0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(5):2434–2444
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doi: 10.1210/en.2004-1550

Stimulation of Human Trophoblast Invasion by Placental Growth Hormone

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A critical step in establishment of human pregnancy is the invasion of the uterus wall by the extravillous cytotrophoblast (EVCT), a process regulated by multiple autocrine and paracrine factors. Hormones belonging to the GH/prolactin family are expressed at the maternofetal interface. Because they are involved in cell motility in various models, we examined the possible regulatory role of human placental GH (hPGH) in EVCT invasiveness. By using an *in vitro* invasion model, we found that EVCT isolated from first-trimester chorionic villi and cultured on Matrigel secreted hPGH and expressed human GH receptor (hGHR). These data were confirmed by *in situ* immunohistochemistry. EVCT expressed the full-length and truncated forms of hGHR, and the Janus kinase-2/signal transducer and activator of transcription factor-5 signaling pathway was activated in EVCT by hPGH treatment. Strong hPGH and hGHR expression was observed when EVCT invaded Matrigel and moved through the pores of the filter on which they were cultured. hPGH stimulated EVCT invasiveness, and this effect was inhibited by a Janus kinase-2 inhibitor. Interestingly, hPGH was more efficient than pituitary GH in stimulating EVCT invasiveness. These results offer the first evidence for a placental role of hPGH and suggest an autocrine/paracrine role of hPGH in the regulation of trophoblast invasion. (*Endocrinology* 146: 2434–2444, 2005)

THE HUMAN PLACENTA produces a GH [human placental GH (hPGH)] specific to this organ. hPGH is the product of the GHv gene and shows a high degree of identity with pituitary GH (hGH), from which it differs by only 13 amino acids and an *N*-glycosylation site (for review see Refs. 1, 2). Expressed by the syncytiotrophoblast layer, bordering the floating villi (3, 4), hPGH is released into the maternal bloodstream. Its concentration increases as pregnancy progresses, whereas that of pituitary GH falls to a nadir. Human PGH is an important regulator of maternal metabolism. Through its nonpulsatile secretion and its modulation of IGF-I expression, hPGH is considered as a major mediator of the insulin resistance observed during pregnancy and has been shown to directly augment insulin resistance in transgenic mice (5). Human PGH displays classical GH biological effects through its binding to the GH receptor (GHR), a type I cytokine receptor. GHR possesses a single transmembrane domain and lacks intrinsic kinase activity. Binding of one GH molecule to predimerized GHR induces conformational changes in the receptor complex (6), leading to its activation and signaling through activation and phosphorylation of Janus kinase-2 (JAK2), a receptor-associated protein (7). Two alternatively spliced transcripts of hGHR have been reported in different human tissues, generating truncated isoforms of the hGHR devoid of signaling activity (hGHRtr) (8, 9).

Although GHR is expressed in third-trimester placentas (10, 11), the involvement of hPGH in placental development and trophoblast differentiation has not yet been documented.

The human placenta is a villous hemochorial placenta characterized by extensive invasion of the maternal uterus by the trophoblast, placing the trophoblast in direct contact with maternal blood. This invasion is ensured by extravillous cytotrophoblasts (EVCT). At the base of anchoring villi, EVCT form clusters of proliferating cells (proliferating extravillous cytotrophoblasts, PEVCT) known as columns and then differentiate into nonproliferative highly invasive cells (interstitial extravillous cytotrophoblasts, IVECT) that colonize the decidualized endometrium and the proximal third of the myometrium. Some EVCT invade the uterine spiral arteries and modify their walls, replacing endothelial cells and intercalating within the muscular tunica (12, 13). This artery remodeling results in low-resistance, high-capacitance vessels capable of ensuring the increased blood flow necessary for placental development. Decidual invasion by EVCT results from classical steps of cellular invasion, including attachment to the basement membrane followed by detachment and proteolysis of the basement membrane before its

First Published Online February 17, 2005

Abbreviations: CK7, Cytokeratin 7; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; EVCT, extravillous cytotrophoblast; FCS, fetal calf serum; GHR, GH receptor; GHRtr, truncated GHR; h, human; HLA, human leukocyte antigen; IEVCT, interstitial EVCT; IRMA, immunoradiometric assays; JAK2, Janus kinase-2; LHRE, lactogenic hormone response element; mAb, monoclonal antibody; PEVCT, proliferating EVCT; PGH, placental GH; PO, acidic ribosomal phosphoprotein; PRL, prolactin.

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penetration. EVCT leaving the proliferative cell cluster acquire an invasive phenotype characterized by a switch in their adhesion molecule expression (14–16) and the production of a set of proteases, metalloproteinases, (17–20), serine proteases (21), and cathepsin (22), which degrade the extracellular matrix. Decidual cells restrain this degradation by secreting metalloproteinase inhibitors (TIMP) (23–25). They also produce a set of growth factors, cytokines, and chemokines that either inhibit or promote EVCT invasion (23, 26–29). EVCT invasiveness is also modulated by hormones, growth factors, cytokines, and protease inhibitors produced by EVCT themselves (25, 26, 30). Receptors for some of these factors are expressed in decidual cells or in EVCT (23, 26). Consequently, EVCT invasiveness is regulated not only by decidual cells-EVCT paracrine dialogue but also by autocrine regulation loops acting on EVCT (23, 31, 32). The cellular matrix and fibroblast of the villous mesenchyme are also involved in this invasion process (33, 34). Any disruption of these autocrine-paracrine regulations may potentially affect the course of pregnancy, leading to disorders such as preeclampsia and intrauterine growth retardation that are characterized by trophoblast shallow invasion (13).

GH is involved in the regulation of cell motility and spread in various cellular models (35–38). In addition, hPGH is expressed by extravillous trophoblasts located in the placental basal plate (39). We therefore investigated the potential effect of hPGH on human trophoblast invasiveness in our *in vitro* model of extravillous cytotrophoblastic cell isolation and invasion (40).

Materials and Methods

Reagents

Recombinant human pituitary GH (hGH) was from Pfizer (Montrouge, France). The JAK2 inhibitor AG490 was from Euromedex (Souffelweyersheim, France). Monoclonal antibodies (mAb) directed against the following antigens were used: cytokeratin 7 (CK7; Dako, Trappes, France), human PGH/hGH (mAb 5B4; Biocode, Sclessin, Belgium), human GHR (mAb 263; American Diagnostica, Andresy, France), fibroblast-specific antigen (clone ASO2; Dianova, Hamburg, Germany), human leukocyte antigen (HLA) class I (HLA-A, -B, -C, -G, and -E, W6/32; Leinco Technologies Inc., St. Louis, MO), and α5-integrin subunit (CD49e; Immunotech, Marseille, France). IgG controls were from Coulter (Fullerton, CA) for mouse IgG1 and from Immunotech for mouse IgG2a. Fluorescein isothiocyanate-conjugated donkey antimouse IgG (H+L) was from Jackson ImmunoResearch Laboratories (West Grove, PA). Culture medium, fetal calf serum (FCS) (Myoclone), penicillin, streptomycin, and DNA size markers (ϕ-X-174 RF/*Hae*III) were from Life Technologies, Inc. Invitrogen (Cergy Pontoise, France). Trypsin was from Difco Laboratories (Detroit, MI), DNase type IV was from Sigma (Saint-Quentin Fallavier, France), Percoll was from Amersham Biosciences (Orsay, France), and Matrigel was from BD Biosciences (Le Pont de Claix, France).

