Drug-induced Differentiation of a Rat Glioma in Vitro

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SUMMARY

The effects of amethopterin, actinomycin D, and 5-bromodeoxyuridine on the differentiation of a cloned line of rat glioma is described. Amethopterin caused spongioblasts to develop rapidly into cells resembling astroblasts in culture. In time, these cells progressively differentiated into sheets of intertwining astrocytes that are morphologically like astroglia from normal rat brain grown under similar conditions. Exposure of glioma cells to low concentrations of actinomycin D resulted in the accumulation of glycogen in the cell bodies and their process without cell degeneration. 5-Bromodeoxyuridine caused a striking change in the surface membrane of the tumor cells. Many desmosomes and close junctions were found. These findings indicate that further morphological differentiation of this rat glioma can be induced by manipulating the environment in which the cells are grown, in this case, by exposure to appropriate antitumor agents.

INTRODUCTION

Many of the tumors that have been established in culture continue to express some of the differentiated chemical and morphological properties of the normal determined cells from which they originated (2, 3, 10, 21, 24, 25). Although little is known about the mechanisms that regulate differentiation in normal or malignant eukaryotic cells, it is apparent that sequential morphological differentiation is repressed in most tumors. However, in vitro, reexpression of differentiated properties, including chemical differentiation, can be induced by altering the environment in which tumor cells are grown. This can be accomplished, e.g., by varying the serum concentration or the substrate, or by adding metabolic inhibitors (11, 20, 21). There are, of course, a number of tumor cell lines that continue to synthesize differentiated products without having to alter the environment radically (2, 7, 10, 23, 25).

Cell lines have been derived from rat and human gliomas which express differentiated properties of normal glia (3, 24). They synthesize a specific glial protein, S-100 (16) and can be induced with hydrocortisone to synthesize an enzyme found in the soluble cell fraction, glycerol-3-phosphate dehydrogenase (6). The cells of some lines are morphologically like astroblasts or maturing astroglia (17, 24).

The purpose of this study was to determine whether some inhibitors of DNA and RNA synthesis would induce further morphological differentiation of a cloned rat glioma line, C-6. The effects of these inhibitors on the synthesis of a specific glial protein, S-100, will be reported later.

MATERIALS AND METHODS

The C-6 strain, a cloned line of rat glioma, was obtained from Dr. Gordon Sato. This line had been grown in Medium F10 with 15% horse serum and 2.5% fetal calf serum. Three months after the cell line was received, and after 14 subcultures in our laboratory, the medium was changed to one composed of 3X amino acids in Eagle’s basal medium and 2X concentration of vitamins, 20% fetal calf serum, and 200 units of penicillin per ml. The cells were propagated in this medium for 8 months before experiments were performed. Stock cells were trypsinized in 0.05% crystalline trypsin and subcultured in Pyrex T-30 flasks. For most bright-field or phase studies, 3 to 4 X 10^6 cells were explanted to 35-mm diameter plastic Petri dishes containing 22 X 22-mm glass coverslips in 2 ml of medium. At the end of the growth period, the cells were fixed in glacial acetic acid:70% ethyl alcohol: formaldehyde, 5:85:10 (9), stained with hematoxylin and eosin or toluidine blue, dehydrated, and mounted in DPX resin. For electron microscopy, the cells were grown directly on the plastic and were fixed at room temperature for 45 min in 2.5% glutaraldehyde in Hanks’ solution adjusted to pH 7 with 1 N NaOH. They were rinsed in Hanks’ solution, postfixed in 1% osmium tetroxide in Hanks’ solution for 30 min, gently scraped as a layer from the plastic dish with a wide rubber policeman, dehydrated, and embedded in Epon 812 as previously described (8). Thin sections were stained with uranyl acetate and lead citrate and observed in a Philips 200 or RCA 3G electron microscope.

Amethopterin (4.4 X 10^-8 g/ml), actinomycin D (5 X 10^-8 g/ml), or BUdR (5 X 10^-6 g/ml), was added for 24 to 48 hr to the medium of cultures growing in log phase. Cultures were either fixed or replaced with normal growth medium and observed for 1 to 21 days.

