ULTRASTRUCTURAL EVIDENCE OF HUMAN CHORIONIC GONADOTROPIN ON TROPHOBLASTIC SURFACE

KOH KAWAGOE, MOTOYASU SUGASE* AND RIKUO MACHINAMI

Department of Pathology, Faculty of Medicine and Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Hongo, Tokyo 113*

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Human chorionic gonadotropin (HCG) was proved to exist on the cell surface of a syncytiotrophoblast by the immuno-electron microscopic technique. The cell surface, including microvilli and micropinocytosis vesicles, was positive. Two types of HCG were observed; one loosely and the other tightly connected to the cell surface. The latter was closely related to the acid mucopolysaccharide of the cell surface coat, revealed by ruthenium red staining. These ultrastructural findings suggest that HCG acts as a local immunosuppressant on the syncytiotrophoblast cell surface, preventing partial maternal immunological reactions against the fetus.

Human chorionic gonadotropin (HCG) was originally reported in 1937 (14) to be produced by the placenta. Since then, various efforts have been devoted towards the determination of localization of this hormone at the cellular level (11, 21, 22, 25, 27, 28). In 1962, Midgley and Pierce used the direct immunofluorescence method to show that HCG was localized exclusively in the syncytiotrophoblastic layer (19). This finding was confirmed by others using the light microscope. (8, 10, 15). Recently, Okudaira et al. (20) demonstrated with the immuno-electron microscopic technique that HCG was synthesized in the rough endoplasmic reticulum of a syncytiotrophoblast, and was secreted as a secretory granule into the maternal blood via the Golgi apparatus.

Besides its well-known gonadotrophic effects, HCG has been shown to exhibit the biological property of immunosuppressive effect (1, 9, 24), and has been thought to play an important role in preventing maternal immunologic reactions against the fetus. While the local effect of HCG as an immunosuppressant has recently been pointed out (2), the surface mucin of a human trophoblast was also thought to suppress the immunological reaction masking the surface antigen (3). The authors have attempted to examine both the surface HCG and mucin of a human trophoblast by electron microscopy in the present study.

MATERIALS AND METHODS

Seven placentas of normal pregnancies from six to ten weeks gestation were obtained by operation of dilatation and curettage.

The tissues for routine electron microscopic study were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), post-fixed with 1% osmium tetroxide, stained on a block with 2% uranyl acetate, dehydrated in a graded alcohol sequence and embedded in Epon 812.

For ruthenium red staining, the tissues were fixed for one hour in a mixture of the following solutions; 0.5 ml of 3.6% glutaraldehyde, 0.5 ml of 0.2 M cacodylate buffer (pH 7.3) and 0.5 ml of ruthenium red stock solution (1500 ppm in water). After washing in two to three changes of 0.1 M cacodylate buffer, the tissues were post-fixed in a mixture of 0.5 ml of 5% osmium tetroxide, 0.5 ml of 0.2 M cacodylate buffer (pH 7.3) and 0.5 ml of ruthenium red stock solution for 3 hrs. The tissues were rinsed briefly with the buffer, dehydrated through the standard ethanol series and embedded in Epon 812 (16).

For the immunoperoxidase method, the tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) or with Mclean's PLP fixative (18) for 5 to 30 min. After rinsing in phosphate-buffered saline (PBS) 3 times during a 30 min period, the tissues were incubated in PBS containing 100× diluted anti-HCG rabbit serum (obtained from Mochida Seiyaku Co., Tokyo, Japan) for 15 to 30 min. The tissues were rinsed in PBS three times during a 30 min period then incubated in PBS containing 10× diluted peroxidase conjugated anti-rabbit IgG goat serum (Cappel Laboratories, Downington, U.S.A.) for 15 min. The tissues were subsequently washed three times with PBS, treated with diaminobenzidine (DAB) reagent (DAB 3 mg, in 10 ml of PBS with 0.1 ml of 1% H₂O₂) for two to three min, and washed three times with PBS again. The tissues were then post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a graded alcohol sequence and embedded in Epon 812. The anti-human IgG rabbit serum (Cappel Laboratories, Downington, U.S.A.) was used rather than the anti-HCG rabbit serum for the control tissues.

