Growth Control and Adenosine 3':5'-Cyclic Monophosphate Levels in Clonal Cells of Rat Glioma

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Growth regulating effect of adenosine 3':5'-cyclic monophosphate (cAMP) or its derivatives, especially dibutyryl adenosine 3':5'-cyclic monophosphate (DBcAMP), on cultured mammalian cells has been reported since 1968.^{4,7),10) 15)}. The action of cell differentiation of these substances has also attracted much attention of many investigators^{11),12),17)}.

Recently, the correlation between those actions of growth inhibition and cell differentiation has been becoming a research project.^{3,16),18)}. The estimation of intracytoplasmic level of cAMP correlated with the growth stage or the cell cycle phase of cultured cells seems to be an important method to analyse the mechanism of action of cAMP.

Neuronal cells have been observed especially from the view point of cell differentiation because of their characteristic indicators for the differentiated grades, i.e. the formation of cell process,^{6),14)}. We used a well established clone of rat glioma—C-6^{1),2)}.

2×10^6 cells were plated in each Falcon Maxi-dish and incubated in CO₂-incubator (5%CO₂ & 95%air) at 37°C. Dulbecco modified Eagle's minimal essential medium was used with 20% fetal calf serum. As exogenous administration, the dibutyryl derivatives of cyclic AMP were used, in the expectation that it would penetrate cell membrane more effectively than the unsubstituted one and might be less susceptible to the hydrolytic action of phosphodiesterase inside the cell. And moreover, the action of theophylline which is an inhibitor of phosphodiesterase was also studied. The final concentrations of 10^{-3} , 10^{-4} and 10^{-5} M of those substances were added in the culture medium. The medium was changed every 3 days, 12 hours before the measurement. The morphological changes of the cells were observed every day by phase contrast microscope.

The measurements of intracytoplasmic level of cAMP were performed on the 1st, 4th, 7th and 10th days after the plating of the cells. The procedure of the preparation for these measurements is as follows: The cells were washed 3 times with physiological saline. Added 2 ml of 5%TCA. Removed the cells from dishes with Rubber Policeman. Homogenized the collected cells by Turrax homogenator for 2 minutes in room temperature. And they were centrifugated 15,000 rpm at 4°C. The supernatant was used for the measurement of cAMP according to Gilmen's method,⁸⁾ while the sediment for the soluble protein estimation by Lowry's method¹³⁾.

The cell number was counted at the same time by following method: The cells were trypsinized by 10ml of 0.25% trypsin for 15 min. in 37°C, centrifugated 1,000

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Fig. 1 C-6 cells, cultured for 10 days after plating. (Non-treated). The cells are in confluence with piling up. (Phase contrast $\times 160$).

Fig. 2 C-6 cells, cultured for 10 days after plating. 10^{-8} M DBCAMP was administered also for 10 days. Elongation of cell bodies is remarkable. Long fine cell processes are forming net-works. (Phase contrast $\times 160$).

Fig. 3 C-6 cells, cultured for 10 days after plating. 10^{-3}M theophylline was administered for 10 days. Between the elongated cells, a lot of round cells remain. (Phase contrast $\times 160$).

Table 1. Intracytoplasmic level of adenosine 3':5'-cyclic monophosphate in C-6. (pM/mg. protein)

Days after plating	1	4	7	10
Control	90.0	14.5	34.4	120.6
10^{-3}M DBcAMP	290.0	40.5	74.6	175.3
10^{-4}	88.0	15.2	—	120.0
10^{-5}	67.4	14.0	—	52.5
10^{-3}M theophylline	103.0	22.3	—	166.1
10^{-4}	89.1	9.3	92.3	101.5
10^{-5}	123.1	15.1	61.2	—

rpm in room temperature, and suspended in medium and counted the number by Coulter Counter each 3 times.

By the action of 10^{-3}M DBcAMP, the cells are elongated and the fine long cell processes are increased forming net-works. These changes begin between 3-12 hours after the administration and accomplished within 24-36 hours. And even in the stationary stage of culture, they do not pile up, but keep their characteristic glial feature (Fig. 2); while the non-treated control cells grow rapidly without contact inhibition phenomenon as shown in Fig. 1. 10^{-3}M theophylline acts in similar manner, although it is weaker than DBcAMP. In Fig. 3 a lot of remaining round cells are noticed. By the administration of the lower concentration of both substances, these morphological changes could not be observed.

The results of the measurement of intracytoplasmic level of cAMP are shown in

Fig. 4

table 1. Also in this study, lower concentrations (10^{-4} , 10^{-5} M) of DBcAMP and theophylline did not act as a growth inhibitory factor. Our data in 1st and 10th days reveal remarkably high level, but those of 4th and 7th days show low level which agree with other reports^{5),9)}. The measurements of control solution of 10^{-6} , 10^{-7} M of DBcAMP and theophylline by the same method did not show significant counting of H^3 cAMP. Therefore, contamination of DBcAMP in the medium through the washing procedure must not be considered.

