall. EVCTs proliferate to form multilayered columns of cells and then invade the decidua up to the upper third of the myometrium. EVCTs also specifically invade the uterine arterioles through

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Abbreviations: CG-R, Chorionic gonadotropin receptor; DAPI, 4′,6′-diamino-2-phenylindole; EVCT, extravillous cytotrophoblast; hCG, human chorionic gonadotropin hormone; HhCG, hyperglycosylated hCG; MMP, matrix metalloproteinase; PPAR, peroxisome proliferator-activated receptor; ST, syncytiotrophoblast; VCT, villous cytotrophoblast.

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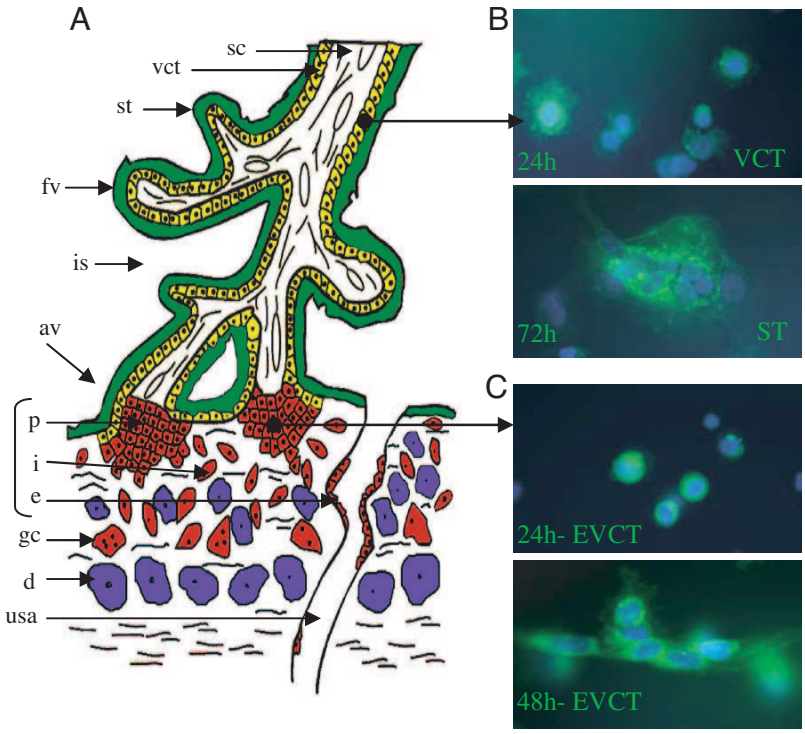

FIG. 1. Detection of hCG in human first-trimester cytotrophoblasts *in vitro*. A, Scheme of anchoring villi. Sc, Stromal core; vct, villous cytotrophoblast; st, syncytiotrophoblast; fv, floating villi; is, intervillous space; av, anchoring villi; evct, extravillous cytotrophoblast; p, proliferative EVCT; i, invasive EVCT; e, endovascular EVCT; gc, giant cells; d, decidual cells; usa, uterine spiral artery. B, Immunodetection of hCG in 24-h-cultured VCTs isolated from first-trimester human placenta and in ST after 72 h of culture. C, Immunodetection of hCG in 24- and 48-h cultured EVCTs isolated from first-trimester human placenta. B and C, For hCG immunodetection, fluorescein isothiocyanate-conjugated antibodies were used and nuclei were counterstained with DAPI. Magnification, ×300.

endovascular or perivascular routes and replace the endothelial lining and most of the musculoelastic tissue, leading to low-resistance vessels. This invasion process and remodeling of the uterine arterioles is essential to provide an adequate supply of maternal blood necessary for fetal growth (1, 2, 12, 13).

We recently reported that in addition to the endocrine ST, invasive EVCTs expressed α- and βhCG and secrete large amounts of hCG *in vitro* (14), suggesting a potential autocrine role for hCG secreted by EVCTs in the modulation of trophoblast invasion. Here we investigated the biological activity and role in trophoblast invasion of hCG secreted by cultured-primary invasive EVCTs in comparison with endocrine ST isolated from the same chorionic villi of first-trimester human placentas. Next, we analyzed the regulation of hCG gene expression by peroxisome proliferator-activated receptor (PPAR)-γ, a nuclear receptor shown to control human trophoblastic invasion (15).

Materials and Methods

Materials

Placental tissues from patients who voluntarily and legally chose to terminate pregnancy during the first trimester (8–12 wk of gestation) were obtained from Broussais Hospital (Paris, France). Our study was approved by the local ethics committee.

MA-10 is a mouse Leydig tumor cell line and was a gift from Dr. M. Ascoli (Iowa City, IA), secreting progesterone in response to hCG stimulation (16).

HIPEC65 is a cell line established from a primary culture of EVCTs transformed by T-SV40. These are proliferative and highly invasive trophoblasts that have been fully characterized in our laboratory (17).

Immunohistochemistry

Samples (n = 3) from 8- to 12-wk placentas were fixed, embedded in paraffin, and immunostained as described elsewhere (14). The different antibodies used were against hCG (A0231,1:2000, rabbit polyclonal antibody; Dako, Trappes, France), LH/chorionic gonadotropin receptor (CG-R) (H50, 1:50, rabbit polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA), or cytokeratin 7 (1:500, mouse monoclonal antibody; Dako).

