 原 著

Localization of Placental Alkaline Phosphatase in Cultured Choriocarcinoma Cells

Immuno — and Enzyme Cytochemical Observations

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絨毛癌は、由来する胎盤に類似して、human chorionic gonadotropin (HCG) をはじめとする諸ホルモン、ならびに胎盤性 alkaline phosphatase (ALP) 等の酵素を産生、分泌する。しかしながら、これらの特異ホルモン、酵素の細胞内局在に関する研究はあまりなされていない。そこでわれわれは、教室にて樹立された絨毛癌細胞, GCH-nu 株を用い、HCG ならびに ALP の細胞内局在を免疫細胞化学的に検索した。

HCG は、およそ 50~70% の GCH-nu 細胞の細胞質内にその存在が確認され、BeWo 株に比べかなり多くの細胞で HCG 産生が営まれていることがわかった。

また GCH-nu 細胞の産生する ALP は、生化学的ならびに免疫学的検索から胎盤性 ALP であることが証明され、その細胞内局在は主として細胞表面の microvilli 表層の glycocalyx であることが判明した。一方、この胎盤性 ALP に関しては、免疫細胞化学と酵素細胞化学との間で、その発現様式に差が認められた。すなわち、免疫細胞化学的検索ではほとんどすべての GCH-nu 細胞に胎盤性 ALP が認められたのに対し、酵素細胞化学的検索では細胞が pile up している部位を中心に、一部の細胞にのみ活性が確認された。このことは、酵素活性を示さない細胞の中にも抗原性は有するものが存在することを示すものであり、細胞内における酵素等の存在を検索する上では、酵素細胞化学だけではなく免疫細胞化学的検索も行う必要のあることを意味する。

Key words : Choriocarcinoma—Histocytochemistry—Cell line—Alkaline phosphatase

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Introduction

Cell-lines established from human choriocarcinoma has been known to maintain production of varieties of hormones and other biologically active substances, e. g. human chorionic gonadotropin (HCG) and alkaline phosphatase (ALP)^{4,5,12,13}. The present study was undertaken in order to establish the ability of a cell-line established from gestational choriocarcinoma in

our laboratory to produce isoenzyme of ALP which is specific to placenta. The placental ALP was localized on these cells in vitro by means of enzyme cytochemistry and immunocytochemistry. It was found that all the cells possessed antigenetically active placental ALP on their surfaces but the enzymatic activity of placental ALP was found only on the surfaces of the cells which formed mound and the cells dispersed between the mound were negative for the enzymatic activity.

Materials and Methods

1) Cell-line

A human gestational choriocarcinoma cell-line designated as GCH-nu is used in this study. The line was established at our laboratory from a human choriocarcinoma which was first grown in nude mice in May, 1975. The characteristics of this line, including morphology and the cytogenetics, have been reported elsewhere⁷⁾.

2) Preparation of the cells

For the cytochemic studies the cells were cultured in a Lab-Tek chamber 4802 (Miles, Elkhart, Indiana). The medium change and cytochemical observations were carried out at every other day. The cells cultured in Lab-Tek chambers were washed in 0.01 M sodium phosphate buffered saline (PBS), pH 7.2 at 4°C, at least 5 times, for 3 minutes each and fixed with cold 4% paraformaldehyde (E. Merck, Darmstadt, Germany) in 0.1 M sodium phosphate buffer (SPB), pH 7.4 for 60 minutes. The cells were washed by PBS at least 5 times. Each sample was divided into three groups. One of which was used for enzyme cytochemical staining. The second group was used for immunocytochemical staining. The third group was used for combination of enzyme cytochemical and immunocytochemical staining for the placental ALP.

For the determination of ALP enzymatic activity cells were cultured in separate plastic Petri dishes (60 mm in diameter: Falcon Plastics, Oxnard, Calif.) and harvested by rubber policeman. Approximately 1×10^6 – 10^7 cells were used for each enzyme assay.

3) Enzyme cytochemical staining for ALP

The cytochemical staining was carried out by a modified Burstone's azo dye method¹⁾ using naphthol AS-BI phosphate (Sigma Chemical Co, St. Louis Mo.) as substrate at room temperature for 10 minutes. Some cells were treated with heat 65°C for 30 minutes in a 0.5 mM MgCl₂ solution prior to the cytochemical staining.

