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EPIDERMAL GROWTH FACTOR INHIBITS GROWTH WHILE INCREASING THE EXPRESSION OF AN ECTO-Ca^{2+}-ATPase OF A HUMAN HEPATOMA CELL LINE

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SUMMARY: We have obtained a cloned cell line (Li-7A) from primary cultures of a human hepatoma xenograft (Li-7). Li-7A was able to grow in the absence of serum. Growth was stimulated 0-3 fold by addition of newborn calf serum, but was inhibited in DME/F12 media containing nine growth factors. The ectoMg^{2+}-ATPase was 1.5-2 fold higher than the ectoCa^{2+}-ATPase activity in cells grown in media with or without serum. In cells grown in media supplemented with the nine factors, the ectoCa^{2+}-ATPase activity exceeded the ectoMg^{2+}-ATPase, and there was also a 5-10 fold increase in its specific activity. Inhibition of growth was due to epidermal growth factor alone. The increased expression of the ecto-Ca^{2+}-ATPase was absolutely dependent on EGF, but also required hydrocortisone and cholera toxin. The characteristics of Li-7A cells make it a suitable system for studying both the mechanism of action of EGF and plasma membrane ATPases.

Neoplastic transformation of mammalian cells is often accompanied by alterations of cell surface properties (1, 2). In several studies on newborn Wistar rat-derived RL34 cells and its variants, Karasaki et al. found that an oncogenic cell line possessed an ectoATPase with higher Km and Vmax than the ectoATPases present in nononcogenic and marginally oncogenic cell lines (3, 4). These investigators suggested that the high Km ectoATPase, assayed in the presence of both Ca^{2+} and Mg^{2+}, could be a useful indicator reflecting the lineage and cyto-differentiation of oncogenic liver epithelial cells.

In an earlier study from this laboratory, we found that the growth rate of primary cultures of a human hepatoma cell line (Li-7) in a defined medium contain-

ABBREVIATIONS: DME/F12, a 1:1 mixture of Dulbecco's modified Eagles' medium and Ham's F-12 nutrient mixture; NBS, newborn calf serum; EGF, epidermal growth factor; CT, cholera toxin; dFBS, dialyzed fetal bovine serum; Et, ethanolamine; Gl, glucagon; HC, hydrocortisone; In, insulin; Tf, transferrin; Se, selenium.

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ing nine growth factors was 1.5-2 times greater than that in 10% serum. Furthermore, the cells cultured in factor containing media exhibited a higher ectoMg^{2+}-ATPase activity (5). The enhanced enzyme activity could be ascribed either to rapid cell growth or to the action of the growth factors.

In this report, we present results showing that a cloned cell line (Li-7A) has significantly different responses than the primary culture cells to various growth media. In addition, the activities of both ectoMg^{2+}-ATPase and ectoCa^{2+}-ATPase were also affected differently depending on the culture media.

EXPERIMENTAL PROCEDURES

Cell Culture: Li-7 human hepatoma was established as xenografts in the athymic mice at the National Cancer Center in Japan (6). Some of the properties of this tumor have been described (7). The tumor was received as xenograft at its 50th passage at the Athymic Mouse Facility at the University of California, San Diego, and has been passaged serially at the Facility since 1980.

Primary cultures of Li-7 tumor cells were established from explants of the tumor by protease digestion. Cells from the 4th passage were stored in liquid nitrogen. Clones were obtained from these cultures by limiting dilution, and isolated by cloning cylinders. Li-7A cell line was selected after two cycles of cloning.

Li-7A cells were usually maintained in DME/F12-10% NBS. For experiments, cells were harvested with 0.1% trypsin-0.03% EDTA in phosphate buffer saline, suspended in DME/F12-10% NBS, and plated at $5x10^4$-10^5 cells per 35 mm dish in 2 ml media. In most experiments, a 6-well Costar Cluster[6] dish was used for each experimental condition. After 24 hours, the media were removed, and the cells were washed twice with 2 ml of the various media (DME/F12, DME/F12 containing factors or DME/F12-10% NBS). The media were changed again after 48 hours. Unless otherwise indicated, cell counts and ectoATPase activities were determined on duplicate wells of cells on the 5th day after plating.

