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Efficacy of a Recombinant Chimeric Anti-hCG Antibody to Prevent Human Cytotrophoblasts Fusion and Block Progesterone Synthesis

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PROBLEM: A recombinant chimeric antibody against hCG (cPIPP) has been engineered and expressed at high yield in plants. The purpose of this work was to enquire whether this antibody is competent to neutralize the bioactivity of hCG on human trophoblasts.
METHODS: Cytotrophoblast cells, isolated from term placentae were maintained in culture for 3 days in presence or absence of humanized chimeric anti-hCG antibodies. Progesterone secreted was quantitated by ELISA. Fusion and cyto-architecture of the cells was studied by light and electron microscopy. Modulation of E-cadherin was investigated using RT-PCR and immunocytochemistry.
RESULTS: Recombinant chimeric anti-hCG antibody blocked the synthesis of progesterone by trophoblasts. No fusion of cytotrophoblasts to form syncytium took place. E-cadherin, a vital cell adhesion molecule involved in cell-to-cell interaction did not show differentiation related decline in its expression in presence of the antibody.
CONCLUSION: Recombinant chimeric anti-hCG antibody (cPIPP) was effective to neutralize hCG induced bioactivities in the human derived trophoblast cells.

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INTRODUCTION

Human chorionic gonadotropin (hCG) is an early product of conception. It is synthesized by the pre-implantation embryo in culture.[1] It plays an essential role in implantation of the blastocyst; marmoset embryos exposed to anti-hCG antibodies do not implant.[2] Interception of implantation by anti-hCG antibodies is also evidenced in humans, as sexually active women immunized by an anti-hCG vaccine did not become pregnant, so long as the antibody titres remained above 50 ng/mL.[3,4] The 'protected' women had regular menstrual cycles without lengthening of the luteal phase. They ovulated normally, and no systemic side effects ascribable to circulating anti-hCG antibodies were noted. The drawback of the vaccine was that only 60–80%, of recipients generated antibodies above the protective threshold and the duration of the antibody response varied from subject to subject. In order to assure efficacy in all recipients, use can be made of preformed bioeffective antibodies, which can be administered, in adequate dose. We humanized a mouse monoclonal of high affinity and specificity for hCG.[5] This recombinant chimeric antibody has human IgG_1, as constant heavy chain and Kappa as constant light chain fused to mouse variable chains. It is expressed in tobacco leaves with a yield of 20–40 mg/kg fresh weight of leaves.[6] Provided that this antibody is bioeffective, it has potential of use for emergency contraception offering protection for 4–6 weeks without impairment of ovulation, hormonal mood changes or derangement of bleeding profiles. It

may also be of therapeutic utility in some cancers,[7,8] where hCG or its subunits are believed to exercise autocrine growth promoting effect.

This communication reports the evaluation of the bioactivity of this chimeric recombinant antibody in a relevant system investigable *in vitro*, of differentiation and fusion of human cytotrophoblasts derived from placentae. These cells make hCG, which has an autocrine effect on formation of syncytium, with enhanced synthesis of hCG and secretion of progesterone.[9–14] The effect of the chimeric antibody on these processes has been studied.

MATERIALS AND METHODS

Recombinant Humanized Chimeric Anti-hCG Antibody

The chimeric antibody (Ab) was human IgG_1, human Kappa fused to mouse variable chains. It was expressed in plants[5,6] and was purified on a Protein A matrix (Pharmacia) column. Coomasie blue stain of SDS-PAGE showed primarily the bands of heavy and light chain (Fig. 1). The association constant of the antibody for hCG was $Ka = 1.9 \times 10^{10}$ M^{-1}.[15]