4) Immunocytochemical staining for ALP and HCG

The sample fixed with cold 4% paraformaldehyde and washed by PBS were dried at room temperature for 45 minutes. Both the indirect and direct method of the horseradish peroxidase (HRP)-labeled antibody technique was used¹⁰⁾. For the indirect method, the samples were treated with normal goat serum for 30 minutes to prevent nonspecific rabbit antibody deposit on the cell surfaces and then incubated with either rabbit anti-human placental ALP or rabbit anti-HCG for 60 minutes. After incubation samples were washed by PBS for 3 times and incubated with HRP-labeled F(ab')₂ goat anti-rabbit IgG at room temperature for 60 minutes in a moist chamber. The cells were washed by PBS for 3 times and were immersed in Graham-Karnovsky's staining medium³⁾ for 10 minutes. For the direct method, the fixed cells were reacted either with HRP-labeled Fab' of rabbit anti-human placental ALP or Fab' of rabbit anti-HCG for 60 minutes, washed by PBS, and incubated in Graham-Karnovsky's staining medium. As the control, normal rabbit serum was used for the indirect method in place of the specific antisera and for the direct method HRP-labeled Fab' of normal rabbit IgG was used in place of the specific HRP-labeled Fab'.

For the immunoelectron microscopic localization of placental ALP the cells were treated in the similar manner as was for the light microscopic studies, except for following a few differences; (1) the fixative used was 4% paraformaldehyde in 0.1 M SPB pH 7.4 including 8% sucrose at 4°C for 3 hours. (2) the cells were reacted for over night either with HRP-labeled Fab' of rabbit anti-human placental ALP or HRP-labeled Fab' of normal rabbit IgG at 4°C. (3) the cells were washed thoroughly and were refixed in 2% glutaraldehyde in 0.1 M SPB pH 7.4 for 20 min-

utes. The cells were incubated for 30 minutes in Graham-Karnovsky's staining medium from which H_2O_2 was omitted, and then incubated in the fully equipped staining medium for 5 minutes. The cells were osmified in 2% OsO_4 in 0.1 M SPB pH 7.4 for 30 minutes, dehydrated in graded ethanol series and embedded in Epon. Ultrathin sections were prepared with LKB Ultratom III and were observed in JEOL 100 C electron microscope.

5) Biochemical assay for ALP and the effects of inhibitors on ALP activity

For the enzyme assay, the cells were washed in 0.1 M Tris-HCl buffer, pH 7.4 and collected by centrifugation at 2,000 r.p.m. for 5 minutes. The cell pellet was sonicated in the buffer containing 1 mM $MgCl_2$ at 0°C for 60 seconds. The sonicated cells were incubated in the following reaction medium for 20 minutes at 37°C: 2.7 mM p-nitrophenyl phosphate (Wako Pure Chemical Ind., Osaka, Japan) and 1 mM $MgCl_2$ in 25 mM $NaHCO_3$ - Na_2CO_3 , pH 10.0. The reaction was stopped by the addition of 250 μ l of 1 N-NaOH and the amount of p-nitrophenol released was determined by absorbancy at 405 nm.

The heat inactivation test was carried out in the buffer containing 1 mM $MgCl_2$ by heating the cell sonicate at 65°C for 15 minutes and the remaining ALP activity was immediately measured as above and was calculated by comparing with the un-heated sonicate. L-phenylalanine, L-homoarginine and imidazole were also used as inhibitors of the ALP. Five mM of each inhibitor was added to the reaction medium and the remaining enzymatic activity was calculated comparing with the sonicate without any inhibitors.

Results

HCG in GCH-nu Cells

By light microscopy, HCG was demonstrable as dis-

crete dark brown reaction products in approximately 50 to 70 per cent of the GCH-nu cells examined (Figure 1). The rest exhibited little or no HCG staining. Occasionally cells undergoing mitosis displayed intense reactivity. On replacing the anti-HCG antibody with normal rabbit serum (the immunocytochemical control test) the cells were consistently negative for the reaction.

ALP in GCH-nu Cells

The ALP activity of GCH-nu cells has proven to be hardly inactivated by heat at 65°C for 30 minutes in the enzyme cytochemical observation (Figure 2). Biochemical assessment have also shown that 91.2 per cent of initial activity remained after heating at 65°C for 15 minutes (the heat stable enzyme) and that the enzyme is susceptible to L-phenylalanine (Table).

The cells which formed mound displayed positive staining along plasma membrane while cells dispersed between the mound were negative.

Immunocytochemically the enzyme were localized along the cell membrane of all cells (Figure 3). Electron microscopically, the cell surface localization of the ALP was found to coincide with the glycocalyx overlying the microvilli. None of the intracytoplasmic organelles were stained for the enzyme (Figure 5).

The above difference in staining patterns between enzyme cytochemical localization and immunocytochemical localization of placental ALP were further confirmed when the cells stained doubly by enzyme cytochemistry and immunocytochemistry (Figure 4).