RESULTS

Ultrastructurally, microvilli of 0.4–1.8 μ in length were found on the surface of the syncytiotrophoblast. The microvilli were usually slender, but were revealed

FIG. 1. Placenta of 8 weeks gestation. A. syncytiotrophoblast (ST) has long slender microvilli on its surface. There were also numerous micropinocytosis vesicles (arrow).

CT: Cytotrophoblast. $\times 14,500$.

FIG. 2. Surface acid mucoprotein of the syncytiotrophoblast revealed by ruthenium red staining. $\times 26,000$.

FIG. 3. Light microscopic appearance of Epon-embedded tissue for electron microscopy. HCG localization as a fuzzy layer on syncytiotrophoblastic cell (ST) surfaces.

CT: Cytotrophoblast. $\times 900$

FIG. 4. Ultrastructural localization of HCG on the syncytiotrophoblastic cell surface. Reaction products are relatively thick. $\times 7,500$. Inset figure shows the higher magnification of the cell surface. $\times 20,000$.

FIG. 5. A thin but distinct reaction product remained clearly on the syncytiotrophoblastic cell (ST) surface even after washing with 0.1 M phosphate buffer.

CT: Cytotrophoblast. $\times 10,000$.

FIG. 6. A syncytiotrophoblast (ST) and a cytotrophoblast (CT) of a control tissue treated with anti-human IgG rabbit serum instead of anti-HCG rabbit serum. $\times 6,300$.

in some areas as rod-like figures with thickening at the top. There were also numerous micropinocytosis vesicles on the syncytiotrophoblastic cell surface (Fig. 1).

With the ruthenium red staining, there was a relatively uniformly thick ruthenium red stained positive layer on the cell surface, with some micropinocytosis vesicles staining positive as well. In addition, the lipid droplets often showed positive reactions (Fig. 2).

Light microscopic examination of 0.5–1.0 μ thick sections of the Epon-embedded specimen revealed strongly positive reactions for HCG as a fuzzy layer on the syncytiotrophoblastic cell surface. The cytoplasm of both syncytio- and cytotrophoblasts was negative for this hormone (Fig. 3). Using the electron microscope all cell surfaces, including microvilli and micropinocytosis vesicles, were positive. The reactions were seen as a relatively thick layer on the cell surface when the tissues were washed moderately with buffer before fixation (Fig. 4). When the tissues were thoroughly washed with PBS (until the blood was completely removed and the tissue became whitish yellow before prefixation with 2.5% glutaraldehyde), the reactions were seen only as a thin, strongly positive layer on the cell surface (Fig. 5).

No reaction product for human IgG was found on the cell surface of the control trophoblastic cells when the tissues were washed thoroughly with PBS before fixation with 2.5% glutaraldehyde (Fig. 6).

DISCUSSION

The ruthenium red has been used successfully to show acid mucopolysaccharides on a cell coat (6, 17, 23). A ruthenium red staining-positive layer was found on the syncytiotrophoblastic cell surface in the present study, indicating the presence of acid mucoproteins. Histochemical (4) and biochemical (5) analyses of the placenta have shown that the human trophoblast cell surface coat is rich in sulphated acid mucopolysaccharide. Although it is not peculiar that micropinocytosis vesicles showed a ruthenium red positive reaction, the positive reaction of the lipid droplets, reported by Luft (16), may be difficult to explain. The discrepancies of HCG localizations in syncytiotrophoblasts in using light and electron microscopy could be explained as follows. In light microscopic studies, tissues were fixed in Bouin's fluid or formalin (7, 10). These fixatives may destroy the cell membranes through which the antisera enter the cytoplasm. Thus, the syncytiotrophoblast could show positive staining on not only the cell surface but in the cytoplasm as well. On the other hand, when glutaraldehyde or Mclean's PLP are used as fixatives, the cell membranes seemed to be well preserved and the antiserum is unable to enter the cytoplasm. It is interesting to note that Okudaira et al. (20) have demonstrated intracellular HCG of syncytiotrophoblasts by immuno-electron microscopy. They incubated 10 μ frozen sections of formaldehyde fixed tissues in the antisera, whereas in the present study, small pieces of glutaraldehyde fixed tissues and cut by knife were used, reaction products were seen on only the cell membrane.