Tissues

For cell cultures, samples of first-trimester placentas (7–10 wk) were obtained from women undergoing voluntary elective termination of pregnancy at Broussais Hospital (Paris, France). Samples for immunohistochemistry were collected in the Department of Obstetrics and Gynecology at La Citadelle Hospital (Liège, Belgium). In both hospitals, informed consent was obtained from the patients.

EVCT isolation and purification

EVCT were prepared as previously described (40) with minor modifications. Briefly, placental tissue was washed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. Chorionic villi were dissected, carefully removing blood vessels and clots, and then rinsed and submitted to mild enzymatic digestion in Hanks' solution containing 0.125% trypsin, 4.2 mM $MgSO_4$, 25 mM HEPES, and 50 Kunitz/ml DNase type IV for 35 min at 37 C without agitation. After sedimentation, supernatants were collected and the remaining tissue was rinsed four times with Hanks' solution. The sedimentation supernatants were pooled and filtered (100-μm pore size). To avoid cell aggregation, supernatants were supplemented with EDTA (1 mM final concentration) and incubated for 5 min; then trypsin activity was neutralized with 10% FCS. Cells were centrifuged (300 $\times$ g for 10 min) and washed once in Hanks'. Cells were then pelleted, resuspended in Hanks', carefully layered on a discontinuous Percoll gradient, and centrifuged for 35 min at 1000 $\times$ g. The layers corresponding to 40–60% Percoll were collected (60% layer not included) and washed twice with Hanks'/10% FCS. The EVCT preparation was further purified by plating in HAM F12/DMEM (F12/DMEM)/10% FCS in plastic dishes at 20,000 cells/cm^2. After incubation overnight in humidified air/5% CO2 at 37 C, villous trophoblasts, macrophages, and fibroblasts adhered to the plastic dishes. Floating cells consisted mainly of EVCT and a few remaining fibroblasts. EVCT were depleted of fibroblasts by magnetic separation using MACS antifibroblast MicroBeads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, after centrifugation, the cell pellet was resuspended in 80 μl PBS/0.5% FCS and incubated with 20 μl antifibroblast MicroBeads for 30 min at 20 C. The cells were then washed and separated on an MS MACS magnetic column. Three milliliters of PBS/0.5% FCS were applied to the column to wash out EVCT (column eluate), fibroblasts remaining bound to the column. To recover fibroblasts, the column was removed from the magnet and flushed with PBS/0.5% FCS. The fibroblastic nature of the flushed cells was checked by immunocytochemistry (ASO2-positive, CK7-negative). EVCT were centrifuged, and then the pellet was resuspended in F12/DMEM/10% FCS and plated in culture chambers or submitted to RNA extraction (see below).

Insert culture of extravillous cytotrophoblasts

The culture chambers consisted of high-throughput samples (HTS) FluoroBlok inserts (24-multiwell insert system; BD Biosciences). The bottom of the insert is composed of a light-tight polyethylene terephtalate pored filter (pore size, 8 μm) that blocks the transmission of wavelengths from 490–700 nm. Invasive cells can move from the upper to the lower face of the filter through pores and can be visualized by immunofluorescence. The polyethylene terephtalate filter blocks fluorescence emitted by cells present on the reverse face of the filter. EVCT (2.5×10^5 cells) were plated on the filters (1 cm^2) coated with Matrigel (10 μl, 5 mg/ml). Inserts were placed in wells (4 cm^2), and the inserts and wells were filled with culture medium (DMEM/F12, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg /ml streptomycin) supplemented with 10% FCS (200 μl in the insert and 600 μl in the well) and placed at 37 C in a humidified atmosphere of 5% CO_2 in air. In some experiments, plating was interrupted after 30 min or 3 h, and cells were fixed for immunocytochemistry as described below. Otherwise, the medium was replaced after 2 h with the same medium supplemented with 1% FCS and various treatments (see below). For immunocytochemistry, inserts were filled with culture medium supplemented with 2% FCS, and wells were filled with culture medium supplemented with 20% FCS, creating an invasion-promoting serum gradient. After 72 h, cells were washed three times with PBS, fixed for 20 min in 4% paraformaldehyde, and washed again three times in PBS. Inserts containing fixed cells were stored in PBS at 4 C for no longer than 2 d until immunodetection assays.

Invasion assays

For invasion assays, inserts and wells were filled with culture medium supplemented only with 1% FCS (to reduce the effect of growth factors present in FCS on EVCT invasiveness). Various concentrations of hGH, hPGH, or human prolactin (hPRL) were added to the medium on both sides of the filter. The same concentrations were again added to the medium after 40 h of culture, without renewing the medium. In some experiments, a JAK2 inhibitor (AG490) dissolved in dimethylsulfoxide (DMSO) was added to the medium in the same conditions. In these experiments the same concentration of DMSO (1/1000) was added to control wells. This concentration of DMSO did not affect cell viability, as tested by trypan blue exclusion, and no nuclei condensation or fragmentation was observed by 4′,6-diamidino-2-phenylindole (DAPI) staining. After 72 h, culture supernatants were collected from control wells to evaluate hPGH production, and the number of invasive cells present on the underside of the filter (entire surface) was scored visually after CK7 immunolabeling. Briefly, cells were fixed in 4% paraformaldehyde as described in the previous section and permeabilized for 8 min in methanol at −20 C. After 1 h of incubation at room temperature in PBS supplemented with 10% donkey serum and 10% horse serum to reduce nonspecific binding, the cells were incubated with CK7 antibody (1 μg/ml) diluted in PBS/10% donkey serum/10% horse serum for 2 h at room temperature. The cells were washed in PBS/0.1% Tween and incubated with fluorescein isothiocyanate-conjugated antimouse IgG (4.6 μg/ml) for 1 h in the dark and were then washed in PBS/0.1% Tween. The filters were removed from the inserts with a scalpel and mounted on a microscope slide in a drop of mounting medium containing 1.5 μg/ml DAPI (Vectashield, Vector Laboratories, Burlingame, CA), with the underside of the filters facing upward. Invasion was scored by counting the cells on the underside of the filters with an Olympus BX60 epifluorescence microscope. Experiments were repeated at least four times (see figure legends) with EVCT from different placentas, and inserts were prepared in duplicate for each experimental condition. To minimize interexperiment variability, the number of cells scored on the underside of the filters in treated chambers was expressed as a percentage (invasion index) of the number of cells scored on the underside of the filter in the control chamber.

Immunohistology

Paraffin-embedded sections were kindly supplied by Prof. Foidart (Pathology Department of La Citadelle Hospital, Liège, Belgium). Samples were fixed by incubation in 4% formalin for 4 h at room temperature and then embedded in paraffin, dewaxed in xylene, and rehydrated in ethanol/water. Antigen retrieval was done by immersing the slides for 40 min in Dako retrieval solution (pH 6) preheated at 90 C in a water bath. Immunostaining was performed with a universal streptavidin peroxidase immunostaining kit (Dako LSAB+ System horseradish peroxidase). Endogenous peroxidase-like enzyme activity was blocked by incubation for 5 min in a blocking reagent containing 3% H_2O_2. After 1 h of saturation in PBS/10% pig serum/10% horse serum/50 μg/ml human IgG (complemented PBS), sections were incubated with CK7 antibody (1 μg/ml), or mAb 263 (25 μg/ml), or mAb 5B4 (17 μg/ml) at 4 C overnight in complemented PBS. Sections were washed in PBS, treated again with H_2O_2 solution for 5 min, and saturated in complemented PBS for 1 h. Then they were incubated with a biotinylated secondary antibody for 1 h. The sections were washed in PBS, and staining was detected by incubation for 2 min with the 3,3′-diaminobenzidine chromogen. The slides were lightly counterstained with hematoxylin. Controls were performed by incubating sections with nonspecific mouse IgG (same isotype) at the same concentration.