Trophoblast Cells

Placentae were obtained after normal delivery from uncomplicated pregnancies. Villous cytotrophoblasts were isolated by trypsin/DNAse (Sigma-Aldrich, St Louis, MO, USA) digestion and purified using discontinuous Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) as standardized by us previously.[16] The purified trophoblast cells were plated at a density of 1.5×10^6 cells per millilitre DMEM-HG (Gibco BRL; Life Technologies, Gaithersburg, MD, USA) containing 10% heat inactivated FCS (Gibco BRL, Life Technologies) and kept at 37°C in a 5% CO_2 humified atmosphere for 4 days. The culture media was changed after every 24 hr and replaced with fresh media. Purity of the trophoblast cells was judged by immunocytochemistry performed immediately after isolation. Most of the cells (>95%) after Percoll purification were found to express cytokeratin (mouse monoclonal clone PAN-CK from Neomarkers, Fremount, CA, USA), but not vimentin (mouse monoclonal V-9 from Dako Laboratories, Glostrup, Denmark).

Culture of Cells in Presence of the Antibody

After 24 hr of plating, the trophoblast cells were washed with fresh medium and then incubated for various time periods in presence of the antibody at a final dilution of 1:500 and 1:1000. The level of progesterone in the medium in presence or absence of the antibody was estimated by ELISA kit obtained from Immuno-biological Laboratories (IBL), Hamburg, Germany. The sensitivity of the assay was 0.05 ng/mL of progesterone at 95% confidence limit. Effect of neutralization of hCG on cellular morphology was studied by phase contrast microscopy and transmission electron microscopy (TEM) after 48 and 72 hr of culture.

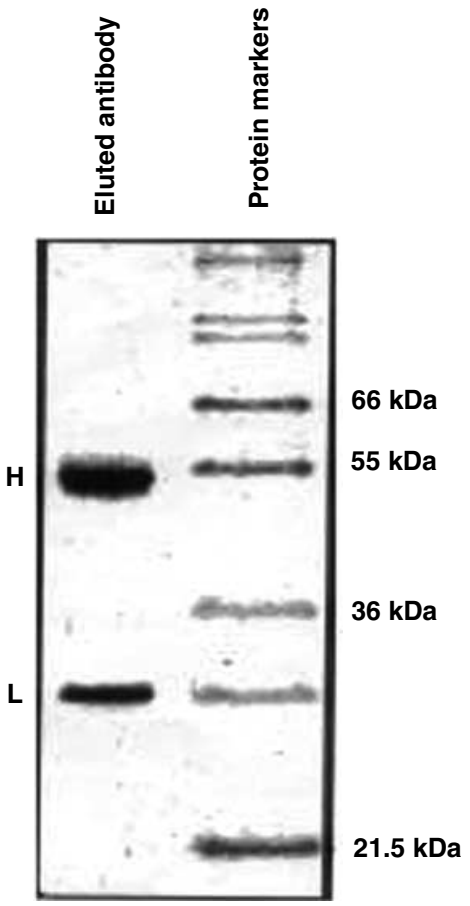

Fig. 1. SDS-PAGE analysis of affinity purified chimeric antibody from transgenic tobacco. 5 μL of purified antibody (2 mg/mL) was resolved on 12% SDS-PAGE. The protein bands were visualized by Coomassie staining. H, Chimeric Heavy Chain; L, Chimeric Light Chain.

RT-PCR for E-Cadherin

Trophoblast cells (2×10^6 cells/well) maintained in culture, in six well tissue culture plates (Griener, Germany), Cat no: 657,160, were exposed to anti-hCG Ab(at a final dilution of 1:500) after 12 hr of plating and incubated for 6, 12, 18 and 24 hr. Cells were harvested in TriZOL reagent (Gibco BRL, Life Technologies) for isolation of total RNA. First strand cDNA was prepared using Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega Corporation, Madison WI, USA), as per manufacturer's

instructions using oligo-dT. Polymerase chain reaction (PCR) was carried out using specific primers for E-Cadherin and GAPDH in an Eppendorf Master cycler. The primer sequences were as follows:

E-Cadherin: F 5′ GCC AAG CAG CAG TAC ATT CTA CAC G 3′
R 5′ GCT GTT CTT CAC GTG CTC AAA ATC C 3′

GAPDH: F 5′ TCA CCA CCA TGG AGA AGG C 3′
R 5′ CAC CAC CTT CTT GAT GTC ATC 3′

The amplified products were resolved on 2% agarose (Biorad Laboratories, Hercules, CA, USA) and single band of 342 bp for E-cadherin and 420 bp for GAPDH were obtained. Band intensities were analysed using a Biorad densitometer and results were expressed after normalizing with GAPDH, used as an internal standard.

