Morphological Changes in Rat Glioma Cells caused by Adenosine Cyclic 3',5'-Monophosphate

Koichi Igarashi, Shuichi Ikeyama, Masao Takeuchi and Yukio Sugino

Biological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Osaka 532, Japan

ABSTRACT. Morphological changes in a cell line from rat glioma induced in vitro by exposure to dibutyryl adenosine cyclic 3',5'-monophosphate (db-cAMP) and other agents were studied. Db-cAMP, adenosine cyclic 3',5'-monophosphate (cAMP) plus theophylline or cholera toxin plus theophylline caused shrinkage of parts of the cytoplasm and converted apparently immature cells to cells with oligodendroglial or astroglial morphology. This morphological alteration induced by db-cAMP proceeded rapidly and was completed within an hour. The morphological alteration induced by db-cAMP and cAMP was reversible, but that mediated by cholera toxin was not reversible. Cytochalasin B also induced a morphological alteration similar to that induced by db-cAMP. Agents such as colcemid counteracted the effects of db-cAMP, cAMP and cholera toxin. These findings suggest that the intracellular level of cAMP may affect the organization of microtubules and result in inducting morphological changes in this glial cell line.

Morphological differentiation is generally repressed in many tumor cells. But the re-expression of their differentiated properties can be induced by altering culture conditions. The morphologies of mouse and rat neuronal and glial tumor cells were altered by varying the serum concentration (19, 20), by X-ray-irradiation (13) and by exposure to dibutyryl adenosine 3',5'-monophosphate (db-cAMP) (6, 14, 17, 19), 5-bromodeoxyuridine (BUdR) (18, 22), amethopterin (22), actinomycin D (22), or conditioned medium from a glial cell culture (9). These morphological phenomena are valuable for studying differentiation in cells of the central nervous system, but little is known about the molecular basis that regulates cellular morphological characteristics.

We here describe morphological changes in a rat glioma cell line induced by db-cAMP and other agents, and present experimental results that suggest that this morphological alteration is correlated with a structural rearrangement of microtubules.

MATERIALS AND METHODS

Cells and culture. The cell line of rat glioma, designated AC cells, used in this study was first cultivated in vitro in October, 1974 by Dr. M. Tanaka (Department of Neurological Surgery, Kobe University, Kobe, Japan) from a brain tumor induced transplacentally with ethylnitrosourea in a SD-JCL rat (4). It was supplied by Dr. K. Fujiwara (Department of Neurological Surgery, Kobe University, Kobe, Japan) in April, 1975. The original tumor was classified as polymorphous oligodendrogliaoma on the basis of its histology. Karyological
analysis showed the presence of long metacentric marker chromosome(s) in all the AC cells tested. The chromosome counts indicated two modalities in hypodiploidy (Chromosome no. 35–40) and in polyploidy (Chromosome no. 70–80) (unpublished observations of Drs. M. Tanaka and K. Fujiwara). Cells were maintained at 37°C in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Flow Laboratories Inc.) and 100 μg/ml of kanamycin. Transfers were performed with 0.125% of trypsin and 0.01% ethylene-diaminetetraacetate. Cloning was carried out with cloning rings. After the AC cells were received in our laboratory, they were cultured for about 800 days with 170 transfers.

**Induction of morphological alterations.** Cells were plated at about 1 × 10^5 cells/35 mm plastic tissue culture dish (Falcon) in 2 ml of growth medium and were allowed to grow for 1 or 2 days. Solutions of 50 mM db-cAMP, adenosine cyclic 3',5'-monophosphate (cAMP), dibutyryl guanosine cyclic 3',5'-monophosphate (db-cGMP), sodium butyrate, 20 mM theophylline, 100 μg/ml of BUdR, 1 mg/ml of cholera toxin, 1.10–2.69 mM colcemid, colchicine or vincristine were made up in phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 8 mM dibasic sodium phosphate and 1.5 mM monobasic potassium phosphate, pH 7.2). Cytochalasin B solution was made up at a concentration of 10.44 mM in dimethylsulfoxide (DMSO). These drugs were added to the medium when cultures were at mid log phase and were mixed by gentle pipetting. The control experiment was performed to ensure that the maximum concentration of PBS or DMSO carried along with the drugs showed negligible effects. To test the reversibility of the effects of these drugs, media containing the drugs were replaced with the normal growth medium and the morphological changes in cells was observed.

**Purification of tubulin.** Tubulin was prepared from fresh porcine brains on the basis of its polymerization and depolymerization (21, 25), and was purified by preparative SDS-gel electrophoresis (8).

