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Glia-Induced Morphological Differentiation in Neuroblastoma Cells

(glial–neuronal interactions)

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ABSTRACT **Glial cells release a factor into their culture medium that induces a high degree of morphological differentiation in neuroblastoma cells under normal growth conditions. This phenomenon is not correlated with a change in intracellular adenosine 3′:5′-cyclic monophosphate or in the rate of cell growth. Media from other cell lines tested induce less morphological differentiation or have no effect.**

Process formation by neuroblastoma cells in tissue culture has been termed morphological differentiation, and its importance as a model for neural differentiation has been pointed out by many authors (1–4). X-Irradiation (5), removal of serum (2), and addition of $N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate (But_2cAMP) (3, 4) or 5-bromodeoxyuridine (BrdU) (6) can induce this process formation. All these treatments are unphysiological, even at low degree of morphological differentiation, and they all interfere with the growth rate of the cells. Considering the theoretical importance of cell to cell interactions in the central nervous system, we tried to influence process formation by using medium from glial cell cultures. We report that such a glial conditioned medium induces a high percentage of morphological differentiation under physiological conditions.

MATERIALS AND METHODS

Neuroblastoma cells, clone NB-2a, derived from the C-1300 tumour cell line (7), and glial cells, clone C-6, derived from a *N*-nitrosomethylurea-induced rat glial tumour (8), were obtained from the American Type Culture Collection, Rockville, Md. Human tumour glial cells, clone 118 INI, derived from 118 MG tumour astrocytes (9) and lines of human fetal normal glia, rat normal glia, mouse fetal normal glia, and human fetal fibroblasts were a gift of Dr. E. H. Macintyre, N.J.H. Research Center, Denver, Colo. 3T3 mouse fibroblasts (10), polyoma (Py 3T3), and simian virus 40 (SV 3T3)-transformed mouse fibroblasts (11), baby hamster kidney cells (12), and H-4-11-E rat hepatoma cells (13) were also used. All the cells were grown routinely in Falcon 60-mm tissue culture dishes in Dulbecco's modified Eagle medium, supplemented with 10% fetal-calf serum (Colorado Serum Co., Denver, Colo.), penicillin G (150 U/ml), and streptomycin sulfate (30 μg/ml) in a humidified atmosphere of 5% CO_2–95% air at 37°. The average doubling time of NB-2a neuroblastoma cells and of C-6 glial cells was 24 hr.

Abbreviations: But_2cAMP, $N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate; SV40, simian virus 40; Py, polyoma (virus).

To determine the percentage of cells undergoing morphological differentiation, 50,000 trypsinized neuroblastoma cells were plated per 60-mm dish. After 16 hr of incubation, the medium was replaced by the medium to be tested, and 48 hr later all cells with and without processes were counted in at least five randomly selected areas of each culture plate. Cells having processes longer than the diameter of the cell body were considered morphologically differentiated. A total of 300–400 cells were counted per dish. The values reported here represent the percentage of differentiated cells determined in three parallel cultures.

For intracellular cAMP determination, the cells were quickly washed with Dulbecco's phosphate buffered saline (pH 7.2, 37°) then fixed with 1 ml of 0.05 N HCl. They were scraped with a rubber policeman and the suspension was heated for 10 min in boiling water. The samples were then neutralized with 0.1 N NaOH to pH 7.3 and treated with 50 μl of 0.17 M $BaSO_4$ and 50 μl of 0.15 M $Ba(OH)_2$. The solution was clarified with a glass-fiber filter and cAMP was determined in triplicate by the method of Gilman (14). For the early time points the cells of two or three plates were extracted in the same 1 ml of 0.05 N HCl. At each time point, three parallel cultures were washed, then solubilized in 0.5% sodium dodecyl sulfate for protein determinations (15).

RESULTS AND DISCUSSION

The induction of morphological differentiation of neuroblastoma cells by glial conditioned medium is illustrated by the micrographs in Fig. 1. The cells extend processes and form a net which connects many cells. The physical nature of these contacts is presently being studied by detailed electron microscopy. Process formation occurs when the neuroblastoma cells are grown on either glass cover slips or plastic tissue culture dishes. The percentage of morphological differentiation obtained with the glial conditioned medium after 48 hr is much higher than with But_2cAMP, BrdU, or removal of serum (Table 1). Unlike those treatments, conditioned medium does not affect the growth rate of the neuroblastoma cells (Fig. 2).