Immunocytology

HLA class I and ASO2 immunodetection was performed on paraformaldehyde-fixed cells. For GHR and hGH immunodetection, cells were permeabilized with methanol as described for CK7 detection. For α5-integrin subunit immunodetection, cells were permeabilized for 4 min in 0.3% Triton X-100/PBS. After 1 h of saturation in PBS/10% donkey serum/10% horse serum/50 μg/ml human IgG, primary antibodies in the same supplemented PBS buffer were added at appropriate concentrations (ASO2, 4 μg/ml; w6/32, 2 μg/ml; mAb 263, 9 μg/ml; mAb 5B4, 6 μg/ml; and CD49e, 1 μg/ml) overnight at 4 C. Cells were then incubated with a secondary antimouse antibody conjugated with fluorescein (4.6 μg/ml), as described for CK7, and were mounted in Vectashield. Control experiments for each antiserum included replacement of the primary antiserum with the appropriate nonimmune mouse IgG (same isotype) at the same concentration.

hPGH immunoradiometric assays (IRMA)

EVCT collected at the end of the purification process were centrifuged and sonicated in 250 μl of 10 mM Tris/HCl, 20 mM sodium molybdate, 0.6 mM KCl, 1 mM EDTA, and antiprotease inhibitor cocktail (set I; Calbiochem, La Jolla, CA). Culture medium (1% FCS) was collected after 72 h of culture, concentrated on Microcon YM-30 (Amicon Millipore Co., Bedford, MA), and evaporated under a gentle stream of nitrogen. The residue was reconstituted with 200 μl IRMA buffer. Known hPGH concentrations extracted in the same way gave a mean recovery of 84 ± 5%. hPGH was measured in duplicate in a specific solid-phase ^{125}I-labeled IRMA (Biocode, Liège, Belgium) using two specific mAbs. Cross-reactivity is 100% for hPGH and less than 0.001% for hGH and human placental lactogen. The detection limit is 200 pg/ml. The intra- and interassay coefficients of variation are 4 and 6%, respectively (41, 42).

Real-time quantitative RT-PCR

Total RNA from EVCT collected before plating (time zero) and after 72 h of culture were prepared by means of the single-step guanidinium-phenol-chloroform method described by Chomczynski and Sacchi (43). The quality of RNA samples was determined by electrophoresis through agarose gel and visualization of the 18S and 28S bands under UV light after staining with ethidium bromide. cDNA synthesis and real-time quantitative PCR were performed as previously described (44). PCR were run in an ABI PRISM 7700 Sequence Detection System with the SYBR Green (Bcl-2 and Ki-67) or TaqMan (GHv and GHR) PCR Core Reagents kits (PerkinElmer Applied Biosystems, Foster City, CA). GHv, hGHR, Bcl-2, and Ki-67 cDNA were amplified with specific forward and reverse primers (Table 1). Transcripts of the constitutive housekeeping gene 36B4, coding for acidic ribosomal phosphoprotein (PO) (44) were measured in each sample to control for sample-to-sample differences in the RNA concentration. Levels of GHv, hGHR, Ki67, and Bcl-2 mRNA were expressed as a ratio of PO mRNA values.

TABLE 1. Oligonucleotide sequence of primers used in the real-time PCR

Genes	Primer	Sequence (5′→3′)	PCR product size (bp)
GHv	Forward primer	AGA ACC CCC AGA CCT CCC T	96
	Reverse primer	TGC GGA GCA GCT CTA GGT TAG	
	Probe	TTT CTG CTG CGT TTT CAC CCT GTT G	
hGHR	Forward primer	GCA ATG GTG GTA CAG TGG ATG A	145
	Reverse primer	CGT GGT GCT TCC CAT CTC AC	
	Probe	CAA CCA GAT CCA CCC ATT GCC CTC A	
PO	Forward primer	GGC GAC CTG GAA GTC CAA CT	149
	Reverse primer	CCA TCA GCA CCA CAG CCT TC	
	Probe	ATC TGC TGC ATC TGC TTG GAG CCC A	
Ki-67	Forward primer	ATT GAA CCT GCG GAA GAG CTG A	105
	Reverse primer	GGA GCG CAG GGA TAT TCC CTT A	
Bcl-2	Forward primer	CCC CTG GTG GAC AAC ATC GC	101
	Reverse primer	AGT TCC ACA AAG GCA TCC CAG C	

RT-coupled hemi-nested PCR

Total GHR transcripts from EVCT were reversed-transcribed using the SuperScript II protocol (Invitrogen) for subsequent amplification. The transcripts corresponding to the full-length hGHR and the truncated form (GHRtr) isoforms were amplified with primers that flank the alternative splice site located in exon 9. Two specific forward primers located in exon 9, designated h9 (5′-ATTTTCTAAACAGCAAAGGA-3′) and h9tr (5′-ATTTTCTAAACAGCAAAGTT-3′), respectively (8), were used in combination with a reverse primer (h10) located in exon 10 (5′-CACTGTGGAATTCGGGTTTA-3′) for the first-round PCR, and a hemi-nested primer (h10′) also located in exon 10 (5′-GATTGTGTTCACCTCCTC-3′) was used for the second round. Both amplification steps were run in a thermal cycler with the following parameters: initial denaturation for 5 min at 94 C, followed by 35 cycles consisting of 30 sec at 94 C, 30 sec at 55 C, and 30 sec at 72 C and by a final extension step at 72 C for 7 min. The amplification products (hGHR, 118 bp; hGHRtr, 92 bp) were detected by electrophoresis on agarose 1%/3% NuSieve gel stained with ethidium bromide. Transcripts from human kidney were amplified as the positive control (8), and water served as the negative control.