Immunocytochemistry

Trophoblast cells grown on coverslips were exposed after 24 hr of plating to anti-hCG antibody (at a final dilution of 1:500) and incubated for another 24 hr. Cells were fixed with 4% paraformaldehyde at 48 hr of culture, the time point when most of the trophoblast cells fuse to form syncytium. After fixing for 1 hr, the cells were washed with 0.1 M phosphate buffer saline (PBS) and treated with 0.1% saponin for 10 min at 25°C to increase the permeability of the antibody into the cell. Endogenous hydrogen peroxidase activity was quenched by treating the cells with 0.03% H_2O_2 for 15 min. After blocking non-specific reactivity with 5% BSA in PBS, cells were incubated with 2 μg/mL of the primary mouse anti human E-cadherin antibody (Neomarkers) for 18 hr at 4°C in a humid chamber. Cells incubated with mouse IgG(1 μg/mL), obtained from Santa Cruz Biotechnologies, CA, USA served as negative control. Cells were then exposed to biotinylated second antibody (Universal LSAB kit, Cat. no. K0690, Dako Laboratories) for 1 hr at room temperature followed by incubation with avidin conjugated horse raddish peroxidase (HRP) for 15 min. The final colour was developed using 3,3′diamino benzidine (DAB; Sigma) at a final concentration of 0.05% in Tris-HCl, pH 7.6, containing 0.1% H_2O_2. The cells were further counterstained with Meyer's hematoxyline (Sigma-Aldrich) and dehydrated in ascending order from 30 to 100% of ethanol followed by Xylene and further mounted with DPX for bright field microscopy.

Transmission Electron Microscopy

Trophoblast cells maintained in six well tissue culture plates for 24 hr were incubated for the next 24 hr with anti-hCG Ab at a final dilution of 1:500. Cells were harvested from the tissue culture plates and fixed in Karnovasky's fixative (1% Paraformaldehyde and 2% glutaraldehyde in 0.1 M Phosphate buffer) for 1 hr at 4°C. These were then washed with 0.1 M PBS to remove the fixative. The samples were then post-fixed in 1%OsO_4, for 2 hr at 4°C, dehydrated and embedded in araldite CY212. Thin sections (50–60 nm) were cut, mounted onto copper grids and constructed with uranyl acetate and lead citrate. The sections were then observed under a Philips CM10 transmission electron microscope (Philips Electron Optics, 500 MD Eindhoven, Netherlands).

Statistical Analysis

Results are expressed as arithmetic mean ± S.D. Statistical analysis was performed using paired *t*-test. $P < 0.001$ and $P < 0.005$ were considered statistically significant. Each experiment was conducted in triplicate on cells collected from one placenta. Reproducibility of each set of experiment was checked thrice on cells collected from three different placentae.

RESULTS

Mononucleated cytotrophoblast cells aggregate and fuse to form multinucleated syncytiotrophoblast in culture. In presence of the chimeric antibody, the cells aggregated, but failed to fuse and form the syncytium (Fig. 2). This was further clearly evident from the transmission electron micrographs, which show that in the presence of the antibody the cells retain distinctly their plasma membrane, while the control cells without the antibody had no plasma membrane separating cytotrophoblasts, a feature characteristic of syncytium formation (Fig. 3). Morphological differentiation of the trophoblast cells in culture is followed by their functional differentiation wherein the syncytial cells as they form, start producing enhanced amounts of hCG,