When DME/F12 was supplemented with the different factors, the concentrations of the various factors were as follows: cholera toxin, 2 ng/ml; dialyzed fetal bovine serum, 0.05%; ethanolamine, 1 μM; EGF, 50 ng/ml; glucagon, 500 ng/ml; hydrocortisone, 50 nM; insulin, 1 μg/ml; selenium, 25 nM; and transferrin, 1 μg/ml.

ATPase Determination: Prior to the determination of ectoATPase activities, the growth media were removed by aspiration, the cells were washed twice with 2 ml of a solution containing 0.1 M NaCl, 0.01M KCl, and 25 mM TrisCl, pH 7.5, then overlaid with 1 ml of the same solution. Ten microliters of 0.5 M $MgCl_2$ or 0.5 M $CaCl_2$ were added, which was followed by the addition of 25 μl of 0.2 M [γ-^{32}P]ATP ($4x10^4$ cpm/μmol). Thus, the final concentration of the divalent ions and ATP were both 5 mM. The dishes were placed immediately in the 37°C incubator. After 30 min, during which time the cells remained attached to the dishes, the reaction mixture above the cell layer was removed by a Pasteur pipette and added to chilled glass tubes containing 0.1 ml of 50% trichloroacetic acid. ^{32}P was determined as described previously (8, 9).

For protein determination, 1 ml of 0.1 N NaOH was added to the dishes after the reaction mixture was removed. The detached cell proteins from the duplicate wells were collected and combined. The wells were rinsed with 1 ml of water and the wash was added to the protein solution. If there was significant turbidity (due to large amounts of DNA and protein), the solution was sonicated briefly. Suitable aliquots of this clarified solution (50-200 μl) were used for protein determination (10) using bovine serum albumin as protein standards.

RESULTS

Results from Experiment 1, Table I, show that DME/F12 media with or without 10% NBS could support the growth of Li-7A cells. The number of cells grown in DME/F12 for 5-7 days ranged from 30-100% of the number of cells grown in DME/F12 containing serum. On the contrary, DME/F12 containing the nine factors were much less effective in supporting cell growth. These cells also exhibited higher ectoATPase activities. While the ectoMg^{2+}-ATPase activity increased by 4 fold, the specific activity of ectoCa^{2+}-ATPase increased by 8 fold.

Results obtained from Experiment 2, Table I, show that (i) EGF alone exerted the same growth inhibitory activity as the combination of nine factors, and (ii) the increase in the activities of the ectoATPases required EGF and some other component(s) in the remaining eight factors, since either alone caused little change in the enzyme activities.

In a search for the minimal number of factors necessary for the increased expression of the ectoATPases, experiments designed at eliminating the unnecessary factors were conducted. An increase in ectoATPase activities was observed when cells grew in media containing hydrocortisone and EGF, in addition to transferrin, insulin, cholera toxin, and dialyzed fetal bovine serum (Table II, Experiment 1). Of the latter four factors, cholera toxin turned out to be the

TABLE I. GROWTH AND ectoATPase ACTIVITIES OF Li-7A CELLS MAINTAINED IN DIFFERENT GROWTH MEDIA

Experiment No.	Growth media	Cell number (x 10^6)	Mg^{2+}-ATPase (μmol Pi/30 min/mg protein)	Ca^{2+}-ATPase (μmol Pi/30 min/mg protein)
1	10% NBS	1.6	1.45	0.9
	DME/F12	1.2	1.13	0.62
	DME/F12 + 9 factors	0.1	4.1	7.47
2	10% NBS	2.1	0.95	0.69
	DME/F12	1.02	0.61	0.41
	DME/F12 + 9 factors	0.06	3.47	6.13
	DME/F12 + 8 factors (no EGF)	0.4	0.32	0.33
	DME/F12 + EGF	0.03	0.38	0.34

In experiment 1, cells were seeded at $5x10^4$ per plate; cell number and ATPase activities were determined on the eighth day. In experiment 2, cells were seeded at $1x10^5$ per plate; cell number and ATPase activities were determined on the sixth day.