Fig. 3 shows the effect of conditioned media taken at different stages of the C-6 growth curve on morphological differentiation of neuroblastoma. The first significant effect is seen after 60 hr. At 72 hr, the monolayer reaches confluency and the ability to induce morphological differentiation reaches its maximum.

The conditioning of medium could be the result of synthesis and secretion of a factor by glial cells. Alternatively, those

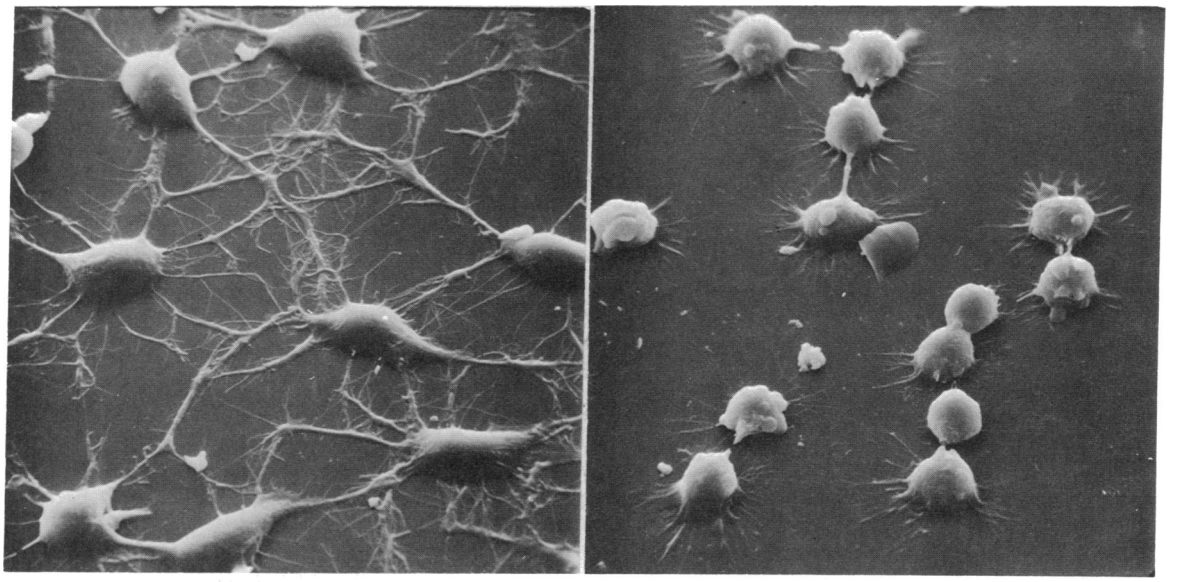

FIG. 1. Morphological differentiation induced by glial-cell conditioned medium. The neuroblastoma cells were plated as described in the *text* in 60-mm dishes containing glass cover-slips. After 16 hr the incubation medium was replaced by glial-cell conditioned medium (72 hr) which had been filtered through a sterile 0.22-μm Millipore membrane (*a*) or by fresh medium (*b*). 48 hr later, the cover slips were taken out, washed twice with Dulbecco's phosphate buffered saline (pH 7.2) and fixed with 1% formaldehyde–2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature (25°) for 1 hr. Post fixation was performed with 1% osmium tetroxide in the same buffer (room temperature, 30 min). The cover slips were dehydrated with ethanol, metal coated, and examined in a scanning electron microscope. Magnification: *a* and *b*, ×900.

cells could modify the medium, e.g., by depletion of a serum factor. To distinguish between these two possibilities, aliquots of concentrated serum-free conditioned medium were tested for activity in the presence of 10% fresh serum (Fig.

