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BIOCHEMICAL CHARACTERIZATION AND MODULATION OF LH/CG - RECEPTOR DURING HUMAN TROPHOBLAST DIFFERENTIATION.

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6 text figures and 1 table

ABSTRACT

Due to the key role of the human chorionic gonadotropin hormone (hCG) in placental development, the aim of this study was to characterize the human trophoblastic luteinizing hormone/chorionic gonadotropin receptor (LH/CG-R) and to investigate its expression using the *in vitro* model of human cytotrophoblast differentiation into syncytiotrophoblast. We confirmed by *in situ* immunochemistry and in cultured cells, that LH/CG-R is expressed in both villous cytotrophoblasts and syncytiotrophoblasts. However, LH/CG-R expression decreased during trophoblast fusion and differentiation, while the expression of hCG and hPL (specific markers of syncytiotrophoblast formation) increased. A decrease in LH/CG-R mRNA during trophoblast differentiation was observed by means of semi-quantitative RT-PCR with two sets of primers. A corresponding decrease (~ 60%) in LH/CG-R protein content was shown by western-blot and immunoprecipitation experiments. The amount of the mature form of LH/CG-R, detected as a 90-kDa band specifically binding ^{125}I-hCG, was lower in syncytiotrophoblasts than in cytotrophoblasts. This was confirmed by Scatchard analysis of binding data on cultured cells. Maximum binding at the cell surface decreased from 3511 to about 929 molecules/seeded cells with a Kd of 0.4 - 0.5 nM. Moreover, on stimulation by recombinant hCG, the syncytiotrophoblast produced less cyclic AMP than cytotrophoblasts, indicating that LH/CG-R expression is regulated during human villous trophoblast differentiation.

INTRODUCTION

Human chorionic gonadotropin (hCG) belongs to a family of glycoprotein hormones, which includes lutropin (LH), thyrotropin (TSH) and follitropin (FSH). These hormones composed of two non-covalently linked subunits, alpha (α) and beta (β), are active on bicatenary form. The α-subunit is common to all glycoprotein hormones, whereas the β-subunits confer the hormonal specificity (Pierce and Parsons, 1981). Alpha hCG is encoded by a single gene and ßhCG by six genes, one of which, *CGß5*, is predominantly expressed in the placenta (Bo and Boime, 1992). HCG is essential for the initiation and maintenance of early pregnancy. After implantation, hCG is produced by the placenta and mainly by the trophoblast (Hoshina et al., 1985; Kliman et al., 1986; Muyan and Boime, 1997, Handschuh et al., 2006). It is used as a diagnostic marker of pregnancy.

The human placenta is characterized by extensive invasion of the trophoblast in the maternal uterus, creating direct trophoblast contact with maternal blood (haemochorial placentation). In early pregnancy, mononuclear cytotrophoblasts (CT) proliferate and invade the maternal endometrium to form the anchoring villi. (Aplin, 1991). Cytotrophoblasts also differentiate into a multinucleated continuous layer known as the syncytiotrophoblast (ST). This cell layer, which covers the chorionic villi, is bathed by maternal blood in the intervillous spaces from early gestation (Richard, 1961; Midgley et al., 1963; Boyd and Hamilton, 1970; Benirschke and Kaufmann, 2000). This syncytiotrophoblast is multifunctional, but its primary functions are exchange of oxygen, nutriments, removal of waste products and hormone production. The syncytiotrophoblast secretes hCG in large amounts, directly into the maternal blood bathing the chorionic villi in the intervillous space.

The mechanisms underlying villous trophoblast differentiation remain largely to be explored. Syncytiotrophoblast formation *in vivo* and *in vitro* arises from villous cytotrophoblast fusion and differentiation. Several factors modulate villous trophoblast differentiation, including EGF (epidermal growth factor) and EGF receptor expression (Morrish et al., 1987; Alsat et al., 1993), hypoxia (Alsat et al., 1996), cAMP-dependent protein kinase (PKA) (Keryer et al., 1998), granulocyte-macrophage stimulating factor (Garcia-Lloret et al., 1994), transforming growth factor β (TGFβ) (Morrish et al.,

1991) and oxidative stress due to overexpression of copper zinc superoxide dismutase (Frendo et al., 2000a, 2001). The molecular mechanisms underlying trophoblast membrane fusion are poorly understood. Proteins involved in cell adhesion (cadherin 11) (Getsios and MacCalman, 2003) and cell-cell communication (connexin 43) (Frendo et al., 2003a) are known to be directly involved. We recently demonstrated the direct involvement of syncytin I, a human endogenous retroviral envelope glycoprotein (Frendo et al., 2003b), and the presence of syncytin 2, restricted to some villous cytotrophoblasts (Malassiné et al, 2006).

Several studies suggest that hCG stimulates villous trophoblast differentiation by acting on the LH/CG-R (Shi et al., 1993; Cronier et al., 1994; Yang et al., 2003). This receptor, which has seven transmembrane domains, belongs to the superfamily of G protein-coupled receptors (Pierce and Parsons, 1981; Loosfelt et al., 1989; McFarland et al., 1989; Minegishi et al., 1990). The LH/CG receptor gene has been cloned in pig, mouse, rat and human; in humans it is composed of 11 exons and 10 introns, and its coding region is over 60 kb long (Segaloff and Ascoli, 1993). HCG binding to its receptor activates adenylate cyclase, phospholipase C and ion channels, which in turn control cellular cAMP, inositol phosphates, Ca^{2+} and other secondary messengers (Gudermann et al., 1992; Hipkin et al., 1992).