TABLE II. GROWTH AND ectoATPase ACTIVITIES OF Li-7A CELLS MAINTAINED IN MEDIA CONTAINING DIFFERENT GROWTH FACTORS

Experiment No.	Growth media	Cell number ($\times 10^6$)	Mg^{2+}-ATPase (μmol Pi/30 min/mg protein)	Ca^{2+}-ATPase (μmol Pi/30 min/mg protein)
1	10% NBS	0.77	1.20	0.81
	DME/F12 + 8 factors (no EGF)	0.60	0.94	0.30
	DME/F12 + 4 factors* + Gl + HC	0.33	0.83	0.23
	" + Gl + HC + EGF	0.03	4.20	6.50
	" + Se + Et	0.82	0.86	0.48
	" + Se + Et + EGF	0.02	2.48	2.91
	" + Gl + HC + Se	0.67	0.53	0.35
	" + Gl + HC + Se + EGF	0.06	3.68	5.56
	" + Gl + Se + Et	0.84	0.56	0.41
	" + Gl + Se + Et + EGF	0.03	1.92	2.61
	" + HC + Et + Se	0.82	0.50	0.32
	" + HC + Et + Se + EGF	0.07	3.01	5.01
2	10% NBS	1.28	1.71	1.09
	dFBS + CT + In + Tf + HC	0.90	0.47	0.31
	dFBS + CT + In + Tf + HC + EGF	0.09	2.53	3.54
	CT + In + Tf + HC	0.83	0.61	0.41
	CT + In + Tf + HC + EGF	0.09	2.62	5.21
	dFBS + In + Tf + HC	1.09	0.8	0.39
	dFBS + In + Tf + HC + EGF	0.07	0.54	0.33
	dFBS + CT + Tf + HC	0.75	0.50	0.34
	dFBS + CT + Tf + HC + EGF	0.09	2.74	3.96
	dFBS + CT + In + HC	0.74	0.45	0.35
	dFBS + CT + In + HC + EGF	0.07	2.0	2.89

*4 factors: Transferrin, insulin, cholera toxin, dialyzed fetal bovine serum.

requisite factor since the increase in the ATPase activities was not observed when cholera toxin was left out of the media (Table II, Experiment 2). While some increase of ATPase activity was obtained with only cholera toxin and EGF, maximal activity was only observed when hydrocortisone was also present (Table III).

Both growth inhibition and increase in ATPase activities were dependent on the concentration of EGF in the media (Figure 1). While the number of cells grown in media containing 50 ng EGF/ml was 10% of the cells grown in the absence of EGF, the protein content of the cells was only reduced by 50%. It is apparent that EGF had differential effects on the ectoCa^{2+}-ATPase and ectoMg^{2+}-ATPase.

DISCUSSION

Many transformed cells have a reduced serum requirement for their growth (11). The human hepatoma Li-7A cell line is distinguished by its ability to

TABLE III. GROWTH AND ATPase ACTIVITIES OF Li-7A CELLS MAINTAINED IN MEDIA CONTAINING HYDROCORTISONE, CHOLERA TOXIN, AND EPIDERMAL GROWTH FACTOR

