IMMUNOCHEMICAL STUDY OF M-HeLa CELLS AS PRODUCERS OF HUMAN PLACENTAL ALKALINE PHOSPHATASE

S. O. Gudima, V. V. Lyakhov, A. V. Sokolov, O. P. Terekhov, S. M. Serov, G. T. Sukhikh, and Yu. Yu. Vengerov

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Human placental alkaline phosphatase (HPAP) is a biochemical marker of pregnancy and of tumor growth [7-9]. Ectopic synthesis of HPAP in some cases is effected by tumors of nontrophoblastic origin (carcinoma of the lung and pancreas, seminoma) [5]. The so-called oncofetal antigen is particularly interesting as a marker protein for monitoring tumor development. Not only can the dynamics of development of tumors in particular locations be estimated from the level of HPAP activity in the secretory form, but the effectiveness of antitumor treatment can also be assessed. Detection of HPAP in the composition of the cell membrane can be used to locate individual cells expressing the marker enzyme, among the complete heterogeneous mass of cells.

In this investigation cell line M-HeLa, obtained from carcinoma of the uterine cervix, was used as the model with which to study HPAP. By means of immunoenzyme systems developed by the writers for determination of HPAP, it is also within the scope of this model to characterize secretory and membrane-bound forms of the enzyme and to study the effect of heat shock on biosynthesis and secretion of HPAP.

EXPERIMENTAL METHOD

The preparation of HPAP used as the standard protein in enzyme immunoassay (EIA) and also for immunization, the method of obtaining specific rabbit polyclonal antibodies against HPAP, and development of systems of endogenous EIA for determining specific HPAP enzyme activity were described previously [1, 10]. In the present investigation a version of endogenous EIA was used in which planchets for EIA were sensitized with affinity-purified antibodies to HPAP, after which the enzyme activity of the phosphatase bound with antibodies was assessed [1, 9]. Secretory HPAP was detected with the aid of endogenous EIA.

A preparation of rabbit IgG from "Calbiochem (USA) was used as the standard preparation of normal (nonimmune) antibodies.

Trophoblastic β_1-glycoprotein (TBG) was purified by a combination of chromatographic methods to a homogeneous state [3]. Specific rabbit polyclonal antibodies against TBG were obtained by affinity chromatography on a sorbent with immobilized TBG. The techniques of chromatography and synthesis of the immunosorbent were similar to those described previously [1].

The M-HeLa strain of human cells used in these experiments is a derivative of the HeLa strain obtained some time ago from carcinoma of the human cervix. Adherent M-HeLa cells were cultured in 25-cm^3 culture flasks ("Costar," USA), suing DMEM medium ("Gibco," England) with 5-10% embryonic calf serum, in a CO_2 incubator ("Haeraus," B-5060 EC/O_2, West Germany) in an atmosphere of 5% CO_2, 10% O_2, 85% N_2 at 37°C. HPAP was extracted from the cells during lysis of washed

V. A. Éngel'gardt Institute of Molecular Biology, Academy of Sciences of the USSR. All-Union Research Center for Maternal and Child Health Care, Ministry of Health of the USSR. All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR. Institute of Applied Molecular Biology, Ministry of Health of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR S. E. Severin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 109, No. 3, pp. 285-287, March, 1990. Original article submitted July 26, 1989.

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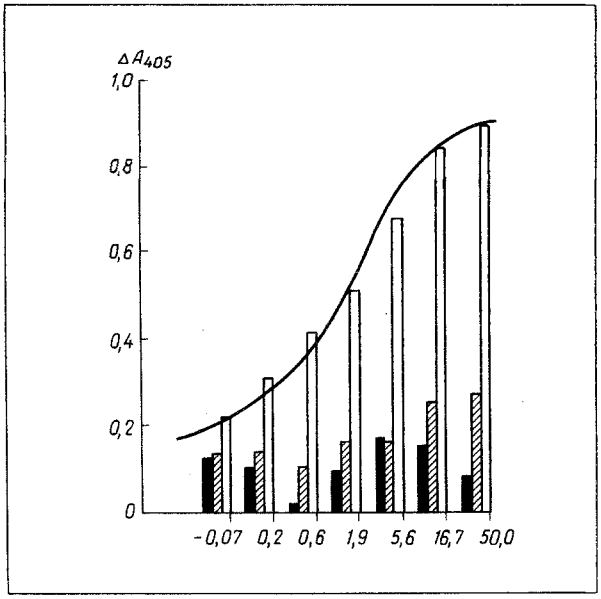

Fig. 1. Detection of membrane-bound HPAP in the composition of M-HeLa cells. Abscissa, IgG concentration, μg/ml; ordinate, ΔA_{405}, optical density, relative units. Unshaded columns — values of ΔA_{405} for binding of anti-HPAP IgG with immobilized cells, shaded columns — ΔA_{405} on addition of non-immune rabbit IgG, black columns — ΔA_{405} on addition of anti-TBG IgG.