Isolation and purification of villous (VCT) and extravillous (EVCT) cytotrophoblasts

VCTs and EVCTs were isolated from the same chorionic villi (n = 30) by differential trypsin digestion according to the methods of Tarrade *et al.* (18) using slight modifications as previously published (14, 19). Purified EVCTs are nonproliferative cells, highly invasive on Matrigel-coated transwells or dishes, and never fuse (15). Purified VCTs aggregate and fuse to form syncytiotrophoblasts from 48 to 72 h of culture. For study of PPARγ effects, EVCTs were stimulated for 48 h with 1 μM of the PPARγ agonist rosiglitazone.

Hormone assays

Samples were tested for free αhCG using an immunoradiometric assay (α-subunit; Immunotech Beckman Coulter Co., Marseille, France). Free βhCG was determined using the Kryptor automated immunofluorescent analyzer (Brahms, Berlin, Germany). The assay used to determine total hCG was described elsewhere (14) and recognizes all the dimeric forms of hCG, *i.e.* intact hCG, hyperglycosylated hCG (HhCG), and nicked hCG. Total hCG was determined using the chemiluminescent immunoassay analyzer ACS-180SE system (Bayer Diagnostics, Westwood, CA). Briefly, two antibodies were used: a capture mouse monoclonal anti-hCG antibody and a labeled polyclonal goat anti-hCG antibody, which were raised against two different epitopes of the β-subunit of hCG. Performance characteristics were as follows: range 2–1000 IU/liter; within-run precision 2.8%, and between-run precision 5.6%; functional detection limit 1 IU/liter. Samples were diluted (1:100) in the reaction buffer when necessary. 2332 IU/liter corresponds to 10^{-8} mol/liter hCG. HhCG was quantified in singleton with an Advantage analyzer (Nichols Institute Diagnostics, San Clemente, CA), using the monoclonal anti-HhCG antibody B152 as capture antibody. Cross-reactivity was less than 8% for other forms of hCG. Progesterone assay was performed as described previously (20).

DNA quantification

DNA was quantified by fluorometry using the fluorochrome Hœchst 33258 as previously described (19).

MA-10 assay and hCG activity

Bioactivity of hCG secreted by cytotrophoblasts. The 2×10^6 MA-10 cells were plated overnight at 37 C on gelatin-coated dishes (bactogelatin 1 g/liter). Cells were washed three times and stimulated with either ST or EVCT supernatants for 4 h at 37 C. Supernatants from four ST and EVCT cultures isolated from the same chorionic villi were assayed for hCG. Supernatants were diluted to obtain increasing concentrations of hCG, from 0.05 to 2.5×10^{-10} mol/liter.

Neutralization of hCG secreted by cytotrophoblasts. MA-10 cells were plated as above and stimulated with either ST or EVCT supernatants containing 0.75×10^{-10} mol/liter hCG. Before stimulation, supernatants (n = 4 ST and n = 4 EVCT) were incubated in the presence or absence of the neutralizing anti-hCG antibody A0231 (1:1000, specific to all dimeric forms of hCG, including HhCG) for 1 h at room temperature. The same supernatants were incubated with isotypic matched-control IgG at the same concentration to test nonspecific effect of the antibody. Regular culture medium was used as a negative control. After stimulation, supernatants were collected to assay progesterone secretion, and cells were counted in each dish. Each condition was tested in duplicate. Results were expressed as progesterone concentration (ng/ml) per 10^6 cells.

Immunocytochemistry

Cells were fixed for 20 min in 4% paraformaldehyde and permeabilized for 4 min in Triton X-100 0.3% in PBS. Saturation of unspecific sites was performed in 7% donkey serum diluted in PBS for 1 h at room temperature. Primary antibodies diluted in PBS containing 1% BSA were added for 2 h at room temperature and amplified with the secondary antibody (fluorescein isothiocyanate-conjugated donkey antirabbit or antigoat; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in PBS for 45 min in the dark at room temperature. A0231 antibody was used to detect hCG (rabbit polyclonal antibody, 1:1000; Dako) and K15 (polyclonal goat antibody, 1:50; Santa Cruz) allowed staining of LH/CG-R. Controls were performed by incubating the cells with a nonspecific IgG at the same concentration as the primary antibody. Cells were mounted in a fluorescent 4′,6′-diamino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Burlingame, CA), examined, and photographed on a BX60 epifluorescence microscope (Olympus, Tokyo, Japan).

Immunoblotting

Cells were trypsinized, harvested, and frozen at −80 C. Cell pellets were sonicated in 1 ml radioimmunoprecipitation assay buffer [150 mM NaCl, 0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 50 mM Tris (pH 8.0)] with antiproteolytic cocktail for 30 sec in ice. In parallel, for each cell lysate, 2 μg of rabbit polyclonal anti-LH/CG-R antibody (H50; Santa Cruz) were incubated for 45 min with 30 μl of the protein G PLUS-agarose immunoprecipitation reagent (Santa Cruz). Cell lysates were then immunoprecipitated with the protein G-H50 complex overnight under rotation at 4 C. Protein G pellets were washed and resuspended in 30 μl of Laemmli $1 \times \beta$-mercaptoethanol 5%. After 10 min at 60 C, the dissociated protein G-H50-LH/CG-R complex was loaded on a 7.5% acrylamide gel. Proteins were transferred onto nitrocellulose membranes and incubated overnight at 4 C with 5% nonfat milk in PBS-Tween 20 0.1%. LH/CG-R was detected with H50 antibody (1:200) followed by horseradish peroxidase-conjugated goat antirabbit IgG antibodies and enhanced chemiluminescence reagents (5).