The presence of LH/CG-R in human placenta was first described by Alsat (Alsat and Cedar, 1974) and has since been confirmed by other authors (Reshef et al., 1990; Lei and Rao, 1992). Inhibition of LH/CG-R expression by specific antisense oligodeoxynucleotides during cytotrophoblast culture results in time- and concentration-dependent inhibition of cytotrophoblast differentiation, showing that hCG, *via* its receptor, is an autocrine and paracrine regulator of human placental syncytiotrophoblast formation (Yang et al., 2003).

Most of the studies actually done, have used transfected cells with cDNA from LH/CG-receptor in rat or mouse models. In human, the characterization and the modulation of LH/CG-R expression during syncytiotrophoblast formation is poorly documented. Here we used the physiological model of cultured primary human trophoblasts (Kliman et al., 1986; Frendo et al., 2000b), in which isolated mononuclear cytotrophoblasts differentiate and fuse to form a syncytiotrophoblast, which secretes large amounts of hCG and other pregnancy-related hormones. We

used various methodological approaches to characterize the hCG/LH receptor, and observed its down-regulation during villous trophoblast differentiation. This was confirmed by *in situ* immunolocalisation of the hCG receptor in sections of human placenta.

MATERIALS AND METHODS

Placental tissue collection and trophoblast cell culture

These studies were performed in agreement with our local ethics committee and with written informed consent of patients. Third trimester placentas were obtained immediately after iterative Caesarian section from healthy mothers delivered at 35-39 weeks of amenorrhea. First trimester placentas (7-12 weeks of gestation) were collected following legal voluntary interruption of pregnancy from women who gave their written informed consent. Cytotrophoblasts were isolated as previously described (Alsat et al., 1993). After sequential trypsin (0.25%)/DNase I digestion followed by Percoll gradient centrifugation (Frendo et al., 2003a), the cells were further purified by negative selection to obtain a trophoblast preparation not contaminated by other cells, by using a monoclonal anti-human leukocytic antigen A, B and C antibodies (W6-32HL, Sera Lab, Crawley Down, UK) according to a published method (Schmon et al., 1991; Cronier et al., 2002). This antibody reacts with most cell types (e.g. macrophages, fibroblasts, extravillous trophoblasts) but not with villous cyto- or syncytiotrophoblast. Cytotrophoblasts were diluted to a final density of $2.7x10^6$ cells in 3 ml of minimum essential medium (MEM) containing 10% fetal calf serum (FCS). Cells were plated in 60-mm plastic dishes (TPP, Trasadingen, Switzerland) and incubated at 37°C in 5% CO_2. Cytokeratin 7 immunocytochemistry was performed to confirm the cytotrophoblastic nature of the attached cells: about 95-98% of the cells were positively stained.

Hormone assay

The hCG concentration was determined in culture medium after 24 and 72 hours of culture by using an enzyme-linked fluorescence assay (Vidas System, BioMerieux, Marcy l'Etoile, France) with a

detection limit of 2 mU/ml. The hPL concentration was determined in 4-fold-concentrated conditioned medium by using a method (Amerlex IRMA, Amersham Pharmacia Biotech) with a detection limit of 0.5 µg/ml. All reported values are means ± SEM of triplicate determinations.

Immunohistochemistry

Placental samples were obtained after first-trimester abortion. They were fixed by incubation in 4% formalin for 4 to 12 h at room temperature and then embedded in paraffin, dewaxed in xylene and rehydrated in ethanol/water. Immunostaining was performed with a universal streptavidin-peroxidase immunostaining kit (Peroxidase, Dako LSAB®+Kit, DAKO©, Glostrup, Denmark). Non-specific binding was blocked by incubation for 5 min in a blocking reagent containing 3% H_2O_2 and then in 3% serum albumin in PBS for 30 min. The sections were incubated with the primary antibody for 30 min at room temperature. The primary antibodies (table 1) were polyclonal anti-human LH/CG-R (LHR-K15, Santa Cruz Biotechnology Inc, CA, USA, at 2 µg/ml), monoclonal anti-cytokeratin 7 (M7018, DAKO©, Glostrup, Denmark, at 1 µg/ml), and polyclonal anti-hCG (A0231 against the beta subunit of hCG, DAKO©, Glostrup, Denmark, at 2 µg/ml). Sections were washed in PBS and incubated with a biotinylated secondary antibody for 15 min. They were then washed three times in PBS and incubated with streptavidin conjugated to horseradish peroxidase for 15 min. The sections were washed in PBS and staining was detected by incubation for 30 seconds with the DAB (3,3'-diaminobenzidine) chromogen. Controls were performed by incubating the sections with nonspecific IgG at the same concentration as the primary antibody. Successive pre-adsorptions of LH/CG-R antibody with trophoblastic cells in culture abrogate LH/CG-R immunodetection.

