Immunohistochemical localization of chorionic gonadotrophin on baboon placenta, dispersed trophoblast cells and those derived from blastocysts grown *in vitro*

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Summary. An immunohistochemical technique using a high specificity antiserum against baboon CG was used to demonstrate the presence of a CG-like material on: (1) fixed baboon placental sections collected between 31 and 39 days of gestation, (2) trophoblast monolayers derived from hatched embryos grown *in vitro* for 15 days and (3) trophoblast cells derived from cells dispersed from placentae collected between Days 31 and 39 of pregnancy. A specific radioimmunoassay was used to detect concentrations of baboon CG in daily spent medium. Immunohistochemical studies showed that material cross-reacting with CG was present on all the three sources of trophoblast. The embryos secreted CG from attachment onwards and immunoactive CG was measurable in daily spent medium collected from placenta-derived trophoblast cultures. It is concluded that baboon CG is localized in the syncytiotrophoblast of fixed placental sections and cellular trophoblast derived from cultured embryos and placental cells.

Keywords: baboon; trophoblast; chorionic gonadotrophin; immunochemistry

Introduction

Chorionic gonadotrophin (CG) is synthesized and secreted by trophoblast cells during human pregnancy. The precise temporal appearance of this hormone in women is not known and estimates for the time of its first appearance range between 6 and 11 days after ovulation (Saxena *et al.*, 1974; Catt *et al.*, 1975; Landesman & Saxena, 1976; Ahmed & Klopfer, 1983; Hay, 1985). Most primates are known to produce a CG-like material during pregnancy (Hearn, 1980; Hodgen, 1980) and rhesus monkey morulae and blastocysts maintained *in vitro* have been shown to secrete a substance capable of inducing luteinization of monkey granulosa cells (Batta & Channing, 1979). In-vitro cultures of baboon (Pope *et al.*, 1980) and marmoset (Summers *et al.*, 1987) trophoblast have been shown to secrete measurable quantities of CG as detected by radioimmunoassay after blastocyst attachment. Evidence of the presence of a CG-like material in preimplantation embryos of several non-primate species such as the mouse and rabbit is also available (Haour & Saxena, 1974; Wiley, 1974; Asch *et al.*, 1978, 1979; Varma *et al.*, 1979).

Histochemical and immunofluorescent techniques coupled with light- or electron-microscopy have shown that hCG is localized predominantly in the human syncytiotrophoblast (Beck *et al.*, 1969; Hamanaka *et al.*, 1971; Genabacev *et al.*, 1972; Genabacev & Sulovic, 1975). However, other workers have shown that human CG can be localized on the cytotrophoblast (Thiede & Choate, 1963; Loke *et al.*, 1972; Tojo *et al.*, 1974) and these results have been mostly obtained in studies utilizing human trophoblast cultured *in vitro*. The baboon produces a CG similar to hCG during pregnancy (Bambra, 1987) and the studies reported herein were carried out to determine its cellular origin on fixed placental sections, placental trophoblast cells cultured *in vitro* and trophoblast derived from embryos grown *in vitro*.

Materials and Methods

Placentae. Placental tissue was obtained from time-mated pregnant baboons at 31–39 days of gestation and fixed in acetone. Blocks were cut from these placentae in a standardized manner, embedded in paraffin wax (MP 56 C) and sectioned at 5 μ m. Serial sections were prepared for staining by immunohistochemical methods to determine the distribution of CG.

Embryo recovery. The procedure was exactly that described by Pope *et al.* (1980). Essentially, an Isaac's endometrial cell sampler, modified to permit bidirectional flow of tissue culture medium, was inserted across the undilated cervix of the anaesthetized female baboon an estimated 6 days after ovulation, as determined by loss of sexual skin turgescence. Then 15 ml ovum culture medium (Flow Laboratories, Irvine, UK) were flushed through the cell sampler, collected in a sterile conical tube, decanted into a 10 $\times$ 35 mm Petri dish and examined for embryos with a Leitz stereomicroscope. Recovered embryos which were at the hatched stage were grown *in vitro* to obtain trophoblast monolayers.