PCR of the LH/CG-R

Total RNA was extracted using TRIzol reagent. Reverse transcription was carried out with random primers and reverse transcription SS III using 5 μg of RNA. PCR was performed using platinum PCR super mix (Invitrogen, Carlsbad, CA) and 1 mM of Xantho primers (21). Cycling conditions were 94 C for 1 min; 35 cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min; and 72 C for 7 min. Because Xantho primers amplify a single exon (exon 11), negative controls were performed using 5 μg of RNA. The expected size for amplified PCR products is 524 bp (21).

hCG depletion

EVCT supernatants were incubated with A0231 (1:100) overnight at 4 C under rotation. Protein A Sepharose was added to the supernatants (0.3 mg/μg of antibody) and incubated under rotation at 4 C for 2 h. After centrifugation (2 min, 13,000 $\times$ *g* at 4 C), the supernatants were removed and hCG was assayed on the ACS analyzer (see above) and by Western blot to check for complete depletion. The protein-A Sepharose was washed, and 200 μl of loading sample buffer was added to the protein A pellet. Nondepleted supernatants, depleted supernatants, or protein A pellets were boiled for 5 min with 2% β-mercaptoethanol and separated by 4–12% SDS-PAGE. After transfer, membranes were blocked overnight at 4 C with 5% nonfat milk in 0.1% PBS-Tween 20. hCG was detected with A0231 antibody (1:500) followed by horseradish peroxidase-conjugated goat antirabbit IgG antibodies and enhanced chemiluminescence reagents (5). The same supernatants were incubated with isotype matched-control antibody at the same concentration, and protein A Sepharose immunoprecipitation was performed as described above.

Invasion assays

Transwell inserts (6.5 mm; Falcon, Oxnard, CA) containing fluorescence-opaque polycarbonate filters with 8-μm pores were coated with 5 mg/ml of Matrigel. HIPEC65 cells (5×10^4) were seeded and stimulated in HAM-F12/DMEM (vol/vol) supplemented with 1% fetal calf serum, 2 mmol/liter glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin during 42 h with EVCT supernatants, hCG-depleted EVCT supernatants, ST supernatants, or EVCT supernatants incubated with an isotypic IgG. Supernatants were prepared to contain 10^{-9} mol/liter hCG. For depleted supernatants, the same volume of nondepleted EVCT supernatant was added. Regular HAM-F12/DMEM-1% fetal calf serum was used as negative control. Nine EVCT and six ST supernatants were tested. Each condition was tested in triplicate. After stimulation, cells were washed and fixed with 4% paraformaldehyde for 20 min at room temperature. Filters were dissected, mounted in a fluorescent DAPI mounting medium, and examined on an Olympus BX60 epifluorescence microscope. For each insert, 10 representative fields were photographed and cells were counted manually.

Quantification of specific transcripts by real-time RT-PCR

Total RNA was extracted from 48-h-cultured primary EVCTs using TRIzol reagent. cDNA synthesis and PCR amplification were performed as described previously (17). α- and βhCG transcripts were amplified using an ABI Prism 7700 sequence detection system (PerkinElmer Applied Biosystems, Foster City, CA) and the Syber Green PCR core reagent kit (PerkinElmer Applied Biosystems). The primers used for this study have already been published (22). The CK7 gene coding for cytokeratin 7, whose expression is specific to cytotrophoblasts, was used as an endogenous RNA control.

Statistical analysis

Values represent the median or 25th to 75th percentile as well as the minimal and maximal values (see Fig. 5D) or the mean ± SD (other graphs). The Mann-Whitney test was used to test differences between groups. Results were considered significant when $P \leq 0.05$.

Results

Comparative analysis of hCG bioactivity in cell supernatants of EVCT or ST primary cultures

Here we isolated, purified, and cultured extravillous and villous cytotrophoblasts from the same first-trimester human chorionic villi, as extensively described (14, 19). EVCTs are invasive cells that do not form syncytium, whereas VCTs