Immunocytochemistry

To detect desmoplakin, LH/CG-R, hCG, cytokeratin 7 and hPL, cultured cells were rinsed with PBS, fixed and permeabilized in methanol at -20°C for 8 min. Alternatively, cultured cells were fixed with 4% paraformaldehyde at 4°C for 20 min. After washing once with PBS, the remaining free aldehyde groups were blocked by adding 50mM NH,Cl for 10 min. A polyclonal anti-desmoplakin (AHP320, Serotec, Oxford, UK at 2.5 µg/ml), two polyclonal anti-LH/CGR (LHR-K15 and LHR-H50, Santa

Cruz Biotechnology Inc, CA, USA, at 2 μg/ml), two polyclonal anti-hCG (A0231, DAKO©, Glostrup, Denmark at 2 μg/ml and SC-7821, Santa Cruz Biotechnology Inc, CA, USA at 2μg/μl), a monoclonal anti-cytokeratin 7 (M7018, DAKO©, Glostrup, Denmark, at 2.6 μg/ml), or a polyclonal anti-hPL (A0137, DAKO©, Glostrup, Denmark, at 1.6 μg/ml) was then applied (table 1), followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG, or fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Jackson Immuno Research, Baltimore, USA at 1:150), or Alexa 488-labeled donkey anti rabbit (Molecular probes Inc, OR, USA at 1:400), or Texas red labeled donkey anti goat (Jackson Immuno Research, Baltimore, USA at 1:400), or Cy™3 goat anti-rabbit IgG, as previously described (Frendo et al., 2001). The controls, which consisted of omitting the primary antibody or applying the non specific IgG of the same isotype, were all negative.

Immunoblotting

Cell extracts were prepared as previously described (Alsat et al., 1996). Protein (70 μg) was solubilized in RIPA (radioimmunoprecipitation) buffer (50 mM Tris, 150 mM NaCl, 1% Triton X100, 1% deoxycholate, 0.1% SDS, pH: 8), and stained markers were submitted to 7.5% SDS-PAGE and transferred to nitrocellulose sheets. Membranes were immunoblotted with two polyclonal antibodies against LH/CG-R, LHR-K15 (goat anti human, Santa Cruz Biotechnology Inc, CA, USA) and LHR-H50 (rabbit anti human, Santa Cruz Biotechnology Inc, CA, USA) at 2 μg/ml each, and the specific band was revealed by chemiluminescence (West Pico Chemiluminescent, Pierce, Rockford, IL, USA) after incubation with an anti-goat or anti-rabbit peroxidase-coupled antibody (Jackson Immuno Research, Baltimore, USA). To detect actin, cytokeratin 7, hCG and hPL, we proceeded as described above, except that proteins were immunoblotted with rabbit polyclonal antibody at 0.7 μg/ml for actin (Sigma-Aldrich, MO, USA), rabbit polyclonal antibody at 0.4 μg/ml for hCG and 0.32 μg/ml for hPL (DAKO©, Glostrup, Denmark) and mouse monoclonal antibody at 0.5 μg/ml for cytokeratin 7 (DAKO©, Glostrup, Denmark). Successive pre-adsorptions of LH/CG-R antibody with trophoblastic cells in culture abrogate LH/CG-R immunodetection in western-blot analysis.

Immunoprecipitation and ligand blotting

Protein G Plus-Agarose (Immuno precipitation Reagent, Santa Cruz Biotechnology Inc, CA, USA) was pre-mixed with a polyclonal antibody against human LHCG-R (K15, Santa Cruz Biotechnology Inc, CA, USA), or without antibody. Cells (1.0×10^6/well) were seeded in six-well plates and cultured as previously described. After 24 hours of culture, cells were washed with PBS and scraped free in ice-cold RIPA buffer. After sonication, the cellular lysate and debris were separated by centrifugation at 10000 *g* for 10 min at 4°C. The supernatant was transferred to the protein G-anti-human LHCG-R immunocomplex and incubated overnight at 4°C on a rocker platform, followed by four washes in RIPA buffer. Protein was eluted by heating at 60°C for 10 min in 1X electrophoresis sample buffer (Bio-Rad laboratories, CA, USA). Aliquots were submitted to 7.5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were exposed to antibody as previously described, or the blots were incubated with ^{125}I-hCG at 10^{-11} M (PerkinElmer Life and Analytical Sciences Inc. MA, USA) for 16 h at 4°C in the absence or presence of excess unlabeled hCG at 10^{-6} M (Organon SA, Puteaux, France). The blots were washed with PBS containing 0.1% Tween 20, then dried. Bound ^{125}I-hCG was visualized by autoradiography and analyzed by Cyclone (Storage phosphorImaging System, Hewlett Packard, France).

RNA extraction

Total RNA was extracted from trophoblastic cells after 24 or 72 hours of culture by using the Trizol reagent (Invitrogen Life Technologies, CA, USA) and was stored at -80°C or at -20°C in 75% ethanol until use. The total RNA concentration was determined at 260 nm and RNA integrity was checked in 1% agarose gel. The relative LH/CG-R mRNA levels were determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The transcript level was normalized to the actin mRNA level (endogenous control).

RT-polymerase chain reaction

RNA samples were pretreated with DNAse I using the RQ1 RNase-Free DNase kit (Promega Inc, WI, USA). Briefly, we used 5 units of RQ1 RNase-free DNase per 5 micrograms of RNA, we then added RQ1 RNase-free 10x reaction buffer and TE buffer. Mixture was incubated at 37°C for 30 min and the digestion was terminated by the RQ1 DNase stop solution. DNase was then inactivated by heating at 65°C for 10 min.