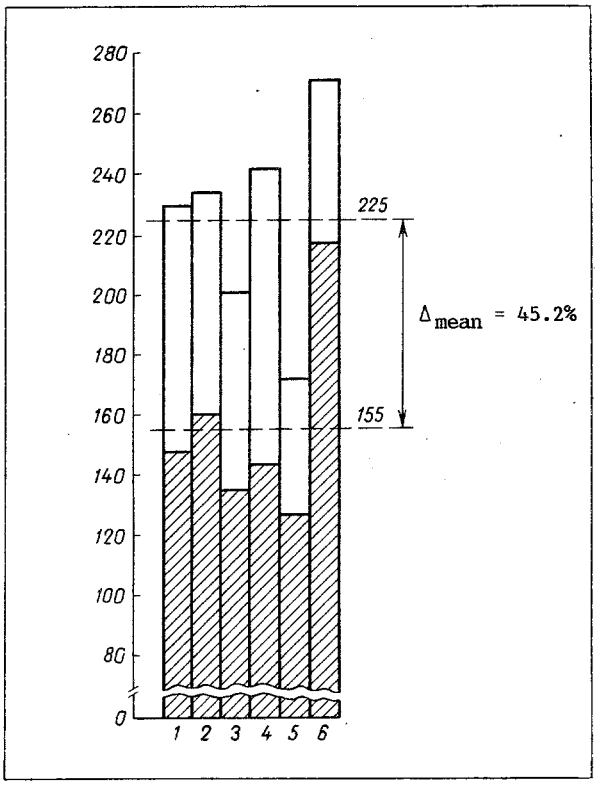

Fig. 2. Effect of heat shock on secretion of HPAP by M-HeLa cells. Abscissa, activity of secretory form of HPAP, U/liter; ordinate, Nos. of samples. Shaded columns denote HPAP activity before heat shock, unshaded columns — HPAP activity after incubation for 15 min at 42°C.

intact cells with extraction buffer [0.01 M K-phosphate buffer containing 0.15 M NaCl, pH 7.4 (buffered physiological saline, BPS), 0.25% Triton X-100]. Heat shock was induced by placing the cells in an incubator at 42°C and incubating them for 15 min.

To determine HPAP in the composition of membranes of whole cells, we developed a modified method of indirect EIA [2]. Planchets for EIA ("Greiner," West Germany) were sensitized with a solution of poly-L-lysine (mol. wt. 78,000 D; from "Sigma," USA) 20 μg/ml in 0.01 M Na-carbonate buffer, pH 9.6, in a volume of 50 μl per well for 17 h at 4°C. At the end, the sample was washed off 5 times with BPS. The M-HeLa cells were removed mechanically from the plate, washed off 5 times with BPS, and the concentration was adjusted with BPS to (1.2-1.4) $\times 10^6$ cells/ml. Next, in volumes of 100 μl this homogeneous suspension was transferred into wells of a 96-well planchet, sensitized with poly-L-lysine. After centrifugation (5 min, 2000 rpm) to form a monolayer of cells on poly-L-lysine, the BPS was accurately withdrawn from the wells. A 0.25% solution of glutaraldehyde in 0.01 M $NaHCO_3$-buffer, pH 9.2, was added to each well in a volume of 75 μl. The samples were incubated for 15 min at room temperature. The liquid was withdrawn from the wells, and the contents were washed 3 times with BPS. The active groups of glutaraldehyde were reduced by the addition of 200 μl of a solution of $NaBH_4$ to the BPS (2 mg/ml). The samples were incubated for 30 min at room temperature and washed twice with BPS. Residual binding sites were additionally blocked with 500 mM glycine, 4 mg/ml bovine serum albumin in BPS for 1 h at room temperature, and then for a further 30 min with the same solution but without Gly, in volumes of 200 μl. Aliquots of 100 μl of solutions of antibodies (to HPAP, to TBG, normal) indifferent concentrations in BPS-BSA (4 mg/ml bovine serum albumin in BPS) were added to the wells previously washed with BPS. The samples were incubated for 1 h at room temperature, washed 3 times with BPS, and then treated with a conjugate of goat IgG ("Sigma," USA) in BPS-BSA. The samples were washed 5 times with BPS after incubation for 1 h and the substrate mixture for peroxidase was added: 100 μl of a solution of ammonium, 2,2′-azino-*bis*-(3′-ethylbenthiazoline-6′-sulfonate; 30 mg/ml in distilled water) to 10 ml of 0.1 M citrate buffer (pH 4.0), containing 0.03% H_2O_2. The results of EIA were recorded on a "Multiscan" scanning spectrophotometer ("Flow Laboratories," England) at 405 nm.

