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Brief Communication

Adenosine 3′,5′-Cyclic Monophosphate and Contact Inhibition

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SUMMARY

Adenosine 3′,5′-cyclic monophosphate (cAMP) and cyclic nucleotide phosphodiesterase were assayed in strain L cells from the 2nd to the 6th day of incubation. cAMP increases markedly when the culture reaches confluency on the 4th day of incubation. The phosphodiesterase increased in a similar but less dramatic manner. cAMP and cyclic nucleotide phosphodiesterase were measured in two nontumorigenic cell lines and five tumorigenic cell lines at confluency. The level of cAMP is higher in the nontumorigenic cultures and is related to the population-doubling time of the cells. It is proposed that contact inhibition of growth may be mediated by activation of adenyl cyclase in the cell membranes with subsequent rise in cellular cAMP.

INTRODUCTION

Cells in culture divide at a markedly reduced rate in the presence of cAMP.[2] This inhibition is readily reversed by removal of the cyclic nucleotide, and no apparent cell damage results from the treatment (10, 16). The inhibitory effect of cAMP varies considerably depending upon the cell type. Tumorigenic cell lines are inhibited 80 to 90% by cAMP, whereas a nontumorigenic cell line was inhibited 13% or less (10). Sarcoma cells regain some of the morphology and growth characteristics of normal fibroblasts when incubated with cAMP (11). Polyoma-transformed cells have a lower level of adenyl cyclase than untransformed cells (6). These experiments suggest that decreased levels of cAMP may be associated with transformation and increased cell division. This investigation reports the intracellular concentration of cAMP and phosphodiesterase in 5 tumorigenic and 2 nontumorigenic cell cultures.

MATERIALS AND METHODS

Cell Lines. The cells used were strain L (NCTC clone 929), HeLa (line 229), HEp-2, FL Amnion, Ehrlich ascites tumor cells, WI-38, and Fibro-5, a primary cell line derived from human foreskin tissue. All the cell cultures were maintained in Eagle's medium as previously described (16).

cAMP Assay. The cAMP level in each cell line was measured by the method of Brooker *et al.* (5). The cells were grown in T-60 culture flasks seeded with 1 million cells/flask in 20 ml of medium. After 4 days of incubation, the medium was removed, and the cell layer was rinsed 4 times with 0.85% NaCl solution and quickly frozen at –90°. After thawing, 2.5 ml of cold 0.6 N perchloric acid containing a tracer amount of cAMP-^{3}H (4.86 Ci/mmole; New England Nuclear, Boston, Mass.) were added. The cell layer was scraped off the flask with a silicone scraper, and the cell suspension was transferred to a centrifuge tube. The flask and scraper were rinsed, first with 2.5 ml of the 0.6 N perchloric acid and then twice with 1.0 ml of water. The rinses were added to the centrifuge tube, and the cell suspension was frozen and thawed 5 times at –90° to lyse the cells. The sample was centrifuged, and the supernatant was used for the assay procedure. Cell counts were determined in flasks grown under identical conditions by detaching the cells with 0.25% trypsin and counting in a Coulter counter.

Cyclic Nucleotide Phosphodiesterase Assay. The amount of phosphodiesterase present in the cell lines was determined by the methods of Butcher *et al.* (7) and Butcher and Sutherland (8). Leighton tubes were seeded with 1×10^5 cells/tube in 2.0 ml of medium. The medium was removed after 4 days of incubation, and the cell layer was rinsed 4 times with cold 0.85% NaCl solution to remove any phosphate contained in the medium. The cells were scraped from the glass with a silicone scraper into 0.7 ml of imidazole buffer (1 mM $MgSO_4$ and 1 mM imidazole, pH 7.5). The suspended cells were frozen and thawed 6 times at –90° to lyse the cells, and an aliquot of the lysate was incubated with cAMP for the analysis. Cells were also incubated without cAMP to determine the 5′-AMP and inorganic phosphate, and this value was subtracted from the total.

Protein Determination. Total cellular protein in the cell cultures was measured with the modification by Oyama and Eagle (14) of the method of Lowry *et al.*

Population-doubling Time. The population-doubling time of each cell line was determined from the rate of growth between the 2nd and 4th days of incubation of a culture inoculated with 8.2×10^3 cells/sq cm. The population-doubling time was calculated by the following formulas.

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[1] Predoctoral USPHS trainee (2T1HD18010).

[2] The abbreviation used is: cAMP, adenosine 3′,5′-cyclic monophosphate.

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$$\text{No. of divisions} = (\log_{10} N_1 - \log_{10} N_0)/\log 2$$

where N_0 is the number of cells found initially and N_1 is the number obtained at a subsequent time.