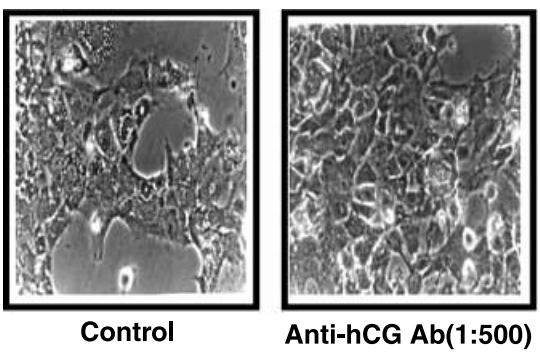

Fig. 2. Phase contrast micrographs depicting an appreciable cellular aggregation but no fusion between the trophoblast cells in the presence of the recombinant chimeric anti-hCG Ab as compared with the control, which shows the normal pattern of syncytilization. Magnification: ×400.

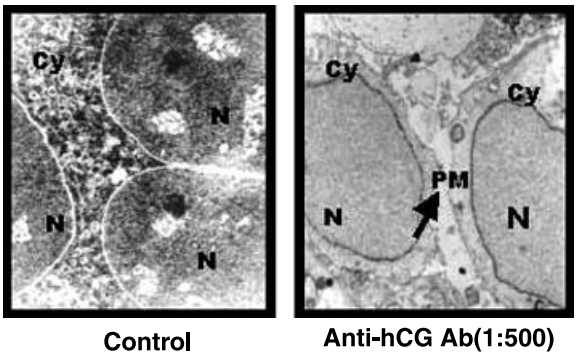

Fig. 3. Transmission electron micrographs of trophoblast cells in culture, showing a distinct plasma membrane separating the two adjoining trophoblast cells in the presence of the Anti-hCG Ab. In the absence of the antibody no plasma membrane is observed separating the cytotrophoblasts, indicating a true syncytium formation. N, nucleus, Cy, cytoplasm, PM, plasma membrane, Magnifacation: ×2650.

which in turn induces progesterone synthesis by an autocrine mechanism.[17] Fig. 4, shows that in presence of the chimeric antibody, the cells in culture fail to produce progesterone as compared with the control cells without antibody, which synthesize increasing amounts of the steroid during the same period. Studies at mRNA level revealed that the expression of E-cadherin, a vital cell adhesion molecule involved in cell-to-cell interaction decreases as the trophoblast cells start to fuse and form the syncytium (Fig. 5). In presence of the chimeric antibody, the expression of E-cadherin however, remained at a higher level than the control cells without the antibody. Immunostaining for E-cadherin further confirmed the RT-PCR results. Weak immunostaining was observed in the control cells while in presence of the chimeric antibody (1:500), the immunostaining for E-cadherin was more intense (Fig. 6).

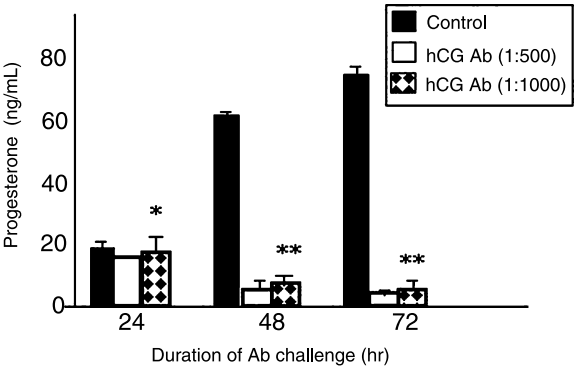

Fig. 4. Quantitation of progesterone levels secreted by trophoblast cells in the presence or absence of the anti-hCG Ab. The antibody was added to the culture medium as described in Materials and Methods. The values are the mean ± S.D. of three experiments performed in triplicate (**$P < 0.001$, *$P < 0.005$).