Placental trophoblast. Placentae were recovered from pregnant baboons between Days 31 and 39 of gestation. Tissues were collected aseptically and cells dispersed using the method of Risbridger *et al.* (1985). Placental tissue was cut into small pieces with scissors and washed thoroughly with ovum culture medium. The cells were allowed to disperse in 0.5% collagenase (Sigma Chemical Co., St Louis, MO, USA) in ovum culture medium for 30 min at 37°C in a 5% CO₂/95% air atmosphere. A further volume of collagenase was added and the cells were redispersed for another two periods of 20 min. The cells were centrifuged at 200 g for 5 min, washed three times with the ovum culture medium and finally resuspended in Hams F10 medium with 20% (v/v) fetal calf serum, 5.0 μ g amphotericin B deoxycholate/ml, 100 μ g gentamycin/ml, 100 units penicillin/ml and 100 μ g streptomycin/ml (all reagents from Flow Laboratories). An aliquant was removed for counting in a haemocytometer. The cells were then cultured and established *in vitro*.

Embryo and placental trophoblast culture. The culture medium was Hams F10 (Flow Laboratories) containing 20% heat-inactivated fetal calf serum and antibiotics in the concentrations given above. Embryos were cultured in flat-bottomed microtitre plate (Falcon 3042) wells in 200 μ l medium under an atmosphere of 5% CO₂/95% air at 37°C. The culture medium was changed daily and the embryo transferred into a fresh well in the same plate using a finely drawn Pasteur pipette under controlled suction. After attachment, the medium was changed daily. Photographs of the embryo were taken daily. Individual spent medium samples were stored at –20°C until required for radioimmunoassay studies.

Trophoblast cells, as identified by their morphology and adherent nature (Zuckermann & Head, 1986) were cultured in 24-well flat-bottomed plates (Nunc, Inter Med. Kamstrup, Roskilde, Denmark) containing 1 ml Hams F10 medium supplemented with fetal calf serum and antibiotics as above under an atmosphere of 5% CO₂/95% air at 37°C. The culture medium was changed daily by withdrawal of medium through a finely drawn Pasteur pipette under controlled suction. Individual spent samples were stored at –20°C until radioimmunoassay. Embryo- and placenta-derived trophoblast tissues were utilized in immunohistochemical studies to localize CG.

Assay of CG. Baboon CG was measured in culture medium using a double-antibody procedure described by Bamba *et al.* (1984). This assay used a rabbit antiserum against baboon CG (2001-102782) kindly provided by Professor V. Stevens (Department of Obstetrics and Gynecology, Ohio State University, Columbus, OH, USA) and was used at a final dilution of 1:3000. The tracer was ¹²⁵I-labelled hCG prepared at the Institute and the Second International hCG Standard (MRC 61/6, NIBSC, London) was used as the standard material. The sensitivity of the assay (B/Bo = 90%) was 0.2 ng/tube. Inter and intra-assay coefficients of variation were 12% and 7.1% respectively. Protease inhibitor (soybean trypsin inhibitor: Sigma) was incorporated in all assay buffers.

Histochemical studies. Placental sections were deparaffinized in xylene and hydrated. All sections were sequentially incubated for 30 min at room temperature with each of the following reagents: (1) a solution of 0.3% (w/v) hydrogen peroxide in methanol to block endogenous peroxidase activity; (2) normal rabbit serum (1:10 dilution) to decrease non-specific background staining; (3) rabbit antiserum against baboon CG as used in the radioimmunoassay (1:20) or hCG (R2001, 1:250) kindly provided by Professor R. Ryan (Department of Cell Biology, Mayo Clinic, Rochester, MN, USA); (4) with diluted biotinylated rabbit antibody (Sigma Chemical Co.); (5) Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, USA). After each incubation the slides were washed with 10 mM-sodium phosphate, pH 7.5, 0.9% saline. The reaction product of the section was developed with a freshly prepared solution containing 3,3'-diaminobenzine tetrahydrochloride (50 mg per 100 ml PBS to which was added 0.02% hydrogen peroxide). Sections were washed with water, counterstained with haematoxylin and mounted in Permount. Hormone antisera and method specificity were tested by substituting an unrelated antibody for the specific antiserum, omitting the antihormone antibody step and absorption of the antibody with the homologous hormone.