Growth media	Cell number (x 10^6)	Mg^{2+}-ATPase (μmol Pi/30 min/mg protein)	Ca^{2+}-ATPase (μmol Pi/30 min/mg protein)
10% NBS	1.12	1.14	0.77
DME/F12	1.11	0.75	0.58
DME/F12 + HC	1.13	0.79	0.58
DME/F12 + HC + EGF	0.04	0.45	0.26
DME/F12 + CT	0.86	0.59	0.55
DME/F12 + CT + EGF	0.05	1.16	1.66
DME/F12 + HC + CT	0.86	0.57	0.48
DME/F12 + HC + CT + EGF	0.04	3.0	3.7

proliferate in media lacking any mitogen after initial plating in serum containing media. It has been hypothesized that these autocrine systems probably utilize ectopically produced growth factors in place of serum mitogens (12). In the case of Li-7A cells, it is likely that the growth factors belong to the class of transforming growth factors (13). We have observed induction of mouse sarcomas in mice carrying the Li-7 human hepatoma xenograft (14), as well as proliferation of mouse fibroblasts in primary cultures of Li-7 cells. A possible

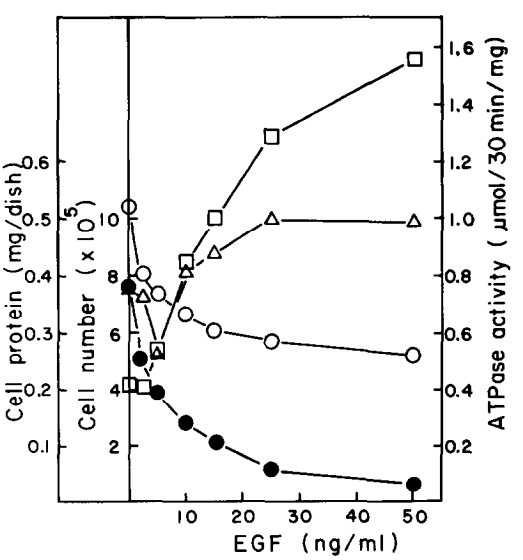

Figure 1. Concentration dependence on EGF of growth inhibition and enhancement of ectoATPase activities of Li-7A cells. In this experiment, the growth media (DME/F12) contained cholera toxin (2 μg/ml), hydrocortisone (50 nM), and the indicated amounts of EGF. Cell number (●-●); cell protein (o-o); ectoMg^{2+}-ATPase, (Δ-Δ); ectoCa^{2+}-ATPase (□-□).

explanation is that transformation of mouse stromal cells occurred in the presence of transformation growth factors manufactured by the human hepatoma cells. Indeed, such activity has been found in the extract of the Li-7 tumors (15).

Li-7A cells have the additional characteristic of growth inhibition by EGF. A notable example of a cell line whose growth is inhibited by EGF is the A-431 epidermoid carcinoma cells. Kawamoto et al. have established a correlation between inhibition of growth by EGF and the number of receptors on the A-431 cells (16). It is interesting to note that the number of EGF receptors of Li-7A cells is ten times greater than that on the parent Li-7 line (17). Thus, Li-7A cells should provide another useful system in the study of the mechanism of action of EGF.

One of the effects of EGF in the Li-7A system is an increase in the ecto-ATPase activities. EctoATPases appear to be ubiquitous. However, no distinction has been made between the ectoMg^{2+}-ATPase and the ectoCa^{2+}-ATPase. Based on the results presented in this report, it appears most likely that these two activities are due to two separate enzymes. We are currently in the process of establishing the relationship of the ectoCa^{2+}-ATPase with the low affinity Ca^{2+}-ATPase which was recently purified from human tumor plasma membranes in this laboratory (9).

With respect to activation of enzyme activities by growth factors, the effect of EGF on the tyrosine protein kinase activity of the EGF receptor is well established (18), which could be dissociated from its growth promoting activity in the A-431 cells (19). It is interesting that in the case of cholera toxin, the induction of some enzymes in a rat hepatoma cell line has been suggested as a result of protein kinase activation by cholera toxin (20). However, the manner in which the combination of EGF, cholera toxin, and hydrocortisone induce an increased activity of the ectoCa^{2+}-ATPase in Li-7A cells remains to be determined.

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