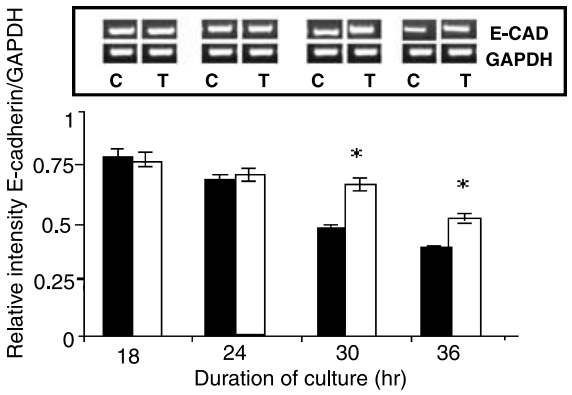

Fig. 5. Effect of anti-hCG Ab on the expression of E-cadherin in trophoblast cells. Densitometric analysis of the RT-PCR of E-Cadherin transcript shows the reduction of E-Cadherin expression with syncytium formation in untreated control cells whereas the expression of E-cadherin remains high in presence of the recombinant chimeric Anti-hCG Ab (1:500). ■ = control; □ = anti-hCG Ab (1:500). Inset shows the corresponding PCR amplified bands resolved in agarose gel. GAPDH was used as an internal control. Each experiment was repeated three times in triplicate and results expressed as mean ± S.D. (*$P < 0.005$, against controls) C, control, T, test, in presence of the anti-hCG antinody (1:500).

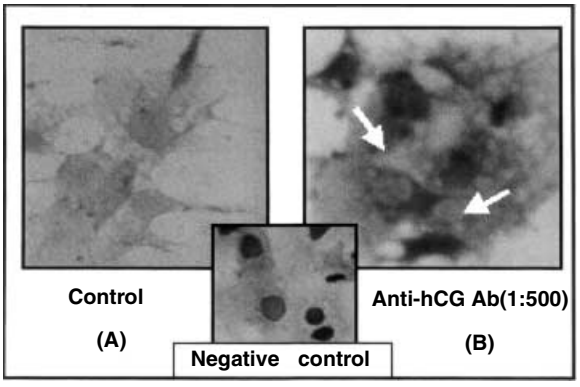

Fig. 6. Immunolocalization of E-cadherin in trophoblast cells in culture without the antibody (A) and in presence of the antibody (B).

DISCUSSION

The aim of this study was to determine whether a recombinant chimeric antibody against hCG is competent to be used for intervention of hCG bioactivity for control of fertility (emergency contraception) or for imaging and therapy of tumours making hCG. Recombinant humanized chimeric antibodies have received drug regulatory approvals for clinical use.

Although rodent Leydig cells were used for bioassay previously,[6] where the ability of antibody to neutralize hCG induced secretion of testosterone was tested, the present studies are on human cells derived from an organ which makes hCG and where hCG plays a critical role in the synthesis of progesterone for

sustenance of gestation. Interestingly hCG made by the cytotrophoblasts acts as an autocrine stimulus for these cells, a property shared by some cancer cells, where hCG or its subunits act as promoters of growth. This is the case with Chago cells derived from a lung cancer patient refractory to available chemotherapeutic drugs.[7] These cells make alpha hCG and antisense RNA blocking its synthesis or antibodies neutralizing the subunits, inhibit the growth of tumour cells *in vitro* and *in vivo* in nude mice.[7] Similar observations were made by Gillot et al.[8] on a bladder cancer cell secreting beta hCG, where anti-beta hCG antibodies were inhibitory to tumour growth.

The data reported here clearly demonstrate that the recombinant chimeric antibody cPIPP prevents the fusion of the human cytotrophoblast to generate syncytium. This is evident from morphology as seen by light microscopy. Electron micrographs reveal the persistence of plasma membrane in cultures containing the antibody, whereas in controls, two or more nuclei share the same cytoplasm with fused plasma membranes. The formation of syncytium represents an important facet of trophoblast differentiation, which is essential for performing its multifaceted functions. Yang et al.[18] have reported a central role of hCG in the formation of human placental syncytium and Shi et al.[9] have observed the novel role of hCG in differentiation of human cytotrophoblast. Clearly cPIPP interferes in these processes.