The correlation between the growth curve of C-6 cells and the intracytoplasmic level of cAMP is demonstrated in Fig. 4. In the control culture, the cells proliferate rapidly and reach in confluence within 6 or 7 days. On the contrary, 10^{-3} M DBcAMP treated cells increase in number slowly, showing almost no logarithmic phase. In 10^{-3} M theophylline treated culture, the cell growth curve is nearly the same to the latter, although a little more rapid than that. The intracytoplasmic level of cAMP in the control culture, showed relatively high level at early stage after plating of the cells, and low level during the logarithmic phase of the growth. And in the confluent stage, it raised up again. In the cultures of those which are treated by 10^{-3} M DBcAMP and 10^{-3} M theophylline, the growth curves do not reveal the definite logarithmic stage. Nevertheless, the intracytoplasmic level of cyclic AMP showed similar tendency to those of control culture, although they are always higher. The rapid proliferation of control cells corresponds to the lower level of intracytoplasmic level of cAMP and the suppressed growth of 10^{-3} M DBcAMP treated cells to the higher level of that, and between them, the curves of 10^{-3} M theophylline treated culture were plotted.

From these results, it might be assumed that in the earlier stage of culture, the intracytoplasmic cAMP would be consumed remarkably. When it reaches the lowest

level, the tumor cells proliferate without any inhibition. The reason why the intracytoplasmic levels of cyclic AMP increase again in the confluent stage has not been cleared out exactly. We could assume that, some intracytoplasmic feed-back mechanism regulated by the balance of adenylylase and phosphodiesterase may exist and make it possible to re-accumulate cyclic AMP inside the cell, and as a result, to lead them to the differentiation rather than the proliferation. Exogenously administered DBcAMP or theophylline might facilitate this re-accumulation and lead the tumor cells to the direction of cell differentiation. The phenomenon of contact inhibition was supposed to be correlated to this mechanism as reported by several authors^{4), 11), 12), 16)}. The effort for solving this problem should be continued further associated with the changes of the cell surface property and the cell cycle time under the influence of exogenously administered cyclic AMP.

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References

- 1) Benda, P., Lightreilly, J., Sato, G., Levine, L. and Sweet, W.: Differentiated Rat Glial cell strain in tissue culture. *Science*, 162:370, 1968.
- 2) Benda, P., Someda, K., Messer, J. and Sweet, W. H.: Morphological and Immunohistochemical studies of rat glial tumors and clonal strains propagated in culture. *J. Neurosurg.* 34:310-323, 1971.
- 3) Burger, M. M., Bombik, B. M., Breckenridge, B. M. and Sheppard, J. R.: Growth control and cyclic alterations of cyclic AMP in the Cell cycle. *Nature New Biol.*, 239: 161-163, 1972.
- 4) Bürk, R. R.: Reduced adenylylase activity in a polyoma virus transformed cell line. *Nature*, 219:1272-1275, 1968.
- 5) Ebadi, M. S., Weiss, B. and Costa, E.: Distribution of cyclic adenosine monophosphate in rat brain. *Arch. Neurol.*, 24:353-357, 1971.
- 6) Furmanski, P., Silverman, D. J. and Lubin M.: Expression of differentiated functions in mouse neuroblastoma mediated by dibutyryl-cyclic adenosine monophosphate. *Nature*: 233:413-415, 1971.
- 7) Gericke, D.: Inhibition of tumor growth by nucleoside cyclic 3',5'-monophosphates. *Hoppe-Seyler's Z. Physiol. Chem.*, 350:1469-1471, 1969.
- 8) Gilman, A. G.: A protein binding assay for adenosine 3':5'-cyclic monophosphate, *Proc. Natl. Acad. Sci.*, 67:305-312, 1970.
- 9) Gilman, A. and Nirenberg, M.: Effect of catecholamines on the adenosine 3',5'-cyclic monophosphate concentrations of clonal satellite cells of neurons. *Proc. Natl. Acad. Sci.*, 2165-2168, 1971.
- 10) Heidrick, M. L. and Ryan, W. L.: Cyclic Nucleotides on cell growth in vitro. *Cancer Res.*, 30:376-378, 1970.
- 11) Hsie, A. W. and Puck, T. T.: Morphological transformation of chinese hamster cells by dibutyryl adenosine cyclic 3':5'-monophosphate and testosterone. *Proc. Natl. Acad. Sci.*, 68:358-361, 1971.
- 12) Johnson, G. S., Friedman, R. M. and Pastan, I.: Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine-3':5'-cyclic monophosphate and its derivatives. *Proc. Natl. Acad. Sci.*, 68:425-429, 1971.
- 13) Lowry, O. L., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193:265-275, 1951.

- 14) Prasad, K. N. and Hsie, A. W.: Morphologic differentiation of mouse neuroblastoma cells induced in vitro by dibutyl adenosine 3':5'-cyclic monophosphate. *Nature* 233: 141-142, 1971.
- 15) Ryan W. L. and Heidrick, M. L.: Inhibition of cell growth in vitro by adenosine 3':5'-monophosphate. *Science*, 162:1484-1485, 1968.
- 16) Seifert, W. and Paul, D.: Levels of cyclic AMP in sparse and dense cultures of growing and quiescent 3T3 cells. *Nature New Biol.*, 240:281-283, 1972.
- 17) Sheppard, J. R.: Restoration of contact-inhibited growth to transformed cells by dibutyl adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci.*, 68:1316-1320, 1971.
- 18) Sheppard, J. R.: Difference in the cyclic adenosine 3',5'-monophosphate levels in normal and transformed cells. *Nature New Biol.*, 236:14-16, 1972.