**Preparation of antiserum.** The antiserum against tubulin was prepared in a rabbit and was purified through a tubulin-Sepharose 4B affinity column as described by Fuller et al. (5).

**Indirect immunofluorescence.** (1) S-100 protein: Cells grown on glass coverslips were fixed with acetone for 5 min at room temperature. After rinsing them with PBS, the coverslips were incubated with 20 μl of the IgG fraction from rabbit antiserum against bovine S-100 protein, a gift from Dr. K. Uemura (Department of Physiology, Saitama Medical College), for 30 min at 37°C in a humid atmosphere. The coverslips were washed thoroughly in PBS and incubated for another 30 min with 20 μl of fluorescein-conjugated goat anti-rabbit IgG antibody (Miles Lab. Inc.). Before mounting them on glass slides in 50% glycerol, the coverslips were washed in PBS. Preparations were observed with a Zeiss fluorescence microscope. (2) Tubulin: Cells grown on glass coverslips were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature (24). Coverslips were immersed on glass slides in 50% glycerol at −20°C and for 1 min in acetone, then washed in PBS to make the samples permeable. These coverslips were incubated with 20 μl of rabbit anti-tubulin antibody for 60 min at 37°C, rinsed in PBS, then incubated with 20 μl of fluorescein-conjugated goat anti-rabbit IgG antibody for 45 min at 37°C. After washing the coverslips in PBS, the cells on them were mounted in 50% glycerol and viewed in a Zeiss fluorescence microscope with vertical illumination.

**Chemicals.** The sources of the agents used were: db-cAMP, cAMP and db-cGMP, Boehringer Mannheim GmbH; sodium butyrate, theophylline and BUdR, Wako Pure Chemical Ind. Ltd.; colchicine and cycloheximide, Sigma Chemical Co.; colcemid, Ciba-Geigy Ltd.; vincristine, Eli Lilly & Co.; cytochalasin B, Aldrich Chemical Co.; DMSO,
Tokyo Chemical Ind. Ltd. Purified cholera toxin was supplied by Dr. T. Miwatani (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan).

RESULTS

General characteristics of AC cells. AC cells, a line of rat glioma, seemed almost homogeneous under gross morphological observation when we received the cell line. They maintained a fibroepithelioid morphology during the exponential phase of growth and many mitotic figures were observed. In the stationary phase, they tended to pile up and were often spherical with short processes. Doubling time was about 24 h and the saturation density was about $8.3 \times 10^4$ cells/cm$^2$ (Fig. 1). These cells grew in a semi-solid medium containing 1.2% methyl-cellulose in vitro and formed tumors in nude mice in vivo when injected subcutaneously. The presence of S-100, a marker protein for glial cells (1, 10, 11), in AC cells was clearly demonstrated by immunofluorescent staining using rabbit antisera against S-100 prepared from bovine brain (Fig. 2).

After 14 subcultures in our laboratory, 6 clonal cells (AC-1 to AC-6) were isolated from the original AC cell line. The morphologies of all these clones were similar to the morphology of the original AC cells. However, one (AC-3) was resistant to db-cAMP, i.e. db-cAMP at a final concentration of 1 mM in medium did not induce morphological change. (The morphological change in AC cells induced by db-cAMP is described below). The mechanisms of this resistance to db-cAMP in clone AC-3 are being investigated.

Effect of db-cAMP or cAMP. When AC cells at log phase were exposed to 1 mM db-cAMP, they underwent morphological change within 20 min. After 60 min, almost all cells had become spherical and had developed highly branched cytoplasmic processes (Fig. 3A-3D). These cells were similar in appearance to oligodendroglial or astroglial cells. Among these changed cells we observed some giant cells, which usually showed typical astroglial morphology, as shown in Fig. 3E. Under these conditions,
the rates of cellular DNA synthesis (data not shown) and multiplication were markedly reduced (Fig. 1).

The effect of cycloheximide was examined to determine whether the morphological change induced by db-cAMP requires protein synthesis. Cycloheximide was added to the culture 30 min before exposure to db-cAMP, but it had little effect on morphological change at concentrations up to 50 µg/ml. This concentration of cycloheximide inhibited the incorporation of ³H-leucine into the protein fraction by more than 96%. These results suggest that the morphological change in AC cells induced by db-cAMP does not require de novo protein synthesis.

Observations with time-lapse cinephotomicrography showed that the morphological change caused by db-cAMP first appeared as a flow of spread cytoplasm toward the center of the cell after which the center portion of each cell gradually rounded up. The retraction of the cytoplasm left the developed processes behind as seen in

Fig. 2. Immunofluorescence demonstration of S-100 protein in AC cells. Cells were fixed and prepared for indirect immunofluorescence as described in MATERIALS and METHODS. ×120.