HCG has been reported to be an immunosuppressant, inhibiting lymphocyte response to PHA (13) and allogeneic lymphocyte (12), and decreasing graft-versus-host disease (24). The effect of this hormone was reversible, noncytotoxic (1) and dose related (26). Although Adcock et al. (1) demonstrated HCG-induced in-

hibition of PHA response at 1–100IU/ml concentration, significant inhibition of PHA, varidase, and mixed lymphocyte responses were found only at HCG concentrations over 1,000IU/ml (9). These findings lead one to think more towards the other mode of *in vivo* effect for HCG than of systemic immunosuppression, because during pregnancy the maternal blood HCG level is approximately 100IU/ml of concentration, even at its peak (26). Borland et al. (2) demonstrated the local effect of HCG in transplantation of trophoblasts and other foetal cells, suggesting its possible role in deciding immunological control capabilities of trophoblastic cells *in vivo*. In the present study, HCG was demonstrated by the immunoperoxidase method on cell surfaces covering the syncytial cells. This finding seems quite reasonable in one sense since HCG is thought to be produced by syncytiotrophoblasts and secreted into the maternal blood flow, as has been previously described. However, in considering the HCG a secretory hormone, it is quite unusual for them to cover the surface evenly to be found on even the microvilli. This finding may be explained as a high concentration of HCG near the cell surface. Based on the observations of the present study, there seem to be two types of HCG binding to the cell surface, i.e., loose and tight. In the case of loose type binding, the HCG could be washed away by a strong jet spray of buffer before fixation, while in tight binding it could not. The distribution of this tightly binded HCG was similar to the mucopolysaccharide surface coat revealed by ruthenium red staining; the tightly binded HCG seems to be closely related to the acid mucopolysaccharides of the cell surface. Furthermore, human IgG, which was abundant in maternal blood, was not found on the syncytiotrophoblastic cell surface. These findings suggest that surface HCG is not derived from the blood stream but produced locally by the syncytiotrophoblast. Thus, the HCG, in addition to its gonadotrophic effect, may play an important role in protection of the human trophoblast from maternal immunological rejection.

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REFERENCES

1. Adcock, E. W. III, Teasdale, F., August, C. S., Cox, S., Meschia, G., Bettaglia, F. C. and Naughton, M. A.: Human chorionic gonadotropin: Its possible role in maternal lymphocyte suppression. *Science* 181; 845, 1973.
2. Borland, R., Loke, Y. W. and Wilson, D.: Immunological privilege resulting from endocrine activity of trophoblast *in vivo*. In *Immunobiology of Trophoblast*, ed. by R. G. Edwards, C. W. S. Howe and M. H. Johnson, Cambridge University Press, Cambridge, 1975, p. 157.
3. Bradbury, S., Billington, W. D., Kirby, D. R. S. and Williams, E. A.: Surface mucin of human trophoblast. *Amer. J. Obstet. Gynec.* 104; 416, 1969.
4. Bradbury, S., Billington, W. D., Kirby, D. R. S. and Williams, E. A.: Histochemical characterisation of the surface mucoprotein of normal and abnormal human trophoblast. *Histochem. J.* 2; 263, 1970.
5. Calatroni, A. and Di Ferrante, N.: The glycosaminoglycans of human term placenta. *Carbohydr. Res.* 10; 535, 1969.