RESULTS

The C-6 glioma cells carried in this laboratory multiply at the same rate as the line described by Benda et al. (3), with a...
doubling time of about 35 hr. Studies with bright field and phase showed that when the cells were subcultured at frequent intervals of 3 to 4 days, they resembled bipolar spongioblasts (Fig. 1) and were similar in morphology to cultures of Tumor 3 described by Benda et al. (4). Many mitotic figures were clustered among these cells. Cultures that were allowed to become very dense contained a small number of astroblasts, with 2 to 8 processes which varied in length and thickness.

Within 24 hr after the addition of amethopterin to cultures in late log phase, many of the spongioblasts developed long processes (Fig. 2). Forty-eight hr after exposure, most of the short, bipolar spongioblastic population was no longer observed. The cultures were then refed with normal growth medium. Between 20 to 30% of the cells degenerated and detached from the substrate following exposure to amethopterin. Most of the surviving population had the morphology of rat astroblasts and astrocytes (17). Cells that then had 2 to 3 long processes developed as many as 20 during the following week in culture. Many of those processes were thick and formed broad-end feet at their terminals. They resembled those of differentiated normal glia and not those of injured epithelial or fibroblastic cells. A similar effect was achieved when cells were incubated for 40 to 48 hr in media with 2 mM thymidine. By the 5th day after removal of the drugs, mitotic figures were again observed among recovering cells (Fig. 3). After the 2nd week, there was a very dense layer of highly differentiated cells with very long processes which had the morphology of normal rat astrocytes (Fig. 4). HeLa cells, normal human fibroblasts, and Chinese hamster fibroblasts did not show this time course change in morphology after exposure to amethopterin.

Cells exposed to actinomycin D, 1 X 10^{-8} g/ml, were lysed within 72 hr. However, when the concentration was reduced to 5 X 10^{-8} g/ml, cell death did not occur. At this concentration of drug, there were no obvious changes in the morphology of the cells except for a reduction in the size of the nucleoli. When the actinomycin D was removed after a 48-hr pulse, cells recovered completely (see “Electron Microscopy” below).

C-6 glioma cells grown in BUdR, 5 X 10^{-6} g/ml, continued to multiply at the same rate as did cells growing in normal media, and there was no striking change in their morphology in bright field. The cultures were not exposed to bright light.

Electron Microscopy. In control cultures, the cells contained many free ribosomes, scattered rough ER, and Golgi complexes similar to those described by Benda et al. (4) and Weinstein and Kornblith (24). Microtubules were prominent in the perikaryon and in the short processes of the cells. There was a dense mat of filaments close to the surface in tangential sections, and many filaments were scattered throughout the perikaryon. No attempt was made to measure the diameters of the filaments accurately.

In cultures treated with amethopterin, the nuclei were slightly enlarged and the chromat was margnated. In addition to the myriads of filaments, there were more microtubules than were found in control cells. The microtubules extended in organized arrays from the cell bodies into their processes, which also contained many free ribosomes and short segments of rough ER. The amethopterin-treated cells had a more extensive Golgi complex. The dilated end feet had tortuous, undulating cell membranes which were separated by a space of about 200 A from the perikarya or processes of other glial cells. The microtubules in the end feet were not organized but were irregularly scattered or aggregated into a tangle of filaments. Surrounding the glial cells was a fine fibrillar matrix in the extracellular space. In bright field, these fibrils stained metachromatically with toluidine blue. These observations at the morphological level support the biochemical findings of Dorfman and Ho (7), who reported that the C-6 glioma cells synthesized mucopolysaccharides.

Only in cells exposed to low levels of actinomycin D were there prominent aggregates of glycogen, an intracellular component of normal glia. These glycogen granules were observed in cells that were not degenerating and that had the same morphology as cells in control media.

Cultures exposed to BUdR, 5 X 10^{-6} g/ml, as they approached confluency showed striking membrane changes which were not observed in cells treated with other drugs. Almost every thin section contained cells that had local thickened evaginations of the surface membrane (Fig. 5), desmosomes, and/or close junctions. Only in the BUdR-treated cultures were so many differentiated close junctions observed (Fig. 6). The mechanism responsible for this inductive phenomenon is unknown, but other workers (1, 14) have shown that BUdR can induce virus production and increased adherence of cells to plastic substrate (20). The appearance of these junctions and the many desmosomes along the membranes of adjacent glial cells would support the observation by Kuffler and Potter (12) that there are low-resistance pathways between glia.