Fig. 3. Morphological differentiation in AC cells induced by db-cAMP and other agents. A. Phase-contrast microscopic appearance of AC cells grown in the standard growth medium for 2 days. ×60. B. The same AC cells as in 3A, to which 1 mM of db-cAMP had been added and cells had been incubated at 37°C for 20 min. ×60. C. The same field as in 3A in the medium containing 1 mM of db-cAMP 40 min later. ×60. D. The same field as in 3A in the medium containing 1 mM of db-cAMP 60 min later. ×60. E. Two giants cells observed among morphologically changed AC cells induced by an exposure to 1 mM of db-cAMP for 60 min. ×60. F. AC cells cultured in the medium containing 1 mM of db-cAMP for 60 min then cultured in standard medium for 60 min. ×60. G. Cells cultured in the medium containing 10 ng/ml of cholera toxin for 2 h. ×60. H. Cells cultured in the medium containing 10 ng/ml of cholera toxin plus 1 mM of theophylline for 2 h. ×60. I. Cells cultured in the medium containing 10 ng/ml of cholera toxin plus 1 mM of theophylline for 2 h, then cultured in standard medium for 24 h. ×60. J. Cells cultured in the medium containing 1 mM of db-cAMP and 2.69 µM of colcemid for 60 min. ×60. K. Cells cultured in the medium containing 1 mM of db-cAMP for 60 min then cultured in the same medium containing 2.69 µM of colcemid for 60 min. ×60. L. Cells cultured in the medium containing 2.69 µM of colcemid for 60 min. ×60. M. Cells cultured in the medium containing 10.44 µM of cytochalasin B for 2 h. ×60.
Morphological Changes in Gila

Thus, the development of processes in the AC cells was generated not by extension of processes but by shrinkage of the cytoplasm.

Antibodies to tubulin and indirect immunofluorescence were used to examine the microtubular system in AC cells. AC cells, in which no morphological alteration was induced, formed microtubular networks distributed throughout the spread cytoplasm (Fig. 4A). But, when morphological change was once induced by db-cAMP, the microtubular networks were crowded around the nuclei and through the cytoplasmic processes of the round cells (Fig. 4B).

AC cells were extremely sensitive to the effect of db-cAMP in that the morphological changes caused by db-cAMP were completed within the very short period of 30–60 min, and the db-cAMP acted at a concentration as low as 0.1 mM.

The same morphological changes were also induced by 1 mM cAMP plus 1 mM theophylline, the inhibitor of phosphodiesterase. In this case, the addition of 1 mM cAMP or 1 mM theophylline alone to the culture induced only slight alterations. In contrast, when AC cells were cultured with 3 mM db-cGMP, 1 mM sodium butyrate or 5µg/ml BUdR for more than 3 days, no obvious changes in morphology were observed.

When a culture medium containing 1 mM db-cAMP or 1 mM cAMP plus 1 mM theophylline, in which morphological changes in AC cells had been induced, was replaced with fresh medium to remove the drug(s), the cells reverted rapidly to their original morphology, i.e. after 60 min cells with long processes were no longer detectable (Fig. 3F), and growth was resumed (Fig. 1). Therefore, the reaction induced by db-cAMP or cAMP was completely reversible.

Effect of cholera toxin. Cholera toxin activates adenylate cyclase and causes an increase in the intracellular levels of cAMP endogenously (2, 12, 16). Therefore, cholera toxin was tested to see if it would be effective for the induction of morphological changes in AC cells. Fig. 3G shows that 10 ng/ml of cholera toxin produced partial morphological change within 2 h. When 1 mM theophylline was added with the cholera toxin, a complete alteration of morphology identical to that induced by db-cAMP was observed (Fig. 3H). However, this morphological alteration induced by combined treatment with cholera toxin and theophylline was not reversible (Fig. 3I). This coincides with the finding that cholera toxin binds to the cell surface ganglioside irreversibly (3).