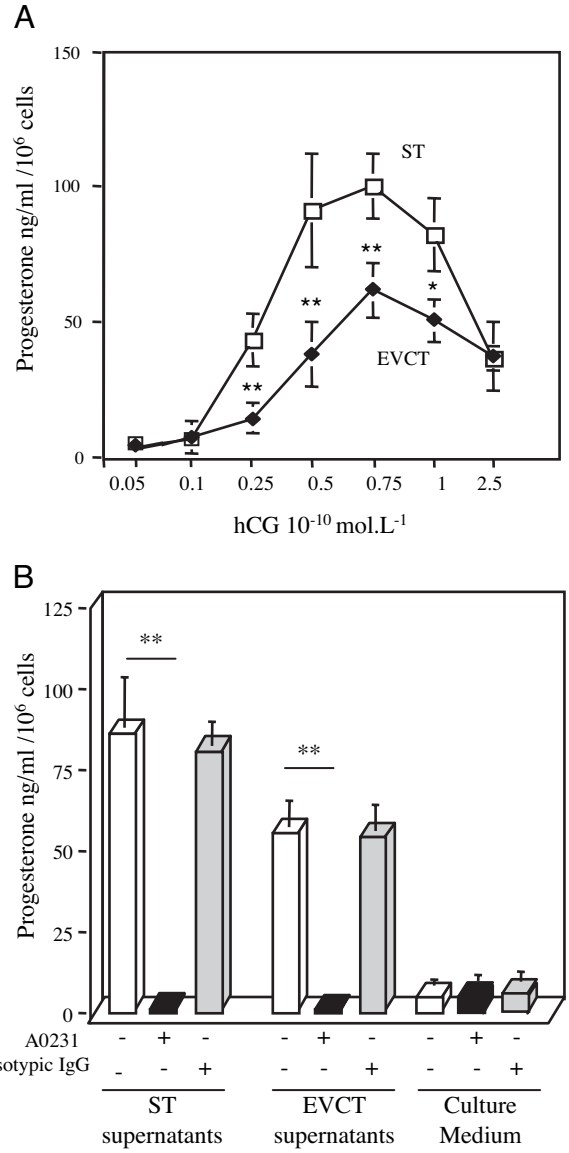

FIG. 2. Bioactivity of hCG secreted by EVCTs and ST. A, Supernatants from either EVCT (υ) or ST (χ) primary culture, obtained from the same chorionic villi (n = 4) were diluted to contain 0.05–2.5 $\times$ 10^{-10} mol/liter of hCG. Progesterone was assayed in MA-10 culture media after 4 h of stimulation, as described in *Materials and Methods*. **, $P < 0.005$; *, $P < 0.05$, EVCT *vs.* ST supernatants. B, EVCT and ST supernatants were diluted to contain 7.5 $\times$ 10^{-10} mol/liter of hCG and incubated with either neutralizing polyclonal anti-hCG antibody (A0231) or isotypic-matched control IgG before stimulation of MA-10 cells. **, $P < 0.005$, treated *vs.* untreated. Each condition was tested in duplicate and results are expressed as progesterone concentration (nanograms per milliliter) per 10^6 cells.

fuse to form syncytiotrophoblast. Figure 1B illustrates the two *in vitro* primary culture models and immunodetection of hCG in both ST (*upper panels*) and EVCTs (*lower panels*). Primary EVCTs secrete large amount of hCG *in vitro*, intermediate quantities between mononucleated VCTs and fused ST, as recently published (14). Whereas hCG secretion increased during VCT differentiation into ST (from 60 to 350 IU/liter/μg DNA), EVCT secretion remained constant and at a high level during the same culture period (160 IU/liter/μg DNA). We tested the ability of hCG secreted by each cultured-trophoblast subtype to stimulate progesterone production through the steroidogenesis MA-10 model (16). hCG was assayed in EVCT and ST supernatants, and dilutions were performed to obtain concentrations of hCG from 0.05 to 2.5 $\times$ 10^{-10} mol/liter. MA-10 cells were stimulated and progesterone was assayed as described in *Materials and Methods*. For both ST and EVCT supernatants, a dose-response curve was obtained reaching a maximal progesterone secretion for 0.75 10^{-10} mol/liter hCG (Fig. 2A). The progesterone response to EVCT supernatant stimulation (range 0.25–1.0 $\times$ 10^{-10} mol/liter) was lower than that elicited by ST supernatant ($P \leq 0.005$). For the highest hCG concentrations (from 0.75 to 2.5 $\times$ 10^{-10} mol/liter), a decrease in progesterone secretion was observed for both EVCTs and ST. To demonstrate that the cell culture-induced increase in progesterone secretion was due to hCG, supernatants containing 0.75 $\times$ 10^{-10} mol/liter hCG were incubated with anti-hCG antibody A0231, which recognizes all the dimeric forms of hCG, including HhCG. Preincubation with A0231 totally abolished the production of progesterone ($P \leq 0.005$), whereas isotype-matched control IgG had no effect (Fig. 2B).

LH/CG-R in cytotrophoblasts in situ and in vitro

The production of a bioactive hCG by EVCTs raised the question of its function at the maternofetal interface. We first investigated the presence of the LH/CG-R at the implantation site at which cytotrophoblasts expressed the cytokeratin 7 marker (Fig. 3C). As showed in Fig. 3B, LH/CG-R was specifically immunodetected in proliferative and invasive EVCTs, compared with isotypic-matched control (Fig. 3D). We confirmed that mononucleated VCTs, but not differentiated ST, was positive to LH/CG-R staining, as published elsewhere (23). hCG was strongly immunodetected in ST and invasive EVCTs (Fig. 3A). The