Villous cytotrophoblasts isolated from the human placenta mimic many of the cellular events associated with *in vivo* processes. Freshly isolated mononucleate cytotrophoblasts when maintained in culture, aggregate and establish extensive interactions with one another through the formation of desmosomes,[19] adherens,[20] and gap junctions.[21] The resulting cellular aggregates promote the terminal differentiation and fusion of the mononucleate cytotrophoblast to multinucleate syncytiotrophoblast. Beside the other important factors, these morphogenetic events are believed to be mediated at least in part, by the regulated expression of the members of the cadherin gene superfamily of calcium-dependent cell adhesion molecules (CAMs). During cytotrophoblast aggregation, E-cadherin has been observed to be localized on the cell surface at points of cell–cell contact but could not be demonstrated following cellular fusion.[22,23] In the present study, higher expression of E-cadherin mRNA observed in cytotrophoblast cells which decreased as aggregates were formed and cells started to undergo differentiation suggesting that temporo-spatial expression of E-cadherin may regulate in part the processes of aggregation and fusion of these cells during their differentiation into syncytial trophoblasts. The effect of cPIPP was studied both at mRNA and protein level of E-cadherin. Higher amount of E-cadherin as seen by mRNA and immunoreactive protein in presence of antibody was noted, as compared with control cells where it gradually decreases during syncytium formation. Higher expression of E-Cadherin in presence of the antibody as compared with the control cells further indicate the absence of syncytium formation in presence of the antibody.

An important action of hCG on trophoblasts is the stimulation of synthesis and secretion of progesterone, which in turn is vital for sustenance of gestation. This increased production of progesterone correlates with formation of syncytium.[17] Chimeric antibody was effective in blocking progesterone synthesis. These observations are consistent with previous studies from our laboratory in which polyclonal antibodies against hCG were seem to inhibit progesterone synthesis.[24]

The ensemble of these studies indicate that the recombinant chimeric antibody cPIPP, engineered to bind with beta hCG and hCG, is effective in blocking hCG induced events of differentiation of human cytotrophoblasts to form syncytium and synthesis of progesterone. In presence of hCG antibody, progesterone synthesis is inhibited as a consequence of absence of syncytium formation and hCG production.

Acknowledgments

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REFERENCES

1. Fishel SB, Edwards RJ, Evans CJ: Human chorionic gonadotropin secreted by pre implantation embryos cultured in vitro. Science 1994; 223:816–818.
2. Hearn JP, Gidley–Baird AA, Hodges JK, Summers PM, Wilbey GE: Embryonic signals during the pre-implantation period in primates. J Reprod Fertil 1988; 36(Suppl.): 49–58.
3. Talwar GP, Singh O, Pal R, Chatterjee N, Sahai P, Dhall K, Kaur J, Das SK, Suri S, Buckshee K, Saraya L, Saxena BN: A vaccine that prevents pregnancy in women. Proc Natl Acad Sci USA 1994; 91:8532–8536.

