THE EFFECT OF CYCLIC AMP AND PROSTAGLANDINS ON THE FIBRINOLYTIC ACTIVITY OF MOUSE NEUROBLASTOMA CELLS

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SUMMARY. The C1300 mouse neuroblastoma cell line was found to produce plasminogen activator which is secreted into the growth medium. The intra- and extracellular activities of this enzyme were markedly increased (up to 14 fold) by treatment with cyclic AMP agents. Prostaglandins E_1 and E_2 and butyric acid were the most efficient inducers followed by propionic acid and dibutyryl cyclic AMP. Theophylline was found to be ineffective. The highest enzyme activities were found in cells exposed simultaneously to prostaglandin E_1 and dibutyryl cyclic AMP.

INTRODUCTION. Many malignant cells grown *in vitro* produce a plasminogen activator which appears to be associated with the transformed phenotype (1-4). This activity is found in transformed cells derived from several tissues and species including human neuroblastoma (5). Since neuroblastoma cells can be induced to "differentiate" and change their enzymatic functions by cyclic AMP agents *in vitro* (6), we decided to study the effects of these agents on the fibrinolytic activity of mouse C1300 neuroblastoma cells. We were particularly interested in the possibility that these agents might influence fibrinolytic activity, since we have recently found that the secretion of plasminogen activator can be induced *in vivo* (7). Also, Wigler et. al. (8) have shown that glucocorticoids can strongly inhibit the fibrinolytic activity of tumor cells.

METHODS. The uncloned C1300 line was kindly provided in passage 62 by J.Z. Finkelstein, Harbor General Hospital, Torrance, Cal. The cells were grown in Eagle's Minimum Essential Medium (GIBCO) supplemented with 10% (v/v) FCS and penicillin and streptomycin (100 units/ml). Cells ($5x10^5$) were seeded into 100 mm tissue culture dishes obtained from Falcon, Oxnard, Cal. The growth

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Abbreviations used: FCS, fetal calf serum; PBS, phosphate buffered saline; dbcAMP, N^6, O^2-Dibutyryl Adenosine 3':5'-cyclic monophosphoric acid; PGE_1 and PGE_2, prostaglandins E_1 and E_2.

medium was changed to test medium 24 hr later. Test medium always contained 10% (v/v) FCS since FCS does not interfere with the fibrinolysis assay but, on the contrary, appears to stimulate protease secretion (7). Control cultures were fed with the growth medium supplemented with calcium and magnesium free PBS (GIBCO) or 96% ethanol respectively at the same concentration as used as solvents for the cyclic AMP-agents. dbcAMP (Sigma), theophylline (Calbiochem), butyric acid and propionic acid were dissolved in PBS and added to the growth medium. PGE_1 and PGE_2 (personal gifts from Dr. J. Pike, Upjohn Co.,Kalamazoo, Mich.), were dissolved in 96% ethanol. Growth medium supplemented with the solvents and compounds respectively was changed after 48 hr incubation at 37^{o}. Extracellular fibrinolytic activity was determined in aliquots of the supernatant taken after 96 hr. At the same time photomicrographs were made of dishes seeded with 10^5 cells and the percentage of "differentiated" cells determined on a minimum sample of 100 cells (9).

After 96 hr of exposure to the compounds the medium was removed and the cell cultures washed once with PBS. The cells were then either trypsinized and counted in a Coulter counter or scraped off the dish with a rubber policeman when they were to be lysed for fibrinolysis assays. Cellular protein levels were determined by scraping the cells directly into cold 5% perchloric acid and determining the protein by the method of Lowry et. al. (10).

Intracellular plasminogen activator was determined in cell lysates prepared with 0.5% Triton X-100 as previously described (7). Lysates(10-20 ul) were incubated with 2.5 ml of freshly prepared 0.1M Tris-HCl pH 8.1 containing 1% acid treated pooled human serum as plasminogen source on [^{125}I] fibrin plates. The appearance of radioactivity in the supernatant was used as a measure of fibrinolytic activity with one unit of activity defined as previously (7). The plasminogen activator secreted by the cells was assayed in 10-20 ul aliquots of growth medium incubated on [^{125}I] fibrin plates exactly as outlined above.