Complementary DNA was synthesized from 5 µg of total RNA. The reaction mixture had a final volume of 20 µl and contained 375 mM KCl, 250 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 0.1 M DTT, 40 U of RNAsin®, 200 U of reverse transcriptase Superscript II (Invitrogen Life Technologies, CA, USA), 10 mM each dNTP and 200 ng of random primers (Invitrogen Life Technologies, CA, USA). Mixture of total RNA, DTT and random primers was heat at 65°C for 5 min. Annealing was run for 10 min at 25°C and primer extension for 50 min at 42°C. An aliquot of the reaction mixture (5 µl) was then made up to 45 µl with Taq polymerase buffer containing 1 unit of Taq polymerase Platinium (Invitrogen Life Technologies, CA, USA). Before heating to 94°C (hot-start), 50 pmol of each specific primer was added. Amplification was run for 40 cycles for LH/CG-R and for 20 cycles for actin, consisting of 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 1 min at 72°C (extension). Oligonucleotide primers specific for the coding sequence of LH/CG-R (NM_000233) were used (Fig. 3A): P1 (+): 5'-CAAGCTTTCAGAGGACTTAATGAGGTC-3'; P1 (-): 5'-AAAGCACAGCAGTGG CTGGGGTA-3'; P2 (+): 5'-TCGACTATCACTTGCCTACC-3'; P2 (-): 5'-GGAGAAGACCTTCGTA ACAT-3'; Actin (NM_001101) (+): 5'-GTGGGGCGCCCCAGGCACCA-3'; Actin (-): 5'-CTCCTTA ATGTCACGCACGATTTC-3'. Amplified products were analyzed by electrophoresis on 1.8% agarose gels and visualized by ethidium bromide staining.

Cloning and DNA sequencing of LH/CG-R from trophoblastic cells

PCR products were eluted from agarose gel by using the Macherey Nagel kit (NucleoSpin Extract II, MN, Hoerdt, France) and purified DNA fragments were cloned into the pCRII-TOPO vector by using the TOPO-TA Cloning kit (Invitrogen Life Technologies, CA, USA). Positive clones were selected by PCR and were sequenced by Genome Express (Meylan, France). Both strands of DNA fragments were sequenced, using Ml3 reverse and Ml3 forward primers.

Intracellular cAMP determination

Cells (1.0 x 10^6/well) were seeded in six-well plates and cultured as described above. After 24 h or 72 h, cells were preincubated with 10mM IBMX (3-isobutyl-1methylxanthine) for 1 hour to prevent cAMP degradation and were stimulated for 20 min with 10^{-8} M hCG (C6322, Sigma-Aldrich, MO, USA). Cells were frozen on dry ice and cAMP was extracted with ice-cold 65% ethanol. The extracts were dried and kept at -20°C until use. Cyclic AMP was assayed after acetylation by using a method (Amersham Biosciences, NJ, USA) based on the competition between unlabelled cAMP and a fixed quantity of ^{125}I-labelled cAMP for binding to a cAMP-specific antibody. Bound antibody was separated from free fraction by magnetic separation with a second antibody Amerlex^{TM}-M preparation that is bound to magnetizable polymer particles. Separation of the antibody bound fraction is effected either by magnetic separation of the Amerlex^{TM}-M suspension or decantation of the supernatant. The concentration of unlabelled cAMP in the sample was then determined by interpolation from a standard curve.

Binding assay and Scatchard analyses

Trophoblastic cells (1.0 x 10^6/well) were seeded in six-well plates and cultured as described above. After 24 h or 72 h of culture the cells were washed five times and cultured in DMEM, 0.1% BSA for 2 hours to dissociate any bound endogenous hCG. The cells were then washed and placed in 1 ml of DMEM containing 0.1% BSA and 1 mM HEPES, pH 7.3. Cells were incubated for 30 min at room temperature with 0.5 nM ^{125}I-hCG and an increasing concentration of unlabelled hCG (from 10^{-12} M to 10^{-8} M, C6322, Sigma-Aldrich, MO, USA) on a shaker platform at 50 cycles/min. At the end of the incubation period the cells were washed and scraped free, and bound radioactivity was counted. Each assay was performed in triplicate. Data were analyzed by using the LIGAND fitting program (version 4.97) (Munson and Rodbard, 1980). For Scatchard analysis, the results showing the number of labeled molecules associated with the cellular membrane were expressed in a number of molecule associated per seeded cells. For comparison between CT and ST experiments, nuclei were counted at 24h and 72h

of culture after staining with DAPI, as previously described in the immunocytochemistry section. We did not observe difference between the number of nuclei at 24h and 72h of culture (CT are non proliferative cells and apoptosis or cellular loss account for about 4% (data not shown).

^{125}I-labeled hCG was prepared using chloramine T as oxidant (Hunter and Greenwood, 1962). In a final volume of 20 µl, hCG (5 µg, 4.4 µM) was added to 0.5 mCi of Na^{125}I (Perkin-Elmer Life and Analytical Sciences, MA, USA; 17.4 Ci/mg, 11.5 µM) neutralized with 0.1 M Mops and poly(ethylene glycol) 1000 (1%). The reaction in 25 mM Mops buffer pH 7.2 was started by adding 100 µM chloramine-T for 3 min at room temperature and was stopped by adding 120 µM sodium bisulfite for 3 min and 2 mM NaI for 1 min. The volume was then adjusted to 0.5 ml with Mops-buffered saline (20 mM Mops, 130 mM NaCl, pH 7.2) containing 1 mg/ml BSA. Iodinated-hCG was desalted on a PD10 Sephadex G25-M column in the same buffer. Specific activity of ^{125}I-hCG was 2.1-2.4 Ci/µmole corresponding to about 1 atom of iodine per molecule hCG.