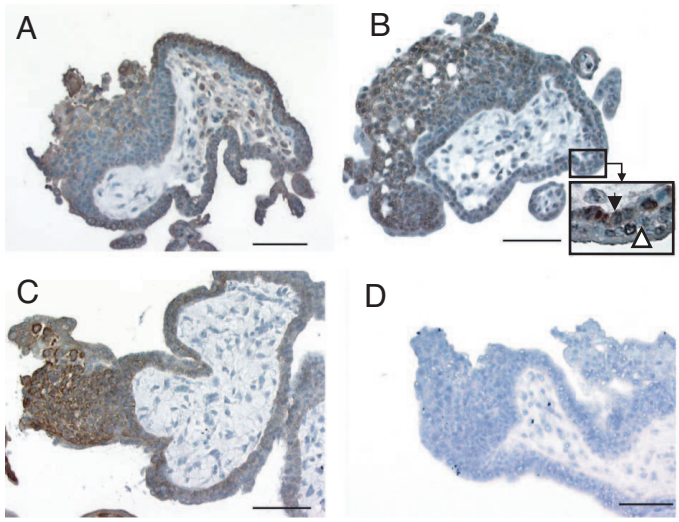

FIG. 3. Immunodetection of hCG and LH/CG-R in VCTs and EVCTs at the implantation site. Samples of first-trimester human placentas were fixed in formalin for 4 h, embedded in paraffin, and sections immunostained for hCG (A), LH/CG-R (B), or CK07 (C) or incubated with nonspecific IgG (D). *Scale bar*, 100 μm. *Inset*, LH/CG-R labeling in VCTs (*black arrowhead*) and absence of labeling in ST (*white arrowhead*).

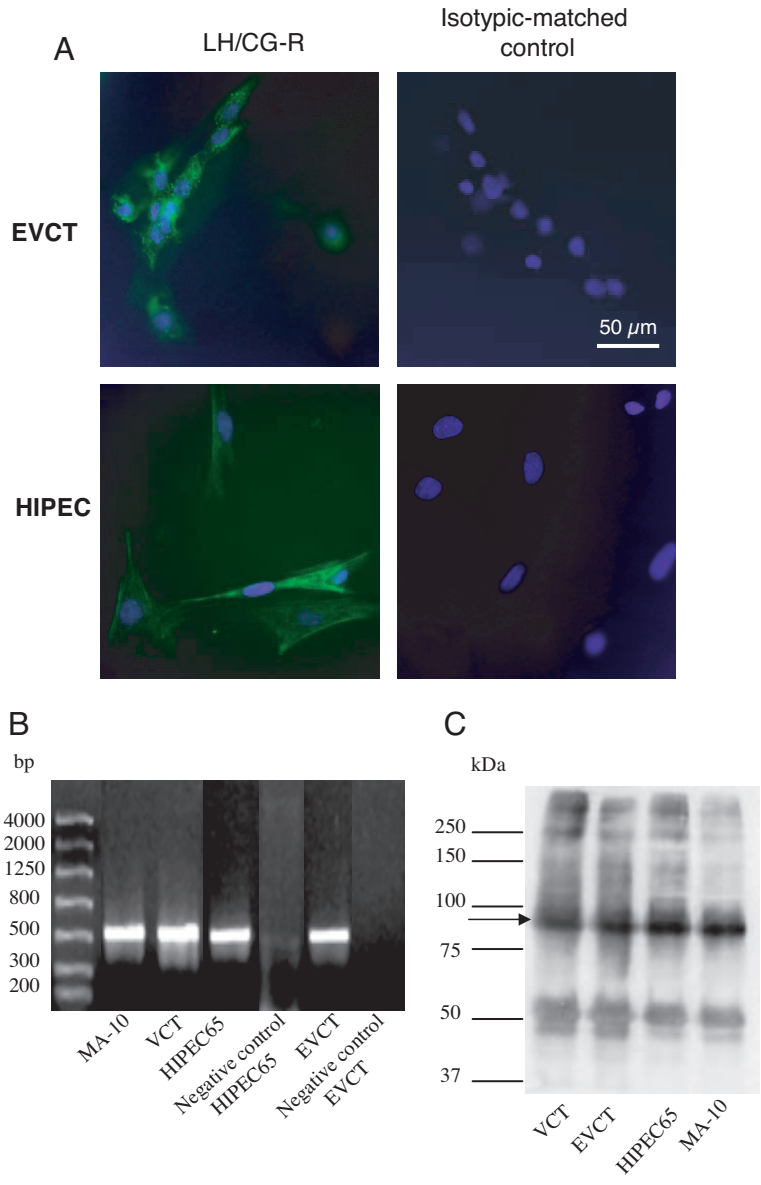

FIG. 4. Detection of LH/CG-R. A, Immunodetection of LH/CG-R in primary EVCTs and HIPEC65 EVCT cell line. LH/CG-R was detected using the K15 goat polyclonal antibody. *Scale bar*, 50 μm. B, Detection of LH/CG-R by PCR. cDNA was amplified using the Xantho primers. Negative controls were performed using RNA in place of cDNA. C, Detection of LH/CG-R by Western blot. LH/CG-R was immunoprecipitated as described in *Materials and Methods* and LH/CG-R was detected using H50 antibody with an apparent molecular mass of 95 kDa (*arrow*). VCT and MA-10 cells are considered as positive controls.