6. Fletcher, J. M., Greenfield, B. F., Hardy, C. J., Scargill, D. and Woodhead, J. L.: Ruthenium Red. *J. Chem. Soc.* 2000, 1961.
7. Gärtner, A., Larsson, L. and Sjöberg, N.: Immunohistochemical demonstration of chorionic gonadotrophin in trophoblastic tumors. *Acta. Obstet. Gynec. Scand.* 54; 161, 1975.
8. Genbacev, O., Robyn, C. and Pantie, V.: Localization of chorionic gonadotropin in human term placenta on ultrathin sections with peroxidase labelled antibody. *J. Microsc.* 15; 399, 1972.
9. Han, T.: Inhibitory effect of human chorionic gonadotrophin on lymphocyte blastogenic response to mitogen, antigen and allogenic cells. *Clin. Exp. Immunol.* 18; 529, 1974.
10. de Ikonicoff, L. K. and Cedard, L.: Localization of human chorionic gonadotropic and somatomammotropic hormones by the peroxidase immunohistochemical method in villi and amniotic epithelium of human placentas (from six weeks to term). *Amer. J. Obstet. Gynec.* 116; 1124, 1973.
11. Jones, G. E. S., Gey, G. O. and Gey, H. K.: Hormone production by placental cells maintained in continuous cultures. *Bull. Johns Hopkins Hosp.* 72; 26, 1943.
12. Kasakura, S.: A factor in maternal plasma during pregnancy that suppress the reactivity of mixed leukocyte culture. *J. Immunol.* 107; 1926, 1971.
13. Kaye, M. D. and Jones, W. R.: Effect of human chorionic gonadotrophin on in vitro lymphocyte transformation. *Amer. J. Obstet. Gynec.* 109; 1029, 1971.
14. Kido, I.: Die menschliche placenta als Produktionsstätte des sogenannten Hypophysenvorderlappenhormones. *Zbl. Gynäkol.* 61; 1551, 1937.
15. Leznoff, A. and Davis, B. A.: The cytological localization of human chorionic gonadotropin. *Can. J. Biochem.* 41; 2517, 1963.
16. Luft, J. H.: Ruthenium Red and Violet. I Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat. Rec.* 171; 347, 1971.
17. Luft, J. H.: Electron microscopy of cell extraneous coats as revealed by ruthenium red staining. *J. Cell Biol.* 23; 54, 1971.
18. McLean, I. W. and Nakane, P. K.: Periodate-lysineparaformaldehyde fixative. A new fixation for immunoelectron microscopy. *J. Histochem. Cytochem.* 22; 1077, 1974.
19. Midgley, A. R. and Peirce, G. B.: Immunohistochemical localization of human chorionic gonadotropin. *J. Exp. Med.* 115; 289, 1962.
20. Okudaira, Y., Hamanaka, N., Ueda, G., Hayakawa, K., Yoshinara, S., Tanizawa, O. and Kurachi, K.: Electron microscopic study on the secretion of HCG using immunocytochemical technique. *Advanc. Obst. Gynecol.* (in Japanese) 24; 14-26, 1972.
21. Pali, K. and Lajos, L.: Histochemische Untersuchungen der innersekretorischen Tätigkeit der Hypophyse und der Plazenta. *Gynaecologia* 133; 77, 1952.
22. Pearse, A. G. E.: Cytochemistry of the gonadotropic hormones. *Nature (London)* 162; 651, 1948.
23. Rambourg, A.: Morphological and histochemical aspects of glycoproteins at the surface of animal cells. *Int. Rev. Cytol.* 31; 57, 1971.
24. Slater, L. M.: Decreased mortality of murine graft versus-host disease by human chorionic gonadotropin. *Transplantation* 23; 103, 1977.
25. Stewart, H. L. Jr., Sano, M. E. and Montgomery, T. L.: Hormone secretion by human placenta grown in tissue culture. *J. Clin. Endocrinol.* 8; 175, 1948.
26. Teoh, E. J., Spellacy, W. N. and Buhi, W. C.: Human chorionic somatomammotropin (HCS): a new index of placental function. *J. Obstet. Gynec. Brit. Cwlth.* 78; 673, 1971.
27. Wislocki, G. B. and Bennett, H. S.: The histology and cytology of the human and monkey placenta, with special reference to the trophoblast. *Am. J. Anat.* 73; 335, 1943.
28. Zilliacus, H.: Polysaccharide structure in normal chorionic villi, hydatidiform mole and chorioepithelioma, with special reference to the chorionic gonadotropic hormone. *Gynaecologia* 135; 161, 1953.