Statistical analysis

We used the StatView F-4.5 software package (Abacus Concepts, Inc., CA, USA). Values are reported as means ± SEM. Significant differences ($p<0.05$) were identified by analysis of variance (ANOVA).

RESULTS

Human villous trophoblast differentiation in vitro

We used the primary cell culture model of villous cytotrophoblasts isolated from term placenta (Kliman et al., 1986; Alsat et al., 1991). Figure 1 shows purified cytotrophoblasts cultured on plastic dishes for 24 and 72 hours. Mononuclear cytotrophoblasts fused and formed multinucleated syncytiotrophoblasts, 72 hours after plating (Kliman et al., 1986). Syncytiotrophoblast formation was associated with a significant increase in hCG and hPL levels in the culture medium (Fig. 1 I). Concomitantly, immunostaining for hCG (Fig. 1 A and B) and hPL (Fig. 1 F and H) showed an increase in intensity during *in vitro* syncytiotrophoblast formation. HPL, expressed mainly by the

syncytiotrophoblast (Handwerger, 1991), was detected by immunostaining at 72 h (Fig. 1 D and H) but not at 24 h (Fig. 1 C and F). Immunostaining of cytokeratin 7, expressed by trophoblastic cells (Blaschitz et al., 2000), was positive at 24 h (Fig. 1 C and E) and 72 h (Fig. 1 D and G).

These results showed that differentiation of isolated cytotrophoblasts into a syncytiotrophoblast is associated with an increase in the expression and secretion of hCG and hPL, hormones mainly synthesized by the syncytiotrophoblast.

Decrease in LH/CG-R protein levels during in vitro trophoblast differentiation

As shown in figures 2 A and B, LH/CG-R was expressed by cultured cytotrophoblasts. The LH/CG-R immunostaining shown in this figure was obtained with the polyclonal antibody LHR-K15. Another antibody (LHR-H50) gave the same results (data not shown). LH/CG-R was expressed in both cytotrophoblasts (24 h) and syncytotrophoblasts (72 h), with punctuate immunolabeling. LH/CG-R immunostaining appeared stronger in cytotrophoblasts than in syncytiotrophoblasts. Double immunostaining for LH/CG-R (LHR-50) and hCG (C-20) of trophoblasts cultured for 48 hours (Fig. 2 C and D respectively, merge Fig. 2 E) illustrated the dynamics of the process. A mononucleated cytotrophoblast (Fig. 2 C arrow head) expressed LH/CG-R, whereas aggregated trophoblasts showed and heterogenous immunostaining of both LH/CG-R and hCG (Fig. 2 E). To validate this observation, western-blot analysis was performed on extracts of cytotrophoblasts (24 h) and syncytiotrophoblasts (72 h) (Fig. 3 A). At 24 h and 72 h of culture, two major bands with molecular masses (estimated from SDS gels) of 65-75 kDa and 85-95 kDa were observed, as described in other cellular models and in mammalian cells transfected with LH/CG-R cDNA. In the literature, the 85-95 kDa band corresponds to the mature LH/CG-R present at the cell surface, and the 65-75 kDa band is the precursor of the cell-surface receptor (for review see Ascoli et al., 2002).

Our results show that the expression of the mature LH/CG-R and its precursor (respectively designated m and p in Fig. 3 A) decreases during cytotrophoblast differentiation. At the same time, actin expression remains constant. Normalization of mature LH/CG-R protein expression to actin expression showed a significant decrease ($58.6 \pm 6.7\%$; $p < 0.0001$) in cell-surface receptor expression. We obtained similar results with the two antibodies used (LHR-K15 and LHR-H50).

Interestingly, in the same cellular extracts, the decrease in precursor and mature LH/CG-R expression coincided with an increase in hCG and hPL expression (Fig. 3 A).

To further characterize LH/CG-R expression during trophoblast differentiation, we performed immunoprecipitation (IP) with anti-human LH/CG-R antibody (K15). Cellular extracts were purified by immobilized anti-receptor antibody (IP) and eluates were analyzed by SDS-PAGE and immunoblotting using the receptor-specific antibody (K15). A 90 kDa band corresponded to the mature form of LH/CG-R (m), and a major band of 75 kDa corresponded to the precursor (p).

To determine which molecular form of the receptor bound the hormone, we used ^{125}I-hCG in ligand-blot experiments (Fig. 3 B). Incubation of the IP blot with ^{125}I-hCG (10^{-11}M) revealed a major band of 90 kDa. This band was absent when the blot was incubated with an excess of unlabeled hCG (10^{-6} M), showing that the 90-kDa LH/CG-R specifically binds the hormone. In these conditions, ^{125}I- hCG binding to the mature form of the receptor (90 kDa) was lower in the syncytiotrophoblast than in cytotrophoblasts.

***Decrease in LH/CG-R mRNA expression during in vitro trophoblast differentiatio*n**

We conducted semi-quantitative RT-PCR experiments with two different sets of primers (P1 and P2) (for primer positions see Fig. 4 A). To avoid contamination by genomic DNA, each primer was located on a separate exon and RNA extracts were pretreated with DNAse I.