Cultures of trophoblast derived from both the placenta and embryo were also subjected to this immunohistochemical method. However, before use the sections were fixed overnight in 70% alcohol and the incubations with various agents were carried out in the tissue-culture plate wells. After washing with water and counterstaining with haematoxylin the cells were covered with a glycerol-PBS mixture before light microscopy.

Results

Radioimmunoassay results

Both the embryos used in this study were recovered at the hatched blastocyst stage (Fig. 1) and were grown *in vitro* for up to 13 days (Fig. 2). They secreted measurable quantities of CG as determined by radioimmunoassay (Table 1). The secretion reached peak levels at Day 10 *in vitro* and continued for the duration of culture. Trophoblast cells were dispersed from the placenta and allowed to grow *in vitro* for up to 5 days (Fig. 3). They also secreted measurable quantities of CG (Table 2).

Fig. 1. Hatched blastocyst recovered by non-surgical uterine flushing. $\times 440$.

Immunohistochemical studies

Placenta. The omission of any one component from the sequential immunoperoxidase method resulted in negative results, as did the use of antiserum which had been previously adsorbed with excess antigen (Fig. 4a). A positive reaction was seen in the villous syncytiotrophoblast with both antiserum to baboon CG (Fig. 4b) and hCG (Fig. 4c); the former antiserum giving the stronger reaction. Cytotrophoblast cells, stroma, endothelia of villous fetal vessels, decidua and endometrial glands gave consistently negative results.

Embryo-derived trophoblast. As in the staining of the placental sections specificity controls gave negative results. A strong positive reaction was obtained with syncytial cells and localization was confined to the cytoplasm (Fig. 5a).

Placenta-derived trophoblast. Once again specificity controls gave negative results. Positive staining was similar to that of the embryo-derived trophoblast in that the positive staining reaction product was restricted to the cytoplasm of trophoblast (Fig. 5b).

Fig. 2. Attached embryo showing extensive trophoblast outgrowth (T) after 14 days in culture. $\times 440$.

Fig. 3. A photomicrograph of placenta-derived trophoblast showing the appearance of the trophoblast (T). $\times 280$.

Table 1. Secretion of CG by 2 baboon embryos

Day in culture	Developmental stage	CG production (i.u./day)
1	Hatched blastocyst	Not measurable
2	Start of attachment	0·10 0·11
3	Attachment	1·50 1·80
4	After attachment	2·10 2·80
5	After attachment	2·50 3·10
6	After attachment	3·30 3·60
7	After attachment	3·50 3·70
8	After attachment	3·60 3·80
9	After attachment	3·50 4·30
10	After attachment	3·90 4·50
11	After attachment	3·70 4·30
12	After attachment	3·90 4·10

Table 2. Secretion of CG by placental trophoblast cells of baboons

Day in culture	CG secretion	
	i.u./well*	mi.u./placenta
1	44.0 ± 1.0	1056.0
2	33.4 ± 0.8	801.6
3	31.9 ± 1.4	765.6
4	27.3 ± 1.2	655.2
5	21.1 ± 1.3	506.4

*Values are mean ± s.e.m. for 10 observations.

Fig. 4. Photomicrographs of placental sections showing (a) lack of staining due to specificity of the antiserum; (b) a positive reaction (arrow) and localization of CG-like material on the villous syncytiotrophoblast; and (c) a positive reaction (arrow) and localization of hCG-like material on the villous syncytiotrophoblast. S = syncytiotrophoblast; C = cytotrophoblast; M = maternal blood space. × 200.