RESULTS. Table 1 shows the effect of these agents on the morphology and growth of C1300 cells. All of the compounds increased the number of cells with extensions ("neurites") after 96 hr exposure. The number of cells with extensions was not simply related to the growth inhibitory effects of the chemicals, since the most inhibitory compound (butyric acid) did not induce a particularly high level of "neurites." Also, dbcAMP which did not severly inhibit growth, caused a higher percentage of extensions. The highest level of "neurite" outgrowth was seen in cultures exposed to both PGE_1 and dbcAMP and these two compounds caused considerable growth inhibition. Table 1 also shows that the cellular protein levels of cells treated with these agents increased when compared to untreated cells. This increase was again greatest in cells exposed to both dbcAMP and PGE_1 and was not simply due to growth inhibition.

The effect of these agents on the fibrinolytic activity of Triton X-100 lysates of C1300 cells is shown as a function of dose in Figure 1. The fibrinolytic activity of the lysates and growth medium was totally dependent on added plasminogen (i.e. acid treated human serum, result not shown). Ex-

Table 1

Effects of Cyclic AMP Agents on Neuroblastoma Cell Morphology and Growth

Treatment	% "Differentiated" Cells		Protein/ Cell. % Control	Cell Number/ Dish. % Control
	24 hr	96 hr		
Control	21	4	-	-
PGE_1 ($3x10^{-5}M$)	27	23	137	70
dbcAMP ($10^{-3}M$)	44	29	121	89
PGE_1 + dbcAMP ($3x10^{-5}M$)($10^{-3}M$)	72	63	175	29
Butyric Acid ($10^{-3}M$)	22	21	110	25
Propionic Acid ($10^{-3}M$)	16	13	118	101
Theophylline ($10^{-3}M$)	27	17	106	87

C1300 cells were treated with the indicated doses of compounds 24 hr after seeding and the percentage of cells with long extensions ("differentiated" cells) counted at 24 and 96 hr after exposure. The cell number and protein content per cell were also determined 96 hr after exposure and compared to control cultures. Control values at 96 hr were about 13 x 10^6 cells/dish with a protein content of 97 ug/10^6 cells.

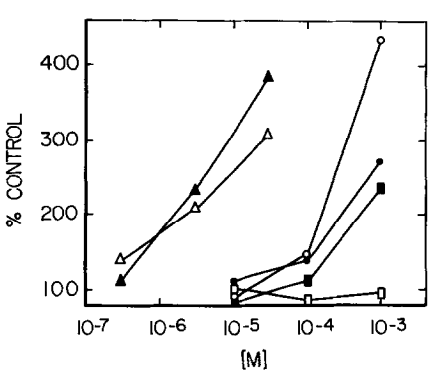

Figure 1. Effect of cyclic AMP agents on the intracellular fibrinolytic activity of C1300 cells. Cultures of C1300 cells were exposed to the agents 24 hr after seeding and the cells harvested after 96 hr treatment. Cell pellets were lysed in Triton X-100, centrifuged and plasminogen activator determined in the supernatant using [^{125}I]fibrin plates incubated with 1% acid-treated human serum. The protein content of the lysates was determined and the specific activity of the enzyme compared to that of untreated controls. The agents used were ; PGE_1 (▲); PGE_2 (△); butyric acid (○); propionic acid (●); dbcAMP (■); theophylline (□). Control values ranged from 5.9 - 14.7 units/mg protein and the points represent the mean values obtained from duplicate dishes in 2-4 separate experiments.