As shown in figure 4 B, amplification of the 647-bp and 282-bp fragments, obtained with primers P1 and P2 respectively, indicated that LH/CG-R mRNA was significantly less abundant in the syncytiotrophoblast (72 h) than in cytotrophoblasts (24 h). No significant difference was noted in the actin mRNA level. We obtained similar results with the two sets of primers. The amplification products were then purified from the agarose gel and cloned into the pCRII-TOPO vector. Sequencing confirmed that both the 647-bp and 282-bp fragments were part of the human LH/CG receptor. Normalization of LH/CG-R mRNA to actin mRNA after RT-PCR with primer sets P1 and P2 showed a significant decrease in LH/CG-R mRNA levels during differentiation (Fig. 4 C). With the P1 primers, LH/CG-R mRNA levels fell from 0.33 ± 0.01 at 24 h to 0.13 ± 0.01 at 72 h ($p < 0.0001$). A similar decrease was observed with the P2 primers (from 0.82 ± 0.02 at 24 h to 0.36 ± 0.01 at 72 h; $p <$

0.0001). Although the amplification product obtained with primers P2 appeared to be at least twice as abundant as that obtained with primers P1 (probably because the P2 amplicon is about half the length of the P1 amplicon), the size of the decrease in LH/CG-R levels at 72 h was similar with the two primer sets (respectively 2.5- and 2.3-fold).

Decrease in ^{125}I-hCG binding to cell-surface LH/CG-R during in vitro trophoblast differentiation

To confirm the decrease in LH/CG-R mRNA and protein levels, we performed binding saturation experiments with iodinated hCG at 24 h and 72 h of culture (Fig. 5). Scatchard analysis of binding data showed that the number of molecules bound per seeded cell at 24 h of culture (cytotrophoblasts) was 3511±693. After differentiation, at 72 h of culture, this number fell significantly (p=0.02) to 929±583. No significant difference in Kd values was observed between 24 h (0.5±0.1 nM) and 72 h (0.4±0.1 nM).

LH/CG-R stimulation during in vitro trophoblast differentiation

In order to confirm the reduction in functional mature hCG receptor expression at the syncytiotrophoblast surface compared to the cytotrophoblast surface, we determined cAMP production in response to an effective hCG concentration for 20 min (Fig. 6). As cAMP is a second messenger for hCG signaling in trophoblastic cells, the decrease in LH/CG-R transcript and protein levels ought to be associated with a decrease in cAMP production. Determination of the most effective hCG concentration was carried out by stimulating trophoblasts with 10^{-12} M to 10^{-6} M hCG; 10^{-8} M hCG was the most effective concentration (data not shown). As shown in figure 6, hCG-stimulated cAMP production by trophoblasts was higher at 24 h than at 72 h of culture (p= 0.0021). Trophoblast stimulation by hCG (10^{-8} M) at 24 h of culture induced at least a 2-fold increase in cAMP production compared to the basal level (p= 0.0016), but did not induce detectable cAMP production at 72 h of culture (p= 0.7644). In contrast, epinephrine (which stimulates camp production and is used as a positive control) induced similar cAMP production at 24 h and 72 h of culture, indicating that the cells were functional and that the decrease in cAMP production observed at 72 h was not due to a defective cAMP pathway.

Immunolocalization of LH/CG-R in villous sections

These *in vitro* findings were confirmed by examining placental LH/CG-R expression *in situ*, on villous sections. First-trimester placenta was chosen because cytotrophoblasts are more abundant than at other stages of pregnancy and form a continuous layer.

LH/CG-R was detected in villous cytotrophoblasts and syncytiotrophoblasts. Use of a polyclonal antibody raised against the extracellular domain of human LH/CG-R showed that LH/CG-R is mainly expressed by the cytotrophoblast layer (Fig. 7 A). Weaker staining was observed in the syncytiotrophoblast (ST). LH/CG-R was also expressed by perivascular cells (VC) of the villous core. We obtained similar results with two other monoclonal antibodies (LHR 29 and LHR 1055) which recognize two different epitopes of the extracellular domain of LH/CG-R (Vuhai et al., 1990; Méduri et al. 1997) (data not shown). No staining was detected in negative control sections (Fig. 7 D). Interestingly, strong hCG immunostaining was observed in the syncytiotrophoblast (Fig. 7 B) while cytokeratin 7 was mainly located in the cytotrophoblast layer (Fig. 7 C).

Taken together, these results strongly suggest that the expression of a functional cell-surface LH/CG-R decreases during cytotrophoblast differentiation into a syncytiotrophoblast.

DISCUSSION

By using several complementary methods and a well-characterized *in vitro* model of human villous trophoblast differentiation, we clearly observed that LH/CG-R mRNA and protein expression is lower in syncytiotrophoblasts than in cytotrophoblasts and that this down-regulation is associated with an apparent decrease of receptor activation by its specific hormone. These results differ from those of two previous studies published by CV. Rao, who described stronger expression of LH/CG-R in syncytiotrophoblasts than in cytotrophoblasts (Reshef et al., 1990; Lei and Rao, 1992). This divergence may come from the use of different tools. Anti-human LH/CG-R antibodies were not available in the early 1990s, and most immunohistochemical and western-blotting studies used

antibodies raised against the N-terminal part of the rat LH/CG receptor. The amino acid sequence identity between the rat and human receptors is 85%, with the strongest similitude in the transmembrane portion of the molecule and not in the N-terminal region (Segaloff and Ascoli, 1993). Moreover, experiments involving radiolabeled probes, such as northern blotting and *in situ* hybridization, used porcine cDNA with 88% sequence identity to the human sequence. In this study, we cloned PCR fragments of the human hCG/LH receptor from villous cytotrophoblasts and used antibodies specific for the human receptor.