LH/CG-R was also detected by immunocytochemistry in invasive primary EVCTs and immortalized HIPEC65 (Fig. 4A). Using the anti-LH/CG-R antibody (K15) raised against a peptide mapping within the internal region of the receptor, we observed a punctiform staining, as frequently described for membrane receptors. Expression of the receptor in invasive EVCTs (primary cells and cell line) was confirmed by RT-PCR (Fig. 4B) with a 524-bp expected band (21) and by immunoblotting (Fig. 4C) with the expected 95-kDa mature form of the membrane receptor, as previously described (24). Twenty-four-hour-cultured VCT and MA-10 were used as positive controls.

hCG from EVCT but not from ST origin stimulates trophoblastic invasion

Because we demonstrated the presence of bioactive hCG and its receptor in the invasive cytotrophoblasts present at the maternofetal interface, we postulated that this hCG from extravillous origin might modulate the cell invasion process. To test this hypothesis, we used the HIPEC65 cell line that we established from an EVCT primary culture. Interestingly and as described above, HIPEC65 cells express the LH/CG-R but secrete no hCG, compared with EVCT primary cells (data not shown). These criteria in addition to the high invasive potential of these cells make HIPEC65 a suitable model to study the modulation of human trophoblastic invasion by exogenous hCG. Nine EVCT and six ST supernatants obtained after independent cultures isolated from first-trimester placenta were used to assay cell invasion. As illustrated in Fig. 5A, EVCT supernatant containing 10^{-9} mol/liter hCG significantly stimulated HIPEC65 invasion (about 10-fold increase), whereas ST-supernatants had no effect. HhCG produced by a choriocarcinoma was described by Cole (25). Using a specific antibody (B152), we assayed HhCG in JEG-3 supernatants and found that HhCG accounted for 20% of total hCG (data not shown). Surprisingly, EVCT supernatants used to

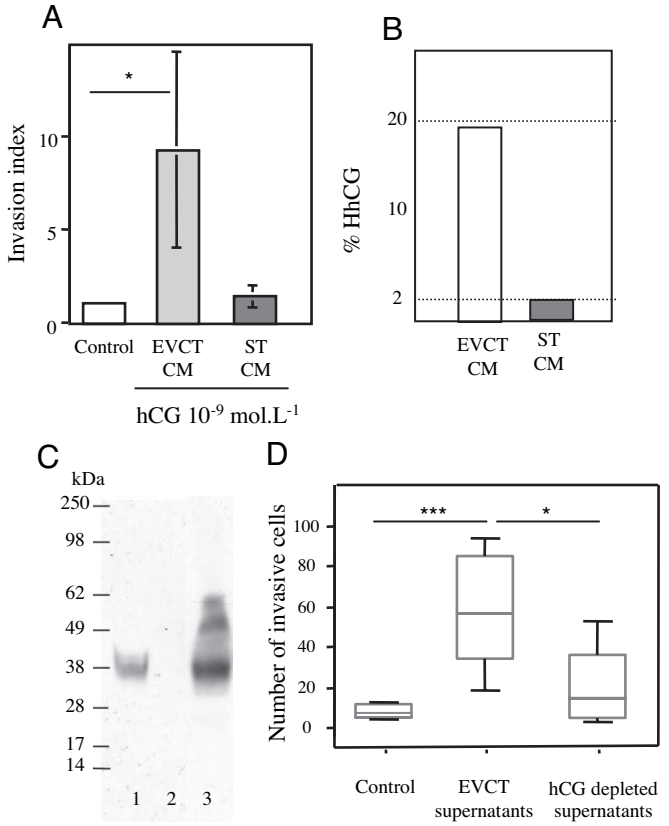

FIG. 5. Invasion assays. A, Invasion assay with different sources of hCG. Culture media (CM) obtained from nine independent EVCT cultures and six independent ST cultures were diluted to contain 10^{-9} mol/liter hCG and used to stimulate HIPEC65 in Boyden chambers. Each condition was tested in triplicate and results are expressed as invasion index. *, $P < 0.05$, EVCT *vs.* control. B, Proportion of HhCG in cell supernatants. HhCG and total hCG were assayed in EVCT and ST culture medium (CM) as described in *Materials and Methods*. Values are expressed as percentage of HhCG in total hCG. C, hCG depletion from EVCT supernatants. EVCT supernatants were incubated with a neutralizing anti-hCG antibody and immunoprecipitated with Protein-A Sepharose, as described in *Materials and Methods*. EVCT supernatants (lane 1), depleted EVCT supernatants (lane 2), and eluted immunoprecipitated hCG (lane 3) were separated by SDS-PAGE. D, Invasion assay with EVCT supernatants. Nine couples of EVCT supernatants and hCG-depleted EVCT supernatants were used to stimulate HIPEC65 in Boyden chambers. Each condition was tested in triplicate. Results are expressed as number of invading cells and represent the median, the 25th to 75th percentile, and the minimal and maximal values. ***, $P < 0.001$, nondepleted *vs.* control; *, $P < 0.05$, depleted *vs.* nondepleted.