Transient transfection of primary invasive EVCT and luciferase assay

Human EVCT isolated and purified as described above (see *EVCT isolation and purification*) were plated (5×10^5 cells per well) on Matrigel-coated Falcon six-well cultures dishes. Transfection was performed after 18 h of culture by using the TransIT-LT1 transfection reagent (Mirus, Madison, WI) as recommended by the manufacturer. Briefly, the cells were cotransfected in complete medium containing 1% FCS for 6 h with 3 μg of the reporter gene and 2 μg of pCH110 (β-galactosidase expression vector, Pharmacia Biotech, Inc., Piscataway, NJ). The reporter gene carries the luciferase coding sequence under the control of a six-repeat sequence of the lactogenic hormone response element (LHRE), followed by the minimal thymidine kinase promoter (45, 46). The sequence of a single LHRE DNA element is 5′-CTGCAGTGTGGACTTCTTGGAATTAAGGGACTTTTGCTGCAG-3′, with the Stat5 consensus binding sequence underlined. hPGH (100 or 500 ng/ml) was then added for an additional 18 h of culture period. Cells were lysed in 200 μl lysing buffer [25 mM Tris/H_3PO_4 (pH 7.8), 10 mM $MgCl_2$, 1 mM EDTA, 15% glycerol, 1% Triton X-100], and luciferase and β-galactosidase activities were measured as follows. For luciferase, 400 μl lysing buffer (without Triton) containing 1 mM dithiothreitol was added to 100 μl cell lysate, and luminescence was measured after addition of 100 μl of the same buffer containing 200 mM ATP and 100 μg/ml sodium luciferin (Sigma) using a luminometer (Lumat LB 9501, Berthold Evry, France). To measure β-galactosidase activity, 250 μl of 60 mM Na_2HPO_4 (2 H_2O), 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ (7 H_2O) (pH 7) containing 3.5 μl/ml β-mercaptoethanol was added to 80 μl cell lysate and incubated at 37 C for 2 h with 100 μl *ortho*-nitrophenyl galactopyranoside (4 mg/ml) in 100 mM phosphate buffer (pH 7) before reading the OD at 420 nm. Arbitrary luciferase units were normalized for β-galactosidase activity, and results were expressed relative to untreated controls.

Statistical analysis

Results are shown as means $\pm$ SEM of at least four independent experiments with individual placentas, each run in duplicate. Statistical analysis was performed using the Stat View F-4.5 software package (Abacus Concepts, Inc., Berkeley, CA). The effects of treatments on the EVCT invasion index were tested using the nonparametric Kruskall-Wallis (ANOVA) test for multiple comparisons and the Mann-Whitney *U* test for pairwise comparisons. *P* values < 0.05 were considered significant.

Results

In vitro model of EVCT invasion (Fig. 1)

Figure 1A (*left*) shows an invasion chamber with EVCT plated on a Matrigel-coated filter. After 30 min of plating, cells were phenotyped as EVCT by immunohistochemical staining with CK7 antibody, W6/32 antibody against HLA class I molecules [including HLA-G, HLA-C and HLA-E expressed by EVCT (47, 48)], and ASO2 antibodies. Purity of the cell population, assessed by positive immunostaining with CK7 (Fig. 1B, e) and W6/32 (Fig. 1B, a) antibodies and negative immunostaining with AS02 antibody (not shown), was more than 95% (95–100% from one experiment to another). Fibroblast contamination was less than 1% (ASO2-positive cells; not shown). After 3 h of plating, including a medium change after 2 h, all these cells expressed α5-integrin subunit (Fig. 1B, i). After 34 h of culture, EVCT started to move to the lower face of the filter (Fig. 1A, *right*). Preliminary migration assays, including in 1% FCS, showed that maximal EVCT invasion occurred at 72 h (not shown), and this time point was used for subsequent experiments.

Autocrine regulation of EVCT invasion (Fig. 2)

EVCT were plated on Matrigel-coated filters (2.5×10^5 cells), and the inserts were placed in three types of wells: normal (control), cell-free wells coated with Matrigel (Matrigel control), and wells coated with Matrigel, on which EVCT from the same preparation had been plated at the same time at two cell densities (1.5 or 4.5×10^5; Fig. 2, *top*). After 72 h of culture, the EVCT migration index was similar in control wells and in Matrigel control wells. The migration index of EVCT grown in inserts placed in EVCT-plated wells was significantly higher than control values. The number of EVCT on the lower face of the filters increased with the number of EVCT plated on the bottom of the wells (Fig. 2, *bottom*; $P < 0.01$).

Expression of hPGH by EVCT (Figs. 3 and 4)

Expression of GHv mRNA in EVCT (Fig. 3A). Total RNA prepared from EVCT collected before plating (0 h) and after 72 h of culture was reverse transcribed and analyzed by real-time quantitative RT-PCR. GHv transcript levels were similar at the two time points.

Immunodetection of hPGH in cultured EVCT. hPGH immunostaining was detected in isolated EVCT at each time of culture considered. Figure 3B (a), shows hPGH signal in invasive EVCT at 72 h of culture. Immunostaining was also detected in EVCT observed after 30 min and 15, 48, and 60 h of culture (data not shown).

Immunodetection of hPGH in situ (Fig. 4)

Paraffin sections of first-trimester anchoring villi were subjected to immunohistochemical analysis with antibody against hPGH. hPGH immunoreactivity was detected in the villous trophoblasts at the syncytiotrophoblast level (Fig. 4B, *left*, *) and in invasive IVECT (Fig. 4B *left*, *white triangles*). A relatively higher level of GH expression was present in the syncytiotrophoblast, compared with invasive EVCT. Very faint, even absent signal, was observed in the PEVCT of the column (Fig. 4B, *left*, *black arrow*). CK7 immunostaining was used as a specific trophoblast marker (Fig. 4A, *left*).

EVCT hPGH content and hPGH production (IRMA). hPGH was detected and quantified in EVCT before plating. The mean