HCG, which is produced in large amounts by the syncytiotrophoblast, plays an important role in cytotrophoblast differentiation into syncytiotrophoblast. An increasing number of studies have investigated the central role of hCG and its receptor in the trophoblastic differentiation process. Many authors have described down-regulation of LH/CG-R expression by increasing concentrations of hCG. Indeed, exposure of ovarian or testicular cells expressing the endogenous LH/CG-R to a high concentration of hCG down-regulates cell-surface receptor expression. This coincides with a decrease in the abundance of LH/CG-R transcripts (Segaloff et al., 1990; Peegel et al., 1994; Hoffman et al., 1991; LaPolt et al., 1990; Hu et al., 1990). It is noteworthy in this respect that hCG is secreted in large amounts during syncytiotrophoblast formation. The decrease in cell-surface receptor expression was confirmed in our study by the clear decrease in cAMP production by the syncytiotrophoblast after stimulation by recombinant hCG. Interestingly, the decrease in cAMP production by the syncytiotrophoblast was not due to a loss of affinity or to weak binding between the receptor and its hormone, as we found no difference in LH/CG-R Kd values between 24 h and 72 h of culture. Moreover, Scatchard plots clearly showed that the maximum number of hCG molecules bound per seeded cell was significantly lower at 72 h of culture than at 24 h (~74%). This result confirms the decrease of LH/CG-R (~60%) observed by western-blot analysis. The difference in LH/CG-R decrease (60% *versus* 74%) may be due to the technical approaches used for the purpose. By western-blot analysis, we quantified the mature form of the LH/CG-R in proteins from total cellular extracts. In binding experiments, we used living cells, meaning that only the mature form of the LH/CG-R present at the cell surface was quantified. Some mature forms internalized or present in the endosome might not be accessible to ^{125}I-hCG.

LH/CG-R desensitization has been described in rat ovary and is accompanied by a transient loss of responsiveness to LH, the receptor being temporarily uncoupled from its Gs protein (Segaloff et al., 1990). We observed here that syncytiotrophoblast stimulation by recombinant hCG (10^{-8} M) did not induce detectable cAMP production although the cAMP pathway was functional as shown by epinephrine stimulation. This loss of responsiveness to recombinant hCG may thus be due in part to cell-surface receptor desensitization.

Western blotting showed that two major species of LH/CG-R with molecular masses of 65-75 kDa and 85-95 kDa were expressed by cytotrophoblasts and by syncytiotrophoblasts. Immunoprecipitation experiments and ligand blot analysis confirmed that the 65-75 kDa band was the intracellular precursor of the cell-surface receptor and that the 85-95 kDa band corresponded to mature LH/CG-R present at the cell surface, as shown by its ability to bind specifically labeled ^{125}I-hCG. Our results show that the expression of the mature LH/CG-R and its intracellular precursor decreased during cytotrophoblast differentiation. Furthermore, the precursor form seemed to be more strongly expressed than the mature form in trophoblastic cells. Most studies of these two forms of LH/CG-R have used mammalian cells transfected with the cDNA for the porcine, rat or human receptor (for review see Ascoli and al., 2002), but as shown here, primary cultured human trophoblasts may be an excellent model for studying the maturation of the intracellular precursor into the mature cell-surface protein. Recently, Pietila et al using transfection models have shown that regulation of the immature form into the mature form might considered be important in LH/CG-R expression (Pietila et al., 2005).

In this study, we characterized for the first time, in a human physiological model, the expression and regulation of LH/CG-receptor. We demonstrate, both *in situ* and *in vitro*, that LH/CG-R is expressed by human cytotrophoblasts and, albeit to a lesser extent, by the syncytiotrophoblast. LH/CG-R expression thus seems to be regulated during villous trophoblast differentiation, and this regulation may involve down-regulation of the receptor by its ligand. Abnormal regulation of this process might be involved in trisomy 21-associated pregnancies, in which we recently observed an abnormal glycosylated form of hCG associated with defective syncytiotrophoblast formation (Frendo et al., 2000b, 2004). Abnormal syncytiotrophoblast formation might lead to complications such as preeclampsia and intrauterine growth retardation.

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FOOTNOTES

§ These two authors contributed equally to the work.

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FIGURE LEGENDS

Fig. 1 ***In vitro*** **human villous trophoblast differentiation. (A)** and **(B):** hCG immunodetection after 24 and 72 hours of culture of villous cytotrophoblasts isolated from term placentas. At 24 hours the cells are sparse or aggregated (A). At 72 hours, they have fused to form the syncytiotrophoblast, characterized by multiple nuclei and a strong positive immunofluorescent staining for hCG (B). Nuclei were labeled with DAPI (blue fluorescence). **(C)** and **(D):** co-immunolocalization of cytokeratin 7 (in green) and hPL (in red) at 24 hours (C) and 72 hours of culture (D). Nuclei are stained blue with DAPI. HPL, known to be expressed mainly by the syncytiotrophoblast, was detected by immunostaining at 72 h **(H)** but not at 24 h of culture **(F)**. Cytokeratin 7 immunostaining, was positive at 24 h **(E)** and 72 h **(G)**. **(I)**: levels of hCG and hPL (expressed respectively in milli-international units per milliliter and micrograms per milliliter of medium) secreted into the culture medium at the indicated times. Since cells were plated in triplicate (see Experimental procedures), hCG and hPL levels were determinated for each plate. **ND**: non detectable. Results are means ± SEM of the three culture dishes. This figure illustrates one experiment representative of three. Scale for pictures A-D: 1 cm = 30 µm. Scale for pictures E-H: 0.5 cm = 30 µm.