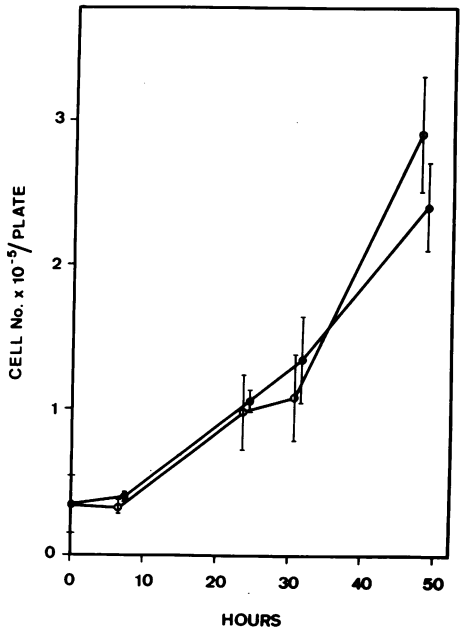

FIG. 2. Growth of neuroblastoma cells in conditioned (○) or normal medium (●). At each time point, three parallel monolayers were washed twice with cold Dulbecco's phosphate buffered saline (pH 7.2), trypsinized, and resuspended in 1 ml of the same buffer. Cell numbers were obtained by hemocytometer readings.

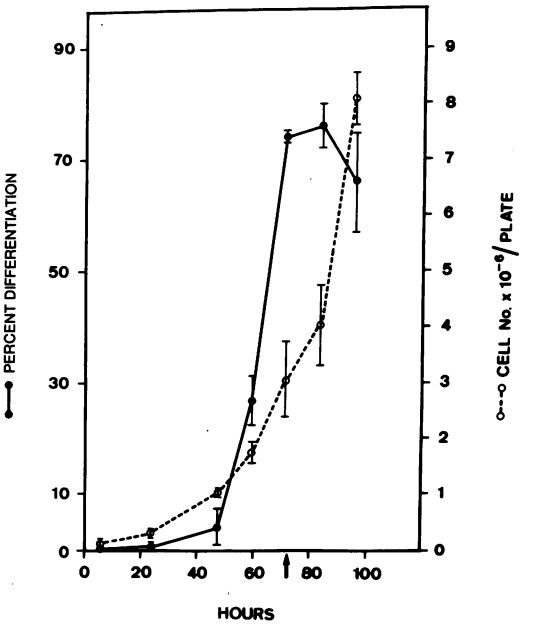

FIG. 3. Appearance of differentiating factor during the C-6 glial cell growth curve. C-6 glial cells were plated at a density of 180,000 cells per 60-mm dish. At each time point the conditioned medium was removed in three parallel dishes and the number of cells was determined after trypsinization (○). The three parallel conditioned media were filtered through sterile 0.22-μm Millipore membranes and kept frozen at −20°. The effect of each medium on neuroblastoma morphological differentiation (●) was tested as described in the *text*. The *arrow* represents the time when the C-6 culture reaches confluency.

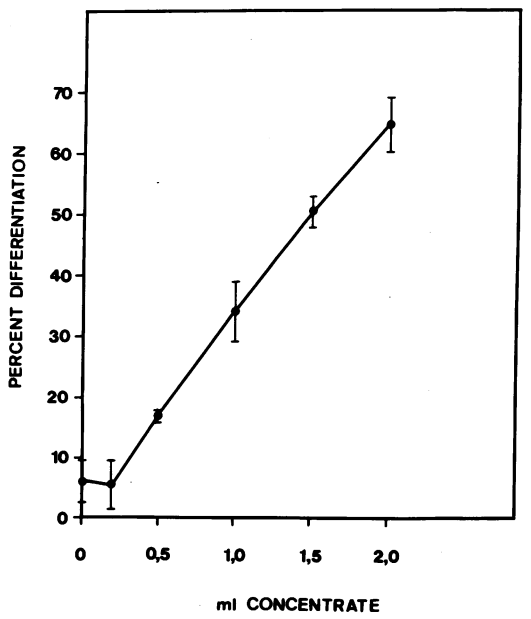

FIG. 4. Release of factor into serum-free medium. C-6 glial cells were plated and grown under the conditions described in the legend to Fig. 3. After 72 hr, when the monolayer reached confluency, medium was removed and replaced by the same amount of fresh serum-free medium. 3 Days later the serum-free conditioned medium was collected and concentrated 10 times with a UM-10 or UM-20 E Diaflo membrane under sterile conditions. Different amounts of concentrate were brought up to 5 ml with 0.5 ml fresh serum and adequate amounts of fresh, serum-free medium. The effect on morphological differentiation (●) was tested as described in the *text*.