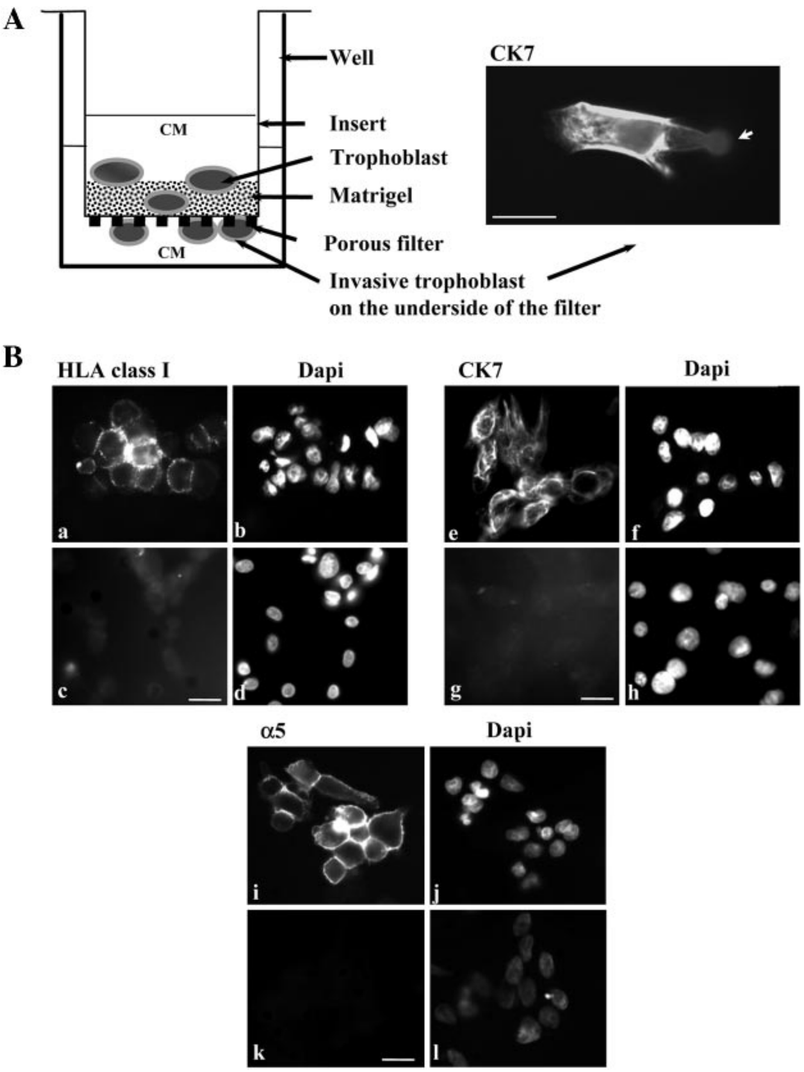

FIG. 1. *In vitro* model of trophoblast invasion. A, *In vitro* model used to study trophoblast invasion (*left*). The culture chamber used for the invasion assay consisted of a plastic well containing an insert with a Matrigel-coated filter. EVCT were plated on the filter and cultured for 72 h (CM, culture medium). Invasive EVCT crossing the filter through pores (*arrow*) were visualized on the underside of the filter by CK7 immunostaining (*right*) and scored. B, EVCT immunophenotyping. Immunodetection of HLA class I (a) and CK7 (e) was performed on cells 30 min after plating. Immunodetection of α5-integrin subunit (i) was performed 3 h after plating. No staining was observed when EVCT were incubated with the IgG isotype control (c, g, and k). Nuclei were stained with DAPI (b, d, f, h, j, and l). *Scale bar*, 30 μm.

cellular hPGH content was 47 $\pm$ 22 pg/10^6 cells (mean $\pm$ SEM; n = 6). hPGH was detected in EVCT culture medium after 72 h of culture without medium changes (mean concentration, 235 $\pm$ 90 pg/ml per 2.5 $\times$ 10^5 cells; mean $\pm$ SEM; n = 6).

hGHR expression by EVCT (Figs. 4 and 5)

HGHR mRNA (Fig. 5A). Real-time PCR was used to detect hGHR transcripts in the same RNA samples as those used for GHv mRNA detection. EVCT expressed hGHR transcripts at both 0 and 72 h of culture. The expression level tended to decrease between 0 and 72 h of culture, but the difference was not significant (GHR mRNA/PO arbitrary units, 6 $\pm$ 2.5 *vs.* 3.7 $\pm$ 0.9, respectively; n = 7).

Expression of the truncated isoform of human GHR in EVCT (Fig. 5B)

RNA isolated from EVCT collected at time zero were reverse transcribed and analyzed by semi-nested PCR. As shown in Fig. 5B, full-length (l; 118 bp) and truncated (tr; 92 bp) hGHR isoforms were detected in EVCT.

Immunodetection of hGHR in cultured EVCT (Fig. 5C)

hGHR-immunostained cells were observed on the upper side of the filter at each culture time studied (30 min and 15, 48, 60, and 72 h; 30 min and 15 and 60 h not shown). Cells were organized as clusters and displayed various labeling intensities. Figure 5C (a) shows such a cluster observed after 48 h of culture; cells leaving the cluster to invade Matrigel were more intensely stained (*arrow*). At 72 h, most immunostained cells were observed on the upper side of the filter; invasive EVCT with pseudopods engaged in pores were strongly stained (Fig. 5C, c). EVCT, which had completely invaded Matrigel and crossed the filter, showed little or no staining (Fig. 5C, e).

Immunodetection of hGHR in situ (Fig. 4)

Paraffin sections of first-trimester anchoring villi were subjected to immunohistochemical analysis with anti-hGHR. GHR immunoreactivity was detected in the villous trophoblasts (cytotrophoblast and syncytiotrophoblast) bordering the villi (Fig. 4C, *left*, *), in PEVCT of the column (Fig. 4C, *left*,

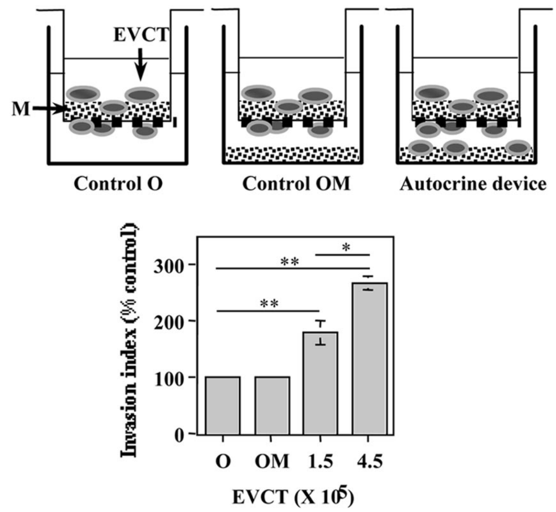

FIG. 2. Autocrine regulation of EVCT invasion. *Top*, EVCT (2.5×10^5 cells) were plated in inserts on Matrigel-coated filters. The inserts were then placed in normal wells (control O), in Matrigel-coated wells without cells (Control OM), or in Matrigel-coated wells with EVCT plated on the bottom (1.5 or 4.5×10^5 cells prepared from the same placenta; Autocrine device). *Bottom*, After 72 h of culture, EVCT that had moved to the underside of the filters were scored after CK7 immunostaining. Results are expressed as the percentage of cells scored on the underside of the filter insert in control wells (invasion index). Values represent the mean of duplicate determinations for four independent cultures established from different placentas. *, $P < 0.05$; **, $P < 0.001$.

black arrow) and in invasive IEVCT (Fig. 4C, *left*, *white triangles*).

Activation of JAK2-Stat5 cascade by hPGH in EVCT (Fig. 5D)

To investigate the ability of hPGH to activate the JAK2-Stat5 signaling pathway in EVCT, we used EVCT transiently cotransfected with the LHRE promoter luciferase construct along with the β-galactosidase construct to correct for variations in transfection efficiency. As shown in Fig. 5D, treatment of these cells with 100 ng/ml hPGH induced a 4-fold increase in luciferase/β-galactosidase activities ($P < 0.05$; n = 3). Higher concentration (500 ng/ml) had no significant effect.

Stimulation of EVCT invasion by hPGH and hGH (Fig. 6)

The effects of hPGH and hGH on cell invasiveness were tested by adding each hormone in culture media, at concentrations ranging from 0.2–100 ng/ml. As shown on Fig. 6, both hPGH (Fig. 6A; n = 7) and hGH (Fig. 6B; n = 5) dose-dependently enhanced the number of cells on the lower face of the filter ($P < 0.01$), with biological responses exhibiting typical bell-shaped curves. Maximal activity was achieved at 20 ng/ml for both hormones. A comparative study of the effect of both hormones at this concentration (20 ng/ml) on EVCT invasion index showed that hPGH was significantly more efficient than hGH (invasion indexes: hPGH, 440 ± 114%, n = 7; hGH, 218 ± 25%, n = 10; $P < 0.05$). hPRL tested at 20 ng/ml (Fig. 5C) and 100 ng/ml (data not shown) had no effect on EVCT invasion. The effect of both hPGH and