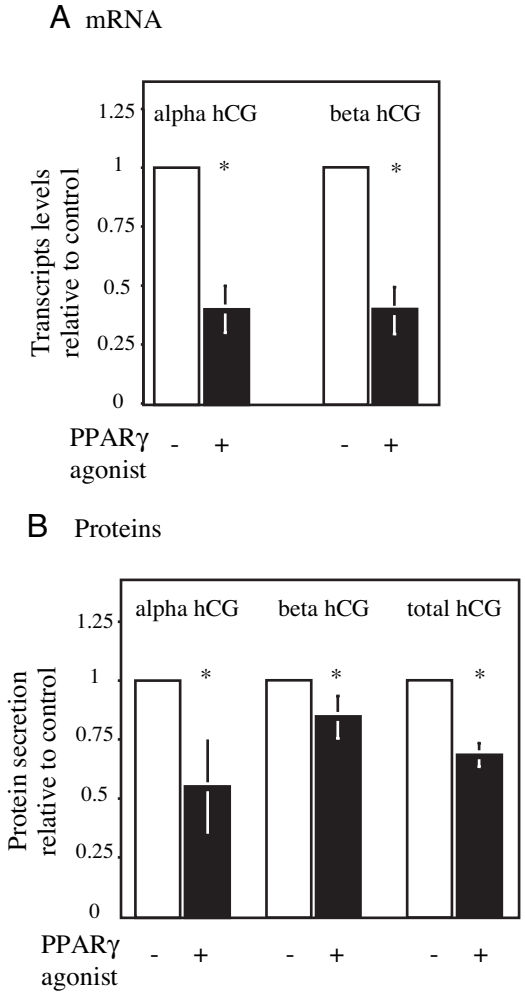

FIG. 6. Effect of PPARγ agonist on hCG expression in EVCTs. A, α- and β-subunits of hCG transcripts were analyzed in 48-h-cultured human EVCTs treated or not with 1 μmol/liter of the PPARγ agonist rosiglitazone. Values represent the mean ± SD of five independent cultures run in duplicate and are expressed relative to untreated cells. B, α- and β-subunits and total hCG secreted proteins were measured in 48-h EVCT culture medium after treatment with 1 μmol/liter of rosiglitazone. Values are expressed relative to untreated cells and represent the mean ± SD of six independent cultures isolated from six different placentas run in duplicate. *, $P < 0.05$, treated *vs.* untreated.

stimulate HIPEC65 invasion contained the same percentage of HhCG as JEG-3 and 10 times more than ST (Fig. 5B). To verify that invasion increase was due to the presence of hCG in EVCT supernatants, hCG was depleted by immunoprecipitation, with an anti-hCG antibody A0231 recognizing all the dimeric forms of the hormone, including HhCG, and using protein A Sepharose. Figure 5C shows that hCG was detected by immunoblotting with an apparent molecular mass of 38 kDa in EVCT supernatants (lane 1) and protein A complex (lane 3). In depleted-supernatant, hCG was no longer detectable, by either immunoblotting (lane 2) or immunoassay (data not shown). In lane 3, the 50-kDa band observed might correspond to the heavy chain of A0231 used for immunoprecipitation detected by the secondary antibody. Nine couples of EVCT supernatants and hCG-depleted EVCT supernatants were used to stimulate HIPEC65 in Boyden chambers. As depicted in Fig. 5D, hCG depletion significantly decreased the ability of EVCT supernatants to stimulate HIPEC65 invasion by about three times. When EVCT supernatants were treated in the same condition used for hCG depletion but with isotypic IgG, no difference in cell invasion was observed, compared with nondepleted EVCT supernatants (data not shown), demonstrating that depletion protocol did not affect EVCT supernatant activity.

PPARγ activation down-regulates hCG expression in invasive EVCT

We previously published that the activation of the nuclear receptor PPARγ inhibits EVCT invasion *in vitro*. We hypoth-

esized that hCG secreted by EVCT might be involved in PPARγ-mediated inhibition of trophoblast invasion in an autocrine manner. We therefore examined in EVCT the regulation of hCG by PPARγ. PPARγ activation by its agonist rosiglitazone led to a significant 60% decrease in both αhCG and βhCG transcript levels (Fig. 6A). When free αhCG, free βhCG, and total hCG were measured in cell supernatants from 48-h-cultured EVCT, we observed a significant rosiglitazone-mediated decrease in secretions of the α-subunit, the β-subunit, and total hCG by 40, 20, and 30%, respectively (Fig. 6B).

Discussion

Trophoblastic invasion during the first trimester is a critical step in establishment of human pregnancy and is controlled by multiple autocrine and paracrine factors, including hormones produced by the invasive EVCT itself, such as the placental GH (26). We recently demonstrated that invasive, but not proliferative, EVCT express α- and βhCG and secrete large amounts of the glycoprotein *in vitro* (14), suggesting a potential autocrine role for hCG of EVCT origin in the modulation of trophoblast invasion. Because the expression and the role of hCG produced by the endocrine syncytiotrophoblast is well established, we compared the biological activity and the role in trophoblast invasion of hCG secreted by EVCT and ST. To obtain hCG from its source of production, we used the sequential enzymatic method (19) permitting isolation, from the same first-trimester chorionic villi, of EVCTs that differentiate *in vitro* into invasive cytotrophoblasts and VCTs that fuse to form the syncytiotrophoblast.