DISCUSSION

Rat glioma cells of a continuously multiplying line with the morphology of spongioblasts can be induced to differentiate into sheets of astroglial cells after a pulse with an antimetabolite, amethopterin, or excess thymidine. Whether this effect is mediated exclusively through the inhibition of DNA synthesis or the interference of other cell functions is unknown. It has been shown, for example, that thymidylate synthetase increases in amethopterin-treated cells (19). Perhaps other repressed gene functions are derepressed, resulting in the development of cells resembling astroglia. S-100, a specific glial protein, also increases after administration of amethopterin (A. Kolber, B. W. Moore, and M. N. Goldstein, in preparation).

Aggregates of glycogen are present in normal glial cells, but rarely observed in the cells of the cloned human or rat glioma (4, 24). However, when the rat glial cells were exposed to low concentrations of actinomycin D, many clusters of glycogen particles were observed in the cytoplasm of a majority of the cells without causing cell degeneration. Actinomycin D at high concentrations inhibits the DNA-directed synthesis of RNA and, at very low doses, inhibits rRNA synthesis. There are, however, reports that it can interfere with normal carbohydrate metabolism by inhibition of respiration and glycolysis (13).
Weinstein and Kornblith (24) reported that a nesux was observed in one of their preparations of human glioma cells, and Benda et al. (4) did not describe any specialization of the glial surface. No desmosomes or close junctions were observed in the membranes in the control cultures of the rat C6 glioma. These membrane specializations, including hemidesmosomes, have been described among glia in the central nervous system (18). Many desmosomes, local evaginations of the cell membrane, and nexuses were observed along the membranes of cells exposed to BUdR. BUdR did not affect the growth rate and the specializations observed along the membranes of drug-treated cells were not seen along membranes of cells in control cultures. Close or tight junctions frequently develop between normal contact-inhibited cells in tissue culture, but occur with much less frequency in transformed cells (15). Silagi and Bruce (22) reported that BUdR inhibited the synthesis of melanin in melanoma cells but at the same time decreased their malignancy. Although BUdR does frequently suppress certain differentiated properties (5), in the case of the melanoma cells and the glioma cells, the membranes may have become more like normal cells. Schubert and Jacob (20) reported that BUdR-treated mouse neuroblastoma cells which grew in suspension rapidly attached to the plastic substrate and proliferated as a monolayer after exposure to BUdR.

The observations presented in this paper indicate that the propitious use of toxic drugs can result in further differentiation of a cloned rat glioma. Recent evidence indicates that it is possible to reexpress normal differentiated properties in tumor cells grown in vitro by drugs or specific growth factors (10, 20).

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REFERENCES

Fig. 1. Confluent patches of spongioblastic cells. The processes are short, and the nuclei occupy most of the perikarya. Many mitotic figures are seen among these cells. Prepared 4 days after subculture on coverslips. Positive phase contrast. H & E, × 300.

Fig. 2. Area similar to that shown in Fig. 1; growing for 24 hr in $4.4 \times 10^{-4}$ g amethopterin per ml. Note the striking elongation of the bipolar processes. Coverslip culture; positive phase contrast. H & E, × 400.
Fig. 3. Exposed for 42 hr to $4.4 \times 10^{-8}$ g amethopterin per ml, then grown in normal medium for 5 days. The perikarya have increased in size and have begun to send out many processes. Note the mitotic figure in the upper right. Coverslip culture; bright field. H & E, \( \times 650 \).

Fig. 4. Exposed for 48 hr to $4.4 \times 10^{-8}$ g amethopterin per ml, then grown in normal medium for 8 days. Multiple long processes extend from these cells, which have the features of normal glia. Expanded end feet are easily seen. T-flask culture; living phase contrast. \( \times 230 \).
Fig. 5. Cells were exposed for 48 hr to $5 \times 10^{-6}$ g BUdR and were grown in drug-free medium for 6 days. Note the local thickened osmiophilic evaginations along the plasma membrane (lower arrows). There are also larger invaginations in the plasma membrane (upper arrow). $\times 55,000$.

Fig. 6. Note the close junction between glioma cells in culture treated with $5 \times 10^{-6}$ g BUdR per ml, as above. Many microtubules are present in the processes of adjacent cells. Focal membrane thickening can be seen in the upper left. $\times 41,000$. 
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