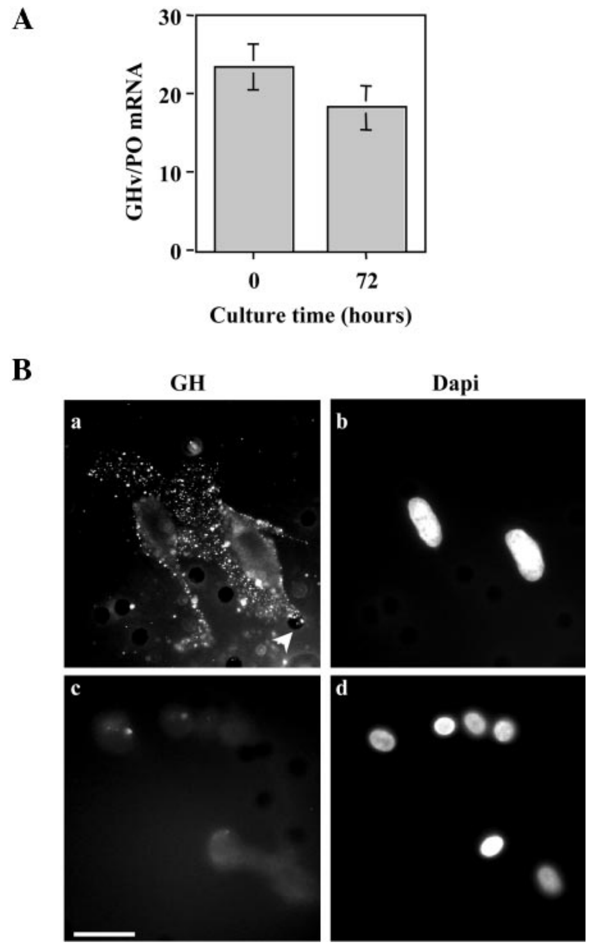

FIG. 3. hPGH expression by EVCT. A, GHv mRNA levels were measured by real-time quantitative RT-PCR in EVCT collected before plating (0 h) and after 72 h of culture. GHv mRNA levels were normalized to PO mRNA levels. Values are the mean of PCR values for seven independent experiments with cells from different placentas. B, Immunodetection of hPGH in EVCT, 72 h after plating on Matrigel-coated filters; a, hPGH expression by an invasive EVCT that has nearly completed crossing the membrane through a pore (*arrowhead*; lower side of the filter visualized). No staining was observed when cells were incubated with the IgG isotype control (c). Nuclei were stained with DAPI (b and d). *Scale bar*, 30 μm.

hGH on EVCT invasion was inhibited by the JAK2-specific inhibitor AG490. Simultaneous addition of 10 μM AG490 and 20 ng/ml hPGH to the EVCT culture medium completely inhibited the effect of hPGH on EVCT invasion ($P < 0.05$; n = 3; Fig. 6D). AG490 alone and DMSO alone had no effect on EVCT migration. In the same conditions, AG490 inhibited the effect of hGH (data not shown). As we have previously reported, EVCT cultured on Matrigel are invasive but not proliferative (40). Because GH is involved in regulating proliferation and apoptosis, we compared Ki-67 and Bcl-2 expression between control and hPGH-treated EVCT by quantitative RT-PCR. EVCT expressed very low levels of Ki-67. hPGH treatment had no effect on Ki-67 expression (Ki67/PO: control 2.1 ± 0.3 *vs.* hPGH 2.9 ± 1; n = 3). EVCT expressed Bcl-2, and hPGH treatment did not affect Bcl-2 expression level (Bcl-2/PO: control 3.13 ± 0.7 *vs.* hPGH 4.3 ± 1.6; n = 3).

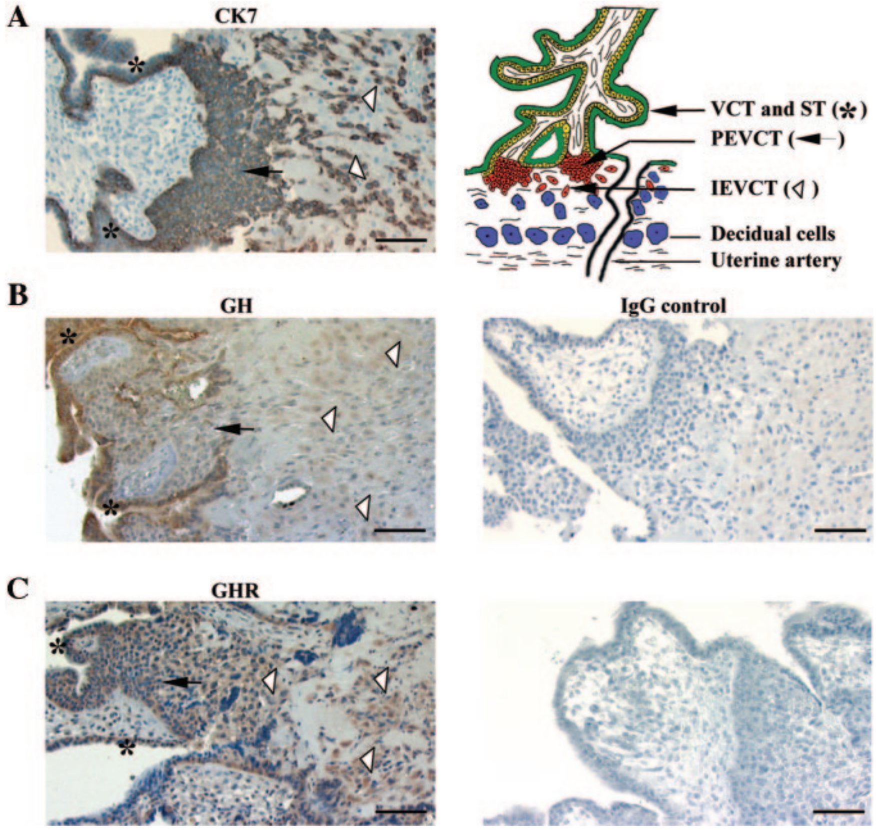

FIG. 4. GH and GHR immunodetection in first-trimester anchoring villi. Paraffin sections of first-trimester anchoring villi underwent immunohistochemical analysis with antibodies against GH, GHR, and CK7. A, *Right*, Scheme of an anchoring villus at the implantation site: villous cytotrophoblasts (*yellow*) and syncytiotrophoblast (*green*) bordering the villi (VCT and ST; ∗), PEVCT (*black arrow*) and IEVCT (*white triangles*); *left*, CK7 antibody was used as a specific trophoblast marker: VCT, ST, PEVCT, and IEVCT showed strong immunoreactivity. B, *Left*, GH immunoreactivity was detected in the ST bordering the villi (∗) and in IEVCT (*white triangles*). Staining was more pronounced in ST than in IEVCT. It was very faint, even absent, in the PEVCT of the column (*black arrow*). C, *Left*, All types of trophoblasts, VCT and ST (∗), PEVCT (*black arrow*), and IEVCT (*white triangle*) stained with GHR antibody. B and C, *Right*, No staining was observed with the IgG isotype controls. *Scale bar*, 100 μm.

Discussion

We investigated the possible involvement of hPGH in the regulation of trophoblast invasion at the fetomaternal interface in early stages of pregnancy. By using an *in vitro* model we found that hPGH and hGHR were expressed by invasive EVCT and that hPGH stimulates EVCT invasiveness.