We first provided evidence that the invasive EVCTs secreted a bioactive hCG, which had the ability to induce, in a concentration-dependent manner, the synthesis of progesterone in the steroidogenesis MA-10 model (16). This effect was nonetheless weaker, compared with hCG produced by ST. Preincubation of cell supernatants with an anti-hCG antibody totally abolished this effect, whereas IgG isotype controls did not. These data suggest that progesterone secretion is mediated through activation of the LH/CG-R signaling by hCG from cytotrophoblast supernatants. Very little is known about the affinity of the different forms of hCG toward the LH/CG-R. On the one hand, a study indicated that alternative glycosylation of CGβ has no detectable effects on either heterodimer formation or binding of the hormone to the receptor (27), supporting previous results indicating that O-linked sugars of the CTP are not involved in receptor binding (28, 29). On the other hand, Cole *et al.* (30) showed that the nicks decreased binding of nicked hCG on the LH/CG-R by 11-fold. To our knowledge, there is no information concerning HhCG affinity to LH/CG-R. We suggest that trophoblast supernatants may contain different forms of hCG that may display different affinity for LH/CG-R.

We then investigated the ability of EVCT supernatants in comparison with ST supernatants to modulate *in vitro* the invasion of the human invasive trophoblast cell line HIPEC65 that we immortalized from primary invasive EVCT and extensively characterized (17). As demonstrated by immunocytochemistry, RT-PCR, and Western blot, HIPEC65 as well as primary EVCT expressed the LH/CG-R, but in contrast to primary EVCT, they secreted no hCG and therefore provided a suitable model to study the effect of exogenous hCG. Incubation of HIPEC65 with EVCT cell supernatants containing 1 nmol/liter hCG induced a 10-fold increase in cell invasion *in vitro*, whereas ST supernatants had no effect. This stimulating effect was significantly decreased by 70% when hCG was depleted from EVCT supernatant with a neutralizing anti-hCG antibody that recognizes all the dimeric forms of the hormone, *i.e.* hCG, HhCG, and nicked hCG. These results show that the EVCT supernatant-induced increase in trophoblast invasion is mainly due to hCG and that EVCT and ST secrete different forms of hCG with different biological functions. hCG is a complex glycoprotein hormone that changes in carbohydrate content during pregnancy and in pathologies such as choriocarcinoma (31, 32) or Down syndrome (20). We used the monoclonal antibody B152 raised against the HhCG produced by a choriocarcinoma (25, 33, 34) to specifically quantify HhCG in supernatants from EVCT and ST primary cultures. We showed that the proportion of HhCG in EVCT supernatants was 10 times higher than in ST supernatants. Our results confirmed a previous study in which HhCG was preferentially detected in first-trimester trophoblast supernatants composed from a mix of EVCTs and VCTs, compared with fused ST supernatants (35). Thus, we suggest that HhCG, but not hCG, may be involved in the stimulation of trophoblast invasion. Nevertheless, because HhCG represented only 20% of total hCG in EVCT supernatants and because hCG depletion induced a 70% decrease in HIPEC65 invasion, we do not exclude that other forms of hCG, not yet described, could be implicated in the trophoblastic invasion process.

The role of hCG in trophoblasts *in vitro* has been reported by using either recombinant or urinary hCG (36–39), but to our knowledge, this is the first report of a comparative study showing the roles of hCG secreted by either invasive EVCT or endocrine ST primary cultures. The regulatory effect of urinary hCG or recombinant hCG in the trophoblastic invasion process was studied, giving contradictory results. Here we showed that hCG produced by invasive EVCT, but not by endocrine ST, promotes invasion. Trophoblast invasion is a very complex process including cytoskeleton remodeling, expression of adhesion molecules, extracellular matrix digestion, and secretion of autocrine factors. Matrix metalloproteinases (MMPs), cytokines, and growth factors are known to stimulate trophoblast invasion. Recently hCG was shown to stimulate MMP-9 (40), although no cAMP response element is described on an MMP promoter. However, hCG was shown to increase vascular endothelial growth factor mRNA and production (41, 42), a factor that stimulates secretion and activity of MMP-2 and MMP-9 (43, 44). According to these data, one can speculate that the effect of hCG on trophoblastic invasiveness might involve MMPs through activation of vascular endothelial growth factor.

Finally, we investigated the regulation of hCG gene expression by PPARγ, a nuclear receptor shown to inhibit the invasion of human primary EVCTs (15). We found that activation of PPARγ by the agonist rosiglitazone significantly

decreased α- and β-subunit transcript levels by about 60% and total hCG secretion by 40% in primary cultured-invasive EVCTs. This is the first evidence of a down-regulation of both α- and β-subunits of hCG by PPARγ. We previously showed that PPARγ/ retinoid X receptor-α heterodimers are functional units during VCT differentiation, and elements located in the regulatory regions of the CGβ gene bind retinoid X receptor-α and PPARγ (45). Our results open a new field of investigation on the transcriptional regulation of hCG subunits and its modulation by PPARγ the invasive extravillous cytotrophoblast.

In conclusion, our results offer the first evidence that hCG secreted by human invasive EVCTs promotes trophoblast invasion, whereas hCG from the endocrine ST does not, and suggest that the source of hCG is essential to understanding its physiological function in human trophoblasts. In addition, we speculate that HhCG may be involved in the PPARγ-mediated inhibition of trophoblast invasion in an autocrine manner.

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