Discussion

A previous study has shown that baboon embryos grown *in vitro* are capable of secreting CG which can be detected by radioimmunoassay (RIA) at or after attachment of the hatched blastocyst to the culture vessel (Pope *et al.*, 1982). Pope *et al.* (1982) used a heterologous RIA and sheep β -subunit LH antiserum (H-26); peak secretion of CG occurred 7–12 days after hatching and continued for up to 27 or more days. The assay results obtained with the 2 embryos growing *in vitro* in the present study are in good agreement with those obtained by Pope *et al.* (1982). A more specific radioimmunoassay utilizing an anti-baboon CG serum was used and concentrations of baboon CG increased with attachment of the embryos to the culture vessels and tended to reach a peak 7 days after attachment. Moreover, it was apparent that measurable quantities of baboon CG were produced by the hatched blastocyst before its attachment. The preimplantation human blastocyst is believed to secrete detectable amounts of CG into the blood (Saxena *et al.*, 1974) and CG may be present on the surface of the free blastocyst or its environment (Hearn, 1980). The marmoset also secretes a CG before attachment (J. P. Hearn, S. Gems, J. K. Hodges & C. Wennink, unpublished data).

Fig. 5. Positive staining for baboon CG in (a) embryo-derived trophoblast and (b) placenta-derived trophoblast. T = trophoblast cells; N = nucleus; arrows = reaction product. $\times 440$.

Trophoblast cells derived from human (Tojo *et al.*, 1974; Huot *et al.*, 1979; Kim *et al.*, 1987), marmoset (Summers *et al.*, 1987) and baboon (Pope *et al.*, 1982) embryos and grown *in vitro* have been reported to secrete CG as measured by RIA. The present findings confirm the baboon data of Pope *et al.* (1982) who utilized trophoblast cells derived from 2 embryos and up to 200 mi.u. CG/day could be measured when the anti-ovine LH serum was used in RIA. The values obtained in the present study are considerably higher and ranged from 506 to 1056 mi.u. CG/day (Table 2). However, the placenta was used as the source of trophoblast cells and these were found to be viable before their utilization in histochemical studies as demonstrated by their histological appearance and continued secretion of CG. As culture time of baboon trophoblast cells increased, the amounts of CG secreted appeared to decline. This has been reported for long-term cultures of human trophoblast; hCG and placental lactogen values were lower than the levels detectable after 10 days *in vitro* (Nelson *et al.*, 1986).

The CG material detected by RIA in the present study was associated with the syncytiotrophoblast in fixed placental tissue and this is in agreement with reports on localization of hCG in human placenta (see Midgley & Pearce, 1962; Ikonicoff & Cedard, 1973; Tabarelli *et al.*, 1983; Al-Timini & Fox, 1986). When hCG antiserum was used instead of baboon CG antiserum a lower intensity of staining was observed on fixed placental sections. This is consistent with reports that β -hCG antisera raised in rabbits and baboons reacts with baboon CG but to a substantially lesser extent than with hCG (Stevens, 1976).

The baboon placental or embryonic trophoblast cells grown *in vitro* were not characterized. It has been reported that most of the embryo- (Pope *et al.*, 1982; Fishel *et al.*, 1984; Summers *et al.*, 1987) or placenta- (Loke *et al.*, 1986; Nelson *et al.*, 1986; Ungar *et al.*, 1987) derived trophoblast cells which establish in culture are cytotrophoblast. It has been shown that hCG is localized on cellular trophoblast in culture (Chung *et al.*, 1969; Fox & Kharkongor, 1970; Loke *et al.*, 1972; Cotte *et al.*, 1980) and thus localization of hCG on cultured baboon trophoblast cells is not surprising.

Based on the facts that: (1) the antibodies used in this study were specific; (2) the method was specific; (3) no reaction was obtained when the specific antibody was substituted with an unrelated

one; and (4) there was abolition of staining by prior absorption with the particular antigens, it can be deduced that the immunoperoxidase reaction noted in the present study showed a specific localization. This, considered in conjunction with the RIA results, indicates that the baboon syncytiotrophoblast cells are the probable site of secretion of this material.

In the marmoset, CG may be an important requirement for attachment and baboon CG may have a similar role.

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