4. Talwar GP, Singh O, Gupta SK, Hasnain SE, Pal R, Majumder SS, Vrati S, Mukhopadhay A, Srinivasan J, Deshmukh U, Ganga S, Mandokhot A, Gupta A: The HSD-hCG vaccine prevents pregnancy in women. Feasibility study of a reversible safe contraceptive vaccine. Am J Reprod Immunol 1997; 37:153–160.
5. Kathuria S, Sriraman R, Nath R, Sack M, Pal R, Artsaenko O, Talwar GP, Fischer R, Finnern R: Efficacy of plant produced recombinant antibodies against hCG. Human Reprod 2002; 17:2054–2061.
6. Kathuria SR, Nath R, Pal R, Singh O, Fischer R, Lohia NK, Talwar GP: Functional recombinant antibodies against human chorionic gonadotropin expressed in plants. Curr Sci 2002; 82:1452–1457.
7. Kumar S, Talwar GP, Biswas DK: Necrosis and growth inhibition of growth of human lung tumour by anti-hCG antibodies. J Natl Cancer Inst 1991; 84:42–47.
8. Gillot DJ, Iles RK, Chard T: The effects of beta human chorionic gonadotrophins on the vivo growth of bladder cancer cell line. Br J Cancer 1996; 73:323–326.
9. Shi QJ, Lei ZM, Rao ChV, Lin J: Novel role of Human Chorionic Gonadotropin in differentiation of human cytotrophoblasts. Endocrinology 1993; 132:1387–1395.
10. Licht P, Lei ZM, Rao CV, Merz WE: The presence and functional relevance of hCG/LH receptors in normal human placentas and choriocarcinomas. In Implantation and Early Pregnancy in Humans, ER Barnea, JH Check, JG Grudzinskas, T Maruo (eds). Carnforth, UK, Parthenon Publishing Group, 1994, pp 235–246.
11. Lei ZM, Rao CV: Gonadotropin receptors in human feto-placental unit: implication of hCG as an intracrine, paracrine and endocrine regulator of human fetoplacental function. Trophobalst Res 1992; 6:213–224.
12. Hoshina M, Boothby M, Hussa RO, Patillo R, Camel HM, Boime I: Linkage of human chorionic gonadotropin and placental laactogen to trophoblast differentiation and tumorigenesis. Placenta 1985; 6:163–172.
13. Hoshina M, Boothby M, Boime I: Cytological localization of chorionic gonadotropin and placental lactogen mRNAs during development of the human term placenta. J Cell Biol 1982; 93:190–198.
14. Paul S, Das C, Jailkhani BL, Talwar GP: Progesterone synthesis by human placental syncytiotrophoblast *in vitro*-preferred precursor and effect of human chorionic gonadotropin. J Steroid Biochem 1981; 14:311–313.
15. Kathuria SR, Nath R, Pal R, Singh O, Fischer R, Lohiya NK, Talwar GP: Functional recombinant antibodies against human chorionic gonadotropin expressed in plants. Curr Sci 2002; 12:1452–1457.
16. Das C, Sanyal M, Chandra Nag T: Localization of nitric oxide synthase in human trophoblast cells. Role of nitric oxide in trophoblast proliferation and differentiation. Am J Reprod Immunol 2000; 43:70–77.
17. Chaudhary J, Bhattacharya S, Das C: Regulation of progesterone secretion in human syncytiotrophoblast in culture by hCG. J Steroid Mol Biol 1992; 42:425–432.
18. Yang M, Lei ZM, Rao ChV: The central role of human chorionic gonadotropin in the formation of human placental syncytium. Endocrinology 2003; 144:1108–1120.
19. Douglas GC, King BF: Differentiation of human trophoblastic cells *in vitro* as revealed by immunocytochemical staining of desmoplakin and nuclei. J Cell Sci 1990; 96:131–141.
20. Getsios S, Chen GTC, MacCalman CD: Regulation of β-catenin mRNA and protein levels in human villous cytotrophoblast undergoing aggregation and fusion in vitro: correlation with E-cadherin expression. J Reprod Fertil 2000; 119:59–68.
21. Cronier L, Bastide B, Herve JC, Deleze J, Malassine A: Gap junctional communication during human trophoblast differentiation: influence of human chorionic gonadotropin. Endocrinology 1994; 135:402–408.
22. Coutifaris C, Kao LC, Sehdev HM, Chin U, Babalola GO, Blaschuk OW, Strauss JF III: E-cadherin expression during trophoblast differentiation of human trophoblasts. Development 1991; 113:767–777.
23. Maccalman CD, Furth EE, Omigbodun A, Bronner M, Coutifaris C, Strauss JF III: Regulated expression of cadherin-11 human epithelial cells; a role for cadherin-11 in trophoblast-endometrium interactions. Development Dyn 1996; 206:201–211.
24. Bhattacharya S, Chaudhary J, Das C: Antibodies to hCG inhibit progesterone production from human syncytiotrophoblast cells. Placenta 1992; 13:135–139.