Various cell culture models have been used to study EVCT invasion. Immortalized trophoblast cell lines are extensively used, but these cells do not express all the markers of primary EVCT (for review see Ref. 49). EVCT used for primary culture are obtained either by trophoblast outgrowth from placental explants, a method that preserves interactions between trophoblasts and adjacent stroma (50), or after enzymatic digestion of villi fragments (51). We used EVCT isolated from first-trimester placenta by a gentle enzymatic digestion procedure (40, 52) modified to further deplete fibroblasts. The EVCT thus obtained were phenotyped by CK7 and HLA class I immunostaining (47–49, 53). Isolated EVCT grown in Matrigel-coated Transwell chambers behave as previously described in anchoring villi explants models; they aggregate in clusters and, at the periphery of these clusters, the cells grew pseudopods and invade Matrigel. As we previously reported, in these culture conditions, EVCT rapidly acquire an invasive phenotype as they express specific markers such as integrin $\alpha5\beta1$ (observed after 3 h of plating in these experiments) and the protooncogene c-erbB2 (40).

We also showed that, in our culture system, isolated purified EVCT regulated their own invasiveness. This up-regulation was not a nonspecific effect explained by a higher concentration of growth factors in the culture medium because of Matrigel spread on the bottom of the well (54). It did not result either from a paracrine interaction with fibroblasts of the mesenchymal core, as described by Aplin *et al.* (33), because our EVCT preparation was depleted of fibroblasts. Because the two populations of EVCT were not in contact, the EVCT plated on the bottom of the wells therefore increased the invasiveness of EVCT plated on filters by the release of soluble factors in the culture medium. There is now evidence that soluble factors produced by EVCT such as IGF-II (55), endothelin-1 (56), urokinase-type plasminogen activator (21) or heparin-binding EGF-like growth factor (28) regulate EVCT migration/invasiveness in an autocrine manner. Among the hormones produced by trophoblasts, hPGH could be involved in an autocrine/paracrine regulation of trophoblast invasiveness, because hGH has been implicated in invasion in various cellular models.

We found that isolated EVCT expressed GHv mRNA. They also produced hPGH (detected in cell extracts) and released it in the culture medium. In our culture model, EVCT expressed hPGH as they acquired an invasive phenotype. PGH staining was observed from the first hours of the culture up to 72 h. We previously found that, 90 min after plating on Matrigel, 42% of the trophoblasts already express $\alpha5\beta1$-integrin (reflecting an invasive phenotype; (40). In this study, we found that after 3 h of culture, all EVCT expressed $\alpha5$-integrin subunit. The strongest hPGH staining was observed in invasive EVCTs that were moving through the filter pores. These data fit with a recent report that autocrine hGH production by mammary carcinoma cells transfected with the hGH gene increases the migration and invasive potential

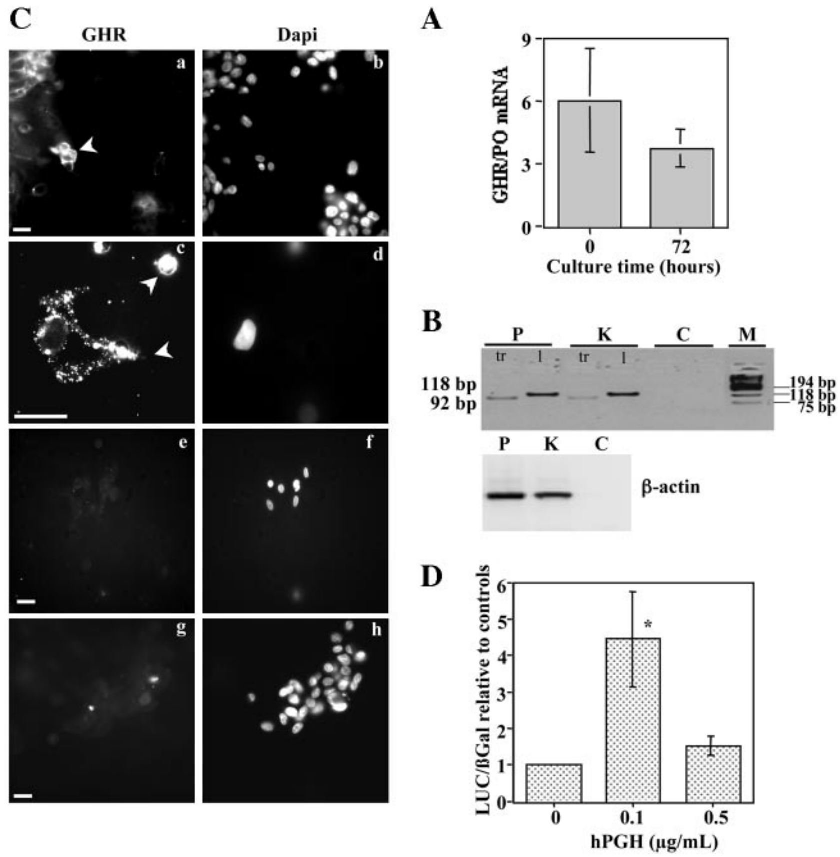

FIG. 5. GHR expression by EVCT. A, GHR mRNA levels were measured by real-time quantitative RT-PCR in EVCT collected before plating (0 h) and after 72 h of culture. GHR mRNA levels were normalized to PO mRNA levels (mean ± SEM; n = 7). B, RT hemi-nested PCR analysis of GHR and GHRtr isoforms in EVCT. RT-PCR products for full-length hGHR (l; 118 bp) and truncated hGHR (tr; 92 bp) were detected on agarose (1%) NuSieve (3%) stained with ethidium bromide. Lanes P and K were loaded with amplified cDNA derived from EVCT and kidney (positive control), respectively, and lane C with specimen control in which water replaced mRNA (M, size markers). Amplification of β-actin was used as procedural control and to check RNA integrity. C, GHR immunodetection was performed on EVCT at 48 and 72 h after plating on Matrigel-coated filters. Invasive EVCT emerging from clusters to invade Matrigel show stronger immunostaining (a, *arrowhead*, upper side of the filter observed at 48 h). c, Higher magnification of GHR expression in an EVCT starting to cross the membrane (upper side of the filter observed at 72 h) through a pore (*arrowhead*). EVCT that had already invaded Matrigel and crossed the filter show poor or no hGHR immunostaining (e, lower side of the filter observed at 72 h). No staining was observed when cells were incubated with the IgG isotype control (g). Nuclei were stained with DAPI (b, d, f, and h). *Scale bar*, 30 µm. D, Activation of the JAK2-Stat5 pathway by hPGH in EVCT. EVCT were cotransfected with a plasmid carrying the luciferase coding sequence under the control of a six-repeat sequence of the LHRE element and the pCH110 vector encoding β-galactosidase used as internal transfection control. Cells were cultured in the presence of hPGH (100 or 500 ng/ml) for 18 h, and luciferase activity was measured and normalized to β-galactosidase activity. Results are expressed relative to controls and are the mean ± SEM of three separate cultures obtained from three different placentas. *, $P < 0.05$.

of these cells (38). Immunohistochemical analysis of first-trimester anchoring villi showed GH staining in the villous trophoblast bordering the villi at the syncytiotrophoblast level and in the invasive IEVCT. This is in keeping with a study of Jara *et al.* (39) in third-trimester placenta, who detected hPGH by immunohistochemical staining in the syncytiotrophoblast and in trophoblasts located in the placental basal plate. GH immunostaining was undetectable in the PEVCT of the column. This could be because of the absence or a very low level of GH expression in these cells and/or because of the lack of sensitivity of mAb 5B4 antibody on paraffin sections (Lacroix, M. C., unpublished observation). But this antibody is the only one available that does not cross-react with the human chorionic somatomammotrophin (58).