4). The morphological differentiation observed is proportional to the amount of concentrate added.

An inverse relationship between growth rate and intracellular cAMP concentration has been established for fibroblasts (16). Since But_2cAMP treatment and other techniques used to induce morphological differentiation also affect the growth rate of the neuroblastoma cells, cAMP could be a causal factor in the regulation of process formation. Our system has the advantage of dissociating process formation from growth inhibition and, therefore, provides an opportunity to test the role of cAMP in the regulation of this phenomenon. Table 2 shows that between 20 min and 48 hr after addition of conditioned medium the cAMP concentration is the same in cells which form processes as in control cells.

Of several cell lines tested (Table 3), C-6 glial cells are the most potent in producing conditioned medium able to induce morphological differentiation. There may be a relationship between the glial factor and glycopeptides from neural membranes which have similar activity in cultures of dissociated chick-embryo spinal ganglia (17). It is not yet established that the effect obtained by some nonneural cell lines is due to the same factor. There are reports that nonneural cells can influence the attachment of ganglionic neuron cells in primary culture (18) and that the physiological environment can induce the differentiation of metastatic human neuronal tumour (19).

We conclude that glial cells in culture release a factor that causes process formation in neuroblastoma cells. This effect is linearly dependent upon the amount of factor used, and it is not correlated with an increase in intracellular cAMP. The observation of this *in vitro* phenomenon suggests a role for glial cells during brain development.

TABLE 1. *Comparison of morphological differentiation obtained by different methods*

Media tested	Cells with processes (%)
Conditioned by astrocytes	68.2 ± 7.2
10% serum	4.1 ± 0.2
10% serum + 10^{-3} M But_2cAMP	32.3 ± 1.8
10% serum + 8×10^{-6} M BrdU	16.2 ± 2.7
0% serum	19.5 ± 1.2

50,000 neuroblastoma cells were plated per 60-mm dish. After 16 hr the medium was replaced with glial conditioned medium, fresh medium containing 10% fetal-calf serum, and the appropriate additions or serum-free medium. The effect on morphological differentiation was tested as described in the *text*.

TABLE 2. *Intracellular cAMP during treatment by conditioned medium*

Hours	cAMP (pmol·mg^{-1} of protein)	
	Normal medium	Conditioned medium
0.3	8.8 ± 1.6	9.2 ± 2.6
1	7.3 ± 4.1	8.0 ± 3.8
2	10.0 ± 3.5	10.7 ± 2.9
5	20.6 ± 1.2	20.5 ± 4.9
24	14.1 ± 6.7	8.5 ± 5.3
48	8.9 ± 2.1	5.9 ± 1.7

50,000 neuroblastoma cells were plated in a 60-mm dish. After 16 hr the medium was replaced with glial conditioned medium or with fresh medium containing 10% fetal-calf serum. Protein and cAMP were determined as described in *Methods*.

TABLE 3. *Effect of medium conditioned by different cell lines on morphological differentiation*

Cell type	Cells with processes (%)
Control fresh medium	7.3 ± 0.4
C-6 glial cells	71.3 ± 3.2
118 INI human tumour astrocytes	21.4 ± 0.2
Human-fetal normal glia	29.2 ± 2.7
Mouse-fetal normal glia	35.4 ± 10.2
Rat normal glia	34.7 ± 5.4
NB-2a neuroblastoma cells	13.8 ± 6.2
H-4-11-E rat hepatoma cells	6.5 ± 2.6
3T3-mouse fibroblasts	7.3 ± 0.7
SV 40 transformed 3T3	14.8 ± 3.1
Py transformed 3T3	16.9 ± 3.2
Human-fetal fibroblasts	34.3 ± 5.3
Baby hamster-kidney cells	42.3 ± 5.3

Cells were plated at the density required for reaching confluency in 72 hr. At that time, the media were removed and stored at −20°. The thawed media were centrifuged under sterile conditions at 2000 rpm (1200 × *g*) for 20 min to remove possible floating cells and tested for effect on morphological differentiation as described in the *text*.

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