Effects of colcemid and cytochalasin B. Agents interacting with cellular microtubules and microfilaments can prevent the transformation of mammalian cells (15). Therefore, we examined the effect of such agents on the morphological change in AC cells induced by db-cAMP. When colcemid, a specific inhibitor of tubulin polymerization, was added to the culture at a concentration of 2.69 µM together with db-cAMP, morphological change was prevented (Fig. 3J). When morphologically altered AC cells, which had been cultured in the presence of 1 mM db-cAMP for 60 min, were exposed to 2.69 µM of colcemid, their morphology reverted to one very similar to that in the control cultures within 60 min (Fig. 3K). The addition of colcemid alone caused no appreciable alteration in cellular morphology (Fig. 3L). The altered morphology of AC cells induced by 10 ng/ml of cholera toxin plus 1 mM theophylline showed a
similar reversion on the addition of colcemid at a concentration of 2.69 μM. These actions did not require protein synthesis, since a simultaneous addition of 50 μg/ml cycloheximide had little effect. Moreover, 2.5 μM of colchicine or 1.1 μM of vincristine also prevented effects due to db-cAMP or reversed these effects like colcemid. The exposure of AC cells morphologically altered by db-cAMP to agents interacting with microtubules caused the microtubular system of the cells to become depolymerized, after which no microtubular networks were observed, in spite of the reversion of cellular morphology (Fig. 4C).

Cytochalasin B, affects the disorganization of cellular microfilaments but not microtubules, and did induce morphological alterations similar to those induced by db-cAMP within 2 h after the addition of 10.44 μM of this agent (Fig. 3 M). This action of cytochalasin B was completely reversible.

**DISCUSSION**

We found that AC cells, a rat glioma cell line, could be changed morphologically to oligodendroglial or astroglial cells after exposure to db-cAMP or cAMP plus theophylline. The alterations occurred so rapidly that almost all the cells developed cytoplasmic processes within 30 to 60 min in cultures containing 1 mM of these agents. This development of the processes in AC cells was caused by the retraction of parts of the extended cytoplasm. This shrinkage phenomenon induced by db-cAMP is common to glial cells (23).

Cholera toxin also induced morphological changes in AC cells. Although the intracellular concentration of cAMP was not measured quantitatively, the addition of this agent may endogenously increase the intracellular levels of cAMP, because theophylline added simultaneously greatly enhanced the action of cholera toxin. However, in contrast to the effects of db-cAMP, reversion of the alteration induced by cholera toxin was not complete when the medium was replaced with a fresh one without toxin and theophylline. These facts suggest that ganglioside GM1, the main binding site of choleragenoid, is present in AC cells and that the binding of choleragenoid is irreversible as previously described (3).

Seeds, N. W. et al. (29) have reported that the axon outgrowth in neuroblastoma cells is completely inhibited by colchicine or vinblastin. In addition, Puck, T. T. et al. (7, 15) have shown that in chinese hamster ovary cells one cellular morphology could be converted to another by db-cAMP, and that an antagonistic effect on this action occurs on exposure to colcemid or cytochalasin B. These morphological alterations do not require protein synthesis. In AC cells, agents known to disintegrate the microtubular system also prevented the effect of db-cAMP or reversed its effect. In this case, cycloheximide had little effect on the alteration. Furthermore, using the antibody to tubulin as the immunofluorescent probe, the microtubular networks observed in morphologically changed cells were crowded around the nuclei and through the processes of the round cells. These results strongly indicate that the morphological alteration in AC cells does not require de novo protein synthesis, but is dependent upon the conformational change of the microtubules. The organization of the microtubular system seems to be regulated by the intracellular levels of cAMP as postulated by Puck, T. T. et al. (15). In AC cells, however, cytochalasin B did induce a morphological alteration similar to that induced by db-cAMP. Therefore, in contrast to the chinese hamster cells (15), the disorganization of microfibrillar system seems to induce the morphologi-
Morphological Changes in Glia

Morphological changes in the AC cells. The mechanisms of this action need to be investigated.

In spite of recent advances that have been made in studies of morphological alterations in cells from the central nervous system, much remains to be learned about the molecular basis of phenotypic expression of morphological characteristics. As AC cells are very sensitive to db-cAMP and are easily handled in cultures, studies using this glioma cell line and its various clonal cell lines will help to clarify the fundamental mechanisms of morphological alteration and the malignant transformation of mammalian cells.

Recently, we found that AC cells spontaneously release a C-type RNA virus, and we showed that this virus is an endogenous C-type virus of rat origin. Evidence for this will be described in detail elsewhere.

Acknowledgements. We thank Drs. K. Fujiwara and M. Tanaka (Dept. of Neurological Surgery, Kobe Univ.) for the generous gift of rat glioma cells and for prepublication data, and Drs. T. Miwatani (Research Institute for Microbial Diseases, Osaka Univ.) and K. Uemura (Dept. of Physiology, Saitama Medical College) for their generous gifts of cholera toxin and antiserum to bovine S-100 protein, respectively. We also thank Mr. M. Koyama of this laboratory for his technical assistance.

REFERENCES


(Received for publication, December 10, 1977)