This is the first report of hGHR mRNA and protein expression by first-trimester EVCT. Other authors have described hGHR mRNA expression in human placenta (10, 11) and also hGHR mRNA and protein expression exclusively in the syncytiotrophoblasts of third-trimester placenta (59, 60). As the culture time increased, we found that only cells leaving clusters and moving to the lower face of filters strongly expressed hGHR. In contrast, EVCT already plated on the lower face showed little or no hGHR immunostaining. hGHR mRNA expression by EVCT was not significantly lower after 72 h of culture than at time zero, probably because EVCT collected at 72 h for mRNA preparation were at different steps of the invasive process. Nevertheless, our results strongly suggest that EVCT hGHR expression is related to acquisition of an invasive phenotype. Immunohistochemical analysis of first-trimester anchoring villi showed GHR staining in the villous trophoblast (cytotrophoblasts and syncytiotrophoblast) bordering the villi, in the PEVCT of the column, and in the invasive IEVCT.

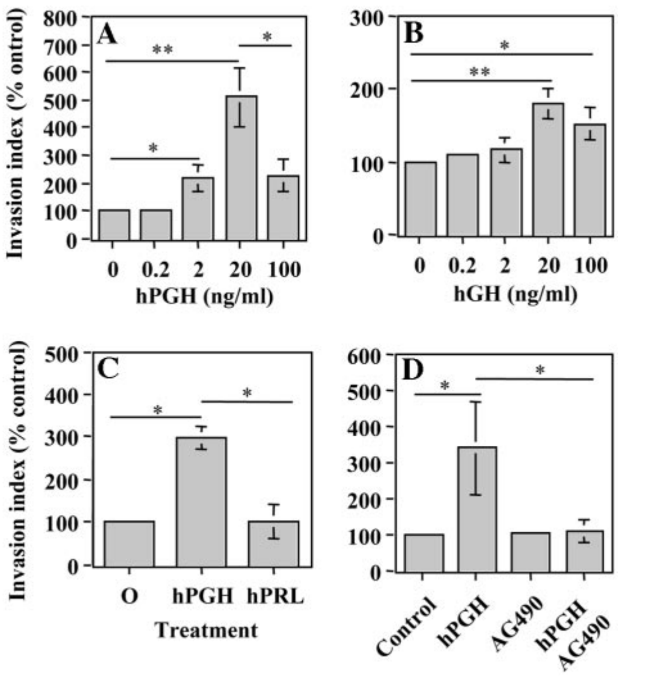

FIG. 6. EVCT invasiveness is potentiated by hPGH and hGH. A and B, EVCT (2.5×10^5 cells) were grown on Matrigel-coated filters for 72 h in the presence or absence of increasing concentrations of hPGH (A) or hGH (B). Both treatments increased EVCT invasion index, calculated as described in Fig. 2. C, Comparative effect of PGH (20 ng/ml) and hPRL (20 ng/ml) 72-h treatment on the EVCT invasion index. hPRL had no effect on EVCT invasion. D, EVCT were cultured for 72 h in the absence (control) or presence of hPGH (20 ng/ml), AG490 (10 μM), or hPGH (20 ng/ml) plus AG490 (10 μM). AG490 inhibited the effect of hPGH on the invasion index. Values are the mean ± SEM of duplicate determinations for seven (A), five (B), four (C), and four (D) independent cultures from different placentas. *, $P <$ 0.05; **, $P < 0.001$.

Alternatively spliced transcripts at exon 9 of the hGHR gene have been reported in human tissues, generating truncated hGHR isoforms devoid of signaling capacity (hGHRtr) (8, 9). We found that first-trimester EVCT expressed both full-length hGHR and hGHRtr. Our results fit with those of Dastot *et al.* (8), who reported that placental hGHRtr expression was higher than in other human tissues, but they extracted mRNA from fragments of whole-term placentas, which therefore included all cell types present in the chorionic villi (trophoblast, fibroblast, macrophage, *etc.*) and in the decidua. Dimerization of hGHRtr with a full-length hGHR inhibits GH signaling (61). Furthermore, hGHRtr generate more soluble GH-binding protein (8), which modulates GH availability for the GHR (62). hPGH binds to GH-binding proteins (63), suggesting that such regulation might take place at the maternofetal interface.

Having shown that hGHR is expressed in EVCT, we examined the ability of hPGH to activate the JAK2-Stat5 signaling pathway by binding to hGHR. We found that hPGH treatment (100 ng/ml) of EVCT transiently transfected with a Stat5 reporter plasmid (LHRE luciferase construct) resulted in transcriptional induction of the reporter gene. High concentrations of hPGH (500 ng/ml) had no effect, probably owing to a self-inhibition of hGHR dimerization by the formation of 1:1 complexes between hormone and receptor molecules (64). These data suggest that the full-length hGHR expressed in EVCT is functional and that the binding of hPGH on this receptor activates Stat5, a classical intracellular protein that is phosphorylated after JAK2 activation. These data are in keeping with the results of a previous study showing that hPGH binding to cell lines expressing GHR results in Stat5b activation (65).

Then we investigated the ability of hPGH to regulate EVCT invasiveness. Addition of hPGH to the culture medium of EVCT significantly increased their invasive potential. Pituitary GH also stimulated invasiveness, but to a lesser extent. Both hormones gave bell-shaped curves in concentration-response assays as classically reported for GH (64). AG490 inhibition of both GH effects on EVCT invasion confirms that these effects were mediated by Janus kinase activation in this cell model. Because GH promotes cell proliferation and inhibits apoptosis, we compared control and GH-treated cells for their expression level of a proliferation marker, Ki-67 (66), and of a cell survival marker, Bcl-2 (67, 68), after 72 h of culture. The results suggested that the increased number of cells invading Matrigel during GH treatment did not result from increased EVCT proliferation or survival.

In our cellular model, hPGH was more efficient than hGH in increasing EVCT invasive potential. Comparative studies of hPGH and hGH binding to somatogenic receptors have given conflicting results. hPGH is equipotent with hGH in binding hGHR or hGH-binding protein (2, 63) and displays similar somatogen biological activities (2). Interestingly, although both GH bind on placental microsomes (10), Igout (70) found that hGH poorly displace radiolabeled hPGH bound to human placental microsomes even at concentrations as high as 450 nM. These data suggest that the human placenta expresses the hGHR but also a second population of GHR more specific for hPGH. It has been reported that the human placenta expresses a unique form of hGHR mRNA deleted for exon 3 (11). However, more recent studies found that the exon 3-deleted hGHR isoform was individual specific and not tissue specific (57, 69). Although the existence of a placenta-specific GHR remains to be demonstrated, our data clearly show that hPGH is more potent than hGH to stimulate cell invasiveness in a homologous primary culture model.

In conclusion, this study provides the first clear evidence for a role of hPGH in the placenta. Our data show that hPGH stimulates EVCT invasiveness and suggests an autocrine role for hPGH at the trophoblast level. Additional studies are needed to elucidate the role of this hormone in early placentation and in pregnancy disorders associated with abnormal trophoblast invasion, such as intrauterine growth retardation or preeclampsia.

Acknowledgments

Our special thanks to Dr. Marie-Laure Sobrier for useful discussions.

Received November 30, 2004. Accepted February 8, 2005.

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