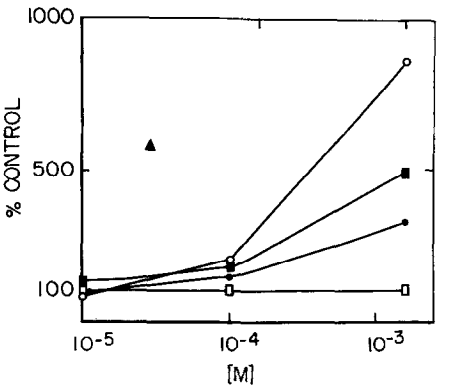

Figure 2. Effect of cyclic AMP agents on the secretion of plasminogen activator by C1300 cells. Cultures of C1300 cells were treated as in Figure 1 and plasminogen activator determined in the culture fluids after 96 hr treatment using [^{125}I] fibrin plates incubated with 1% acid-treated human serum. The cell number present after treatment was also determined and the amount of enzyme secreted was normalized for cell number and then compared to untreated controls. The agents used were; PGE_1 (▲); butyric acid (O); propionic acid (●); dbcAMP (■); theophylline (□). Control values ranged from 9-29 units/10^6 cells and the points represent the mean values obtained from duplicate cultures in 2-3 separate experiments.

periments with lysates of untreated cells showed that the assay was linear with increasing protein concentration up to 75 ug/assay (i.e. 30 ug/ml). Therefore although inhibitors of fibrinolysis might be present in the lysates, they were not measured by this assay and all assays were conducted on aliquots containing less than 75 ug protein (range 20-50 ug/assay). Both PGE_1 and its close relative PGE_2 increased the specific activity of plasminogen activator over the range of concentrations used. Butyric and propionic acids were also capable of inducing large increases in the activity of this enzyme. Since there were substantial differences in the growth inhibitory properties of these acids (Table 1) it seems unlikely that these increases can be due to cytotoxicity. Figure 1 also shows that dbcAMP raised the enzyme activity in C1300 lysates and that theophylline was ineffective over the dose range used. It was found that a combined treatment with PGE_1 (3×10^{-5}M) and dbcAMP (10^{-3}M) caused a 650% increase in intracellular plasminogen activator (not shown).

Figure 2 shows the abilities of these agents to influence the secretion of plasminogen activator into the growth medium. The results are thus similar to those observed for the intracellular enzyme except that dbcAMP was more effective than propionic acid in inducing enzyme secretion. A combined dose of PGE_1 (3×10^{-5}M) and dbcAMP (10^{-3}M) caused a 1400% increase in the level of extracellular enzyme (not shown). The increases shown in Figure 2 could also be detected 24 hr after addition of the agents.

DISCUSSION. The agents which we have used induce many irreversible "differentiated" functions in cultured C1300 neuroblastoma cells including morphological alterations (6) and the activities of several enzymes such as acetylcholinesterase (11), choline acetyltransferase (6) and sialytransferases (12) amongst others. There is a wide range in increase of these enzymes (4 to 40 fold) and our results clearly show that fibrinolytic activity can also be markedly increased in C1300 cells treated with these agents. It is unlikely that the increase is due to decreasing levels of inhibitors of fibrinolysis, since the assays were conducted at protein concentrations which were in the range of linearity of the assay. Although we confirmed the appearance of the morphological changes considered to be indicative of differentiation, correlations between "differentiation" and increased fibrinolytic activity are difficult to establish at this stage.

The observation that short-chain fatty acids can induce large increases in fibrinolytic activity is also of interest. It has been reported that sodium butyrate alters the shape of HeLa cells and induces increases in sialyltransferase activity (13) and membrane-bound alkaline phosphatase activity (14). Also, butyric acid and to a lesser extent propionic acid are potent inducers of differentiation in cultured erythroleukemic cells (15). Butyric acid also increases the endogenous cAMP level of C1300 cells (16). Clearly, the use of butyric acid derivatives of cAMP introduces many problems in the interpretation of the role of cAMP in cells. Thus we cannot at this stage be certain of the active intermediate in the increase of fibrinolytic activity in C1300 cells treated with dbcAMP.

The large increases in fibrinolytic activity seen when C1300 cells are treated with these agents is of interest in view of our observation that the secretion of cell factor can be induced *in vivo* (7). We are therefore currently investigating the biochemical nature of the induction as well as determining whether other cell types respond to these agents.

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