Discussion

HCG in GCH-nu Cells

It is universally recognized that HCG is produced by the syncytiotrophoblast of normal placenta and by

Table Effect of various inhibitors applied on the biochemically assayed ALP activity of GCH-nu cells

	Percentage activity remaining after indicated treatment			
	Heat 65°C, 15min	L-Phenylalanine 5 mM	L-Homoarginine 5 mM	Imidazole 5 mM
GCH-nu cell	91.2	10.7	67.5	83.3

Figure 5. Immunoelectron microscopic localization of the placental ALP in the GCH-nu cells (the direct method). The enzyme is mainly localized on the glycocalyx overlying the surfaces of microvilli. ($\times 19,000$)

malignant trophoblastic cells^{8,9,11,15}). Intracellular localization of HCG in BeWo cells was demonstrated by Yorde et al. through an immunocytochemical technique¹⁶). Their study revealed that 6 per cent of the cells were found to be positive for HCG β , although up to 70 per cent of the cells may be stimulated by dibutyryl cyclic AMP and theophylline to produce HCG β . In the present experiment on GCH-nu cells, the HCG staining was evidenced in approximately 50 to 70 per cent of the cells under the unstimulated condition. Even though one may take account of the differences in the techniques employed, the observation suggest that more cells of GCH-nu cell-

line produce HCG than BeWo cell-line.

ALP in GCH-nu Cells

Normal and tumor trophoblastic cells have been known to elaborate placental ALP^{2,13,14}). In the present study, the localization of this isoenzyme was investigated by the application of both enzyme cytochemical and immunocytochemical techniques. The double staining method with these two techniques were also employed.

The ALP in the choriocarcinoma cells of the GCH-nu line was found to be heat stable and markedly susceptible to L-phenylalanine. This indicate that this ALP would be placental isoenzyme. This assumption

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- Figure 1.** Cells of the GCH-nu line immunocytochemically stained for the HCG (the direct HRP-labeled antibody method). HCG is stained in brown. Approximately 50 to 70 per cent of the GCH-nu cells are positively stained. Note the cells undergoing mitosis displayed intense reactivity (arrows).
- Figure 2.** Enzyme cytochemical staining for the heat stable ALP activity of the GCH-nu cells. A portion of mound. The ALP localization is indicated as fine reddish deposits along the cell membrane. ($\times 480$)
- Figure 3.** Immunocytochemical staining (the direct HRP-labeled antibody method) for the placental ALP of the GCH-nu cells. Cells between the mound. Placental ALP is demonstrated as brownish deposits along the cell membrane. All cells are stained positively. ($\times 480$)
- Figure 4.** The dual staining with the enzyme cytochemistry and immunocytochemistry for the placental isoenzyme of ALP of the GCH-nu cells. The enzyme cytochemical staining for the ALP shown as reddish deposits are only seen on cells which formed mounds (piled up cells) (arrows) are cells in between are unstained. In contrast to this, all cells are stained brown at their surfaces by the immunocytochemistry for the isoenzyme. The cells in the mound are doubly stained (reddish brown in color). ($\times 192$)

was substantiated by the fact that those ALP positive cells were also stained by the immunocytochemical technique for the placental ALP.

It is of interest that all the cells of GCH-nu line were stained by the immunocytochemistry but only limited proportions of the cells were proved to have the ALP activity by the enzyme cytochemistry. The possible explanation for this phenomenon could be as follows; (1) The staining sensitivity of the immunocytochemistry is better than that of the enzyme cytochemistry. (2) The antigenicity of the enzyme is more resistant to cytochemical procedures, especially fixation, than the enzyme activity. (3) The enzyme activity may have been suppressed by some agents, e. g. biologically generated inhibitors, or by lacking the enzyme activating agents such as certain metal cations. In this regard, the liver ALP is known to be inhibited specifically by sialic acid⁶⁾. Regardless of the reason for this discrepancy in staining, the absence of staining with enzyme cytochemical procedure does not indicate the absence of the enzyme as an antigen. Thus in characterizing certain cell-line or tissues for an enzyme, enzyme cytochemical approaches alone can be insufficient to establish absence or presence of the enzyme and may require immunocytochemical approaches as well.

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Summary

Placental alkaline phosphatase was localized in human chorionic gonadotropin producing gestational choriocarcinoma cell-line, designated as GCH-nu, using enzyme cytochemical and immunocytochemical techniques.

Light and electron microscopic observations revealed that the alkaline phosphatase (ALP) specific to placenta was found on the surface of microvilli of the cultured cells. With the enzyme cytochemical method, the heat stable ALP was present on the surfaces of cells which

formed mound. While the cells which was spread in-between the mound were negative for the ALP. On the other hand, with the immunocytochemical method, the surfaces of all the cells including those spread between the mound were positive for placental ALP. These observations suggest that all cells possess placental ALP on their surfaces, although those on the surfaces of cells which were spread between the mound were enzymatically inactive but active as an antigen.

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