EXPERIMENTAL RESULTS

Ectopically synthesized secretory HPAP was found by endogenous EIA in the culture fluid from M-HeLa cells. Activity of the secretory HPAP was proportional to the number of cells and the duration of culture. Besides the existence of secretory HPAP, the presence of membrane-bound HPAP also was demonstrated by indirect EIA on immobilized cells. Specific binding of antibodies to HPAP with immobilized M-HeLa cells is reflected in Fig. 1. In the case of normal rabbit IgG and antibodies to TBG no effective binding was observed. Consequently, unlike HPAP, TBG is not a component of M-HeLa cell membranes. On the basis of these results the M-HeLa cell line can be regarded as a convenient model with which to study biosynthesis, secretion, and regulation of activity of HPAP.

Besides immunochemical determination of HPAP in M-HeLa cells we also studied the effect of heat shock on biosynthesis and secretion of this marker of tumor growth. We used a model of short-term heat shock (15 min at 42°C). The level of specific enzyme activity of HPAP was assessed in the culture fluid and in cell extract (cellular HPAP).

After 15 min at 37°C virtually no change was found in secretory HPAP activity. Under heat shock conditions activity of secretory HPAP increased on average by 45% (Fig. 2). Meanwhile an increase in activity of cellular HPAP (the cytosol and membrane-bound forms of the enzyme) was observed, on average by 37% (Fig. 3), suggesting that the result of short-term heat shock in M-HeLa cells is stimulation of secretion and biosynthesis of HPAP, or that there is some additional modification of the enzyme in the cell, leading to an increase in the specific enzyme activity of HPAP. The immunochemically demonstrated simultaneous existence of HPAP in the form of an enzymically active secretory polypeptide and an integral membrane glycoprotein creates a basis for analysis of the particular features of HPAP function depending on its localization.

It was shown previously [4, 6] that under conditions of hyperthermia, de novo synthesis takes place of heat shock proteins, the function of which are considered to be: 1) organization of the cellular response to heat shock, 2) the maintenance of thermotolerance of the cells, 3) modulation of the enzyme systems [4]. However, heat shock proteins accumulate in effective amounts during a quite long period of time. Rapid synthesis of thermostable proteins (HPAP) in response to the inducing action of high temperature (15 min, 42°C) can be logically regarded as the primary response of the cells. Stimulation of activity of secretory and cellular HPAP can create objective grounds for the effective dephosphorylation of membrane and cytoplasmic proteins, and may suggest that HPAP plays a role in the organization of the primary response of cells to heat shock and in the maintenance of their thermotolerance.

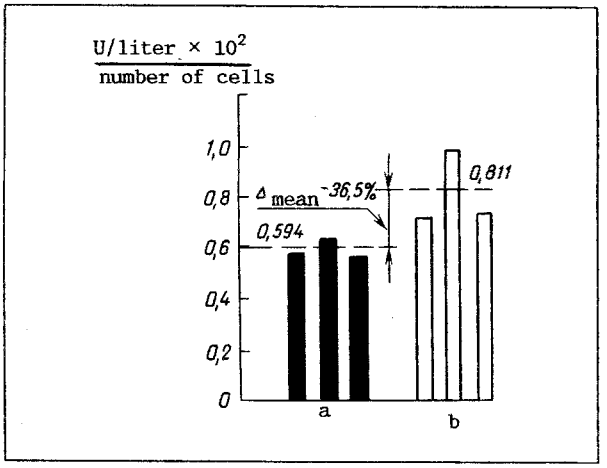

Fig. 3. Effect of heat shock on activity of cellular HPAP in M-HeLa cells. Abscissa, activity of cellular HPAP (U/liter × 10^2) × (number of cells)$^{-1}$; ordinate, a) control samples; b) samples subjected to heat shock.

It is intended later to use HPAP as marker of different states of the M-HeLa model arising as a result of various extra- and intracellular modulating influences.

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