Fig. 2 **LH/CG-R immunodetection during** ***in vitro*** **trophoblast differentiation. (A)** and **(B):** immunostaining for LH/CG-R by using the polyclonal antibody LHR-K15 raised against the human LH/CG receptor. LH/CG-R was expressed in both cyto- (A; 24h) and syncytiotrophoblasts (B; 72h), albeit more strongly in cytotrophoblasts. **(E):** co-immunodetection of LH/CG-R and hCG by using the polyclonal antibodies LHR-H50 (**C**; in green) and hCG-C20 (**D**; in red) respectively at 48 hours of culture. Single trophoblast (arrowed) was stained for LH/CG-R and aggregated trophoblasts were stained for both LH/CG-R and hCG. Nuclei were labeled with DAPI (blue fluorescence). Scale for pictures A and B: 0.5 cm = 30 µm; scale for pictures C-E: 1 cm = 15 µm.

Fig. 3 **LH/CG-R protein expression during** ***in vitro*** **trophoblast differentiation. (A)** and **(B):** Western-blot analyses **(A)** were performed using the same antibody on extracts from cytotrophoblasts

(24 h) and syncytiotrophoblasts (72 h). At 24 h and 72 h of culture, two major bands with molecular masses of 65-75 kDa, corresponding to the precursor (p) of the cell-surface receptor and 85-95 kDa, corresponding to the mature LH/CG-R (m) present at the cell surface, were observed. The histogram presents the normalization of mature LH/CG-R protein expression (m) by actin expression (43kDa) (***: $p < 0.0001$). Results are expressed as the mean ± SEM of three culture dishes. In the same cellular extracts, decrease in precursor and mature LH/CG-R expression was concomitant with an increase in hCG (38kDa) and hPL (22 kDa) expression. **(B):** immuno-precipitation and ligand-blot analysis. Cellular extracts were purified by immobilized anti-receptor antibody. Eluates were analyzed by SDS-PAGE and immunoblotting using the receptor-specific antibody. A 90 kDa band corresponding to the mature form of LH/CG-R (m) and a major band with a molecular mass of 75 kDa corresponding to the precursor (p) were observed. Incubation of the IP blot with labeled ^{125}I-hCG (10^{-11}M) revealed a major radioactive band at a molecular weight of 90 kDa, which was not detected when the blot was incubated with an excess of unlabeled hCG (10^{-6} M). Figures A and B illustrate one experiment representative of five.

Fig. 4 **LH/CG-R mRNA expression during *in vitro* trophoblast differentiation. (A):** diagram showing the seven transmembrane domains of the LH/CG-receptor and the location of the primers sets used in this study. The two sets of primers (P1 and P2) are located on the extracellular domain. P1 amplifies a fragment of 647-bp in the exons 2-9 and P2 amplifies a fragment of 282-bp in the exons 1-5. **(B):** Ethidium bromide-staining gel of one representative of five independent experiments. Semi-quantitative RT-PCR experiments with both the primers P1 and P2 shows respectively a 647-bp and a 282-bp amplified fragment. RT-PCR products were separated on 1.8% agarose gel and analysed by densitometry. Sequencing confirmed that both the 647-bp and the 282-bp fragments are part of the LH/CG receptor. **(C):** histograms represent the normalization of LH/CG-R mRNA by actin mRNA after RT-PCR with primers sets P1 (upper histogram) and P2 (lower histogram). Data are expressed as mean ± SEM of five independent experiments similar to the one shown in B. bp: base pairs; ***: $p < 0.0001$.

Fig. 5 **Scatchard analyses of ^{125}I-hCG binding to trophoblasts during *in vitro* differentiation.** Binding was performed for 30 minutes at room temperature, on cells at 24 hours (□) or 72 hours (●) of culture. The apparent dissociation constants (Kd) and the maximum number of molecules bound per mg of protein at 24 hours and 72 hours of culture were calculated by the LIGAND program (lower table). Results are expressed as the mean ± SEM of three experiments.

Fig. 6 **Intracellular cAMP production after LH/CG-R stimulation during *in vitro* trophoblasts differentiation.** Stimulation of cells at 24 hours and 72 hours of culture was performed with 10^{-8} M of hCG or with epinephrine (used as a positive control) for 20 min and compared to non stimulated cells (0). **: $p < 0.005$ and ***: $p < 0.0001$

Fig. 7 **Immunolocalization of LH/CG-R, hCG and cytokeratin 7 in villous sections. (A):** immunohistochemical staining of LH/CG-R, using the polyclonal antibody (H50) raised against the extracellular domain. Villous cytotrophoblasts (CT), syncytiotrophoblast (ST) and perivascular cells (VC) of the villous core were positively stained. **(B):** a strong immunostaining of hCG was observed in the syncytiotrophoblast. **(C):** immunostaining of cytokeratin 7 was mainly located in cytotrophoblasts layer. **(D):** No staining was observed in control sections treated with non specific isotypic immunoglobulins.