Population-doubling time = hr of growth/no. of divisions

RESULTS

In our preliminary experiments, it became apparent that considerable variation in cAMP occurred that appeared to be related to the age of the culture. Measurable quantities of cAMP were found only in older cultures. For this reason, cAMP was measured each day for 6 days in a strain L culture. The same number of cells were analyzed for each day by pooling the cells from several flasks for the assays on Days 2 and 3. It is apparent from this experiment (Table 1) that the amount of cAMP increases sharply on the 4th day of incubation, which coincides with the development of a stationary population and with confluency of the cell sheet. Phosphodiesterase also increases in a similar but less dramatic fashion.

Because measurable quantities of cAMP were found in strain L cells only after 4 days of incubation, the remainder of the cell cultures were analyzed only on the 4th day. A comparison of the cAMP and phosphodiesterase levels of the tumorigenic and nontumorigenic cell cultures is presented in Table 2. These data demonstrate that there exists an apparent parallel between population-doubling time and the level of cAMP. The shorter the doubling time is the lower is the level of cAMP. Further, it can be seen that the 2 nontumorigenic cell cultures have considerably higher levels of cAMP than the transformed, tumorigenic cell cultures. There is no correlation between the phosphodiesterase and the type of cell or population-doubling time. The phosphodiesterase is interesting because of the wide variation found in the different cell cultures.

DISCUSSION

Abercrombie and Heaysman (1) described contact inhibition of movement, in which fibroblasts are inhibited in their ability to migrate over each other; many malignant cells are not inhibited in this way. Cell cultures, when grown on a glass surface, tend to grow until the culture reaches confluency, after which they divide at a markedly reduced rate. This is known as contact inhibition of growth (19). The various forms of contact phenomena have been reviewed by Weiss (21).

Cells transformed from normal to malignant by polyoma virus, simian virus, carcinogenic hydrocarbons, or X-irradiation demonstrate a loss of contact inhibition and a growth pattern that is more random than that of normal cells (2–4, 17, 18, 20).

The cause of the various forms of contact inhibition is

Table 1

cAMP and phosphodiesterase in strain L cells

The cAMP and phosphodiesterase values are the averages of 5 determinations.

Incubation time (days)	Cell count		cAMP		Phosphodiesterase	
	Cells/L-tube × 10^6	Cells/T-flask × 10^6	μμmoles/cell × 10^6	μμmoles/mg protein	Units/cell × 10^6	Units/mg protein
0	0.100	1.000				
1	0.156				0.0	0.0
2	0.349	2.727	0.0	0.0	0.05	0.34
3	0.860	6.413	0.0	0.0	0.09	0.57
4	1.518	11.530	10.7	55.2	0.36	1.86
5	1.780	13.400	13.8	61.2	0.63	2.80
6	1.875	13.100	13.9	63.1	0.78	2.99

Table 2

cAMP and phosphodiesterase in 4-day-old cultures

The cAMP and phosphodiesterase values are the averages of 5 determinations.

Cell line	Population-doubling time (hr)	cAMP		Phosphodiesterase	
		μμmoles/cell × 10^6	μμmoles/mg protein	Units/cell × 10^6	Units/mg protein
Strain L	20	10.7	55.2	0.36	1.86
Ehrlich ascites tumor	24	16.3	65.1	0.08	0.33
HeLa	25	15.8	59.8	0.21	0.78
FL Amnion	26	19.1	70.7	0.58	2.16
HEp-2	26	23.9	106.7	0.04	0.19
Fibro-5	35	28.5	101.4	<0.01	<0.04
WI-38	55	30.8	80.1	0.35	0.92

unknown, but the following evidence suggests that the cellular concentration of cAMP is related to this phenomenon. Our previous investigations have demonstrated the inhibitory properties of cAMP and its derivatives (10, 15, 16). Bürk (6) has found that transformation of cells by polyoma virus decreases adenyl cyclase. Recently, Johnson *et al.* (11) found that, when sarcoma cells in culture were treated with cAMP or its derivatives, several of the morphological and growth characteristics of normal fibroblasts were regained.

In the present investigation, no measurable quantities of cAMP were found in strain L cells until contact of the cells occurred. Then, at confluency a marked rise in cAMP occurred. In addition, the normal cells with the longest population-doubling time have the highest concentration of cAMP, which also suggests a relationship between the rate of cell division and the cellular level of cAMP.

The increase in cAMP in the cells upon contact may result from activation of the adenyl cyclase of the cell membrane (9, 11). Changes in cell membranes have been described for cells transformed by chemical carcinogens and polyoma virus (21). In addition, embryonic tissue cells and neoplastic cells differ in their surface properties. This difference may be responsible for the altered response to cell contact and their organization (13).

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