Effects of Cytosine Arabinoside and 1,3-Bis(2-chloroethyl)-1-nitrosourea on Human Glial Tumor Cells

Tsu T. Chen and John Mealey, Jr.

Division of Neurological Surgery, Indiana University Medical Center, Indianapolis, Indiana 46202

SUMMARY

The effects of 1-β-D-arabinofuranosylcytosine were examined in cultures of 5 glial tumor cell strains derived from different patients. The growth inhibition by 1-β-D-arabinofuranosylcytosine was assessed by counting the number of cells remaining in microtest plates. A significant drug effect was demonstrated against all 5 cell strains. Inhibition was dependent on dosage and duration of treatment. When doses reached the level of 0.5 μg/ml, however, there were no further increases in inhibitory response. The degree of recovery from growth inhibition likewise became less marked with higher dosage and longer treatment. The surviving cells remained in situ without further proliferation. The cytotoxic effect was shown to be enhanced by combination treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea (8.0 μg/ml).

INTRODUCTION

ara-C, an analog of cytidine, has been shown to inhibit the growth of mammalian cell lines in vitro (8, 22) and has shown antitumor activity against a variety of transplanted mouse tumors in vivo (10, 11, 29). This drug was very effective against intracerebrally inoculated L1210 leukemia cells in mice (17), and was recently shown to have a therapeutic effect against meningeval leukemia in children, with relatively low clinical toxicity (27). Intrathecal administration of ara-C in monkeys (25) and dogs produced neurological toxicity only at dose levels 10 times greater than those used in humans (18). Most of the active antimetabolites show limited ability to cross the blood-brain barrier when given parenterally and prohibitive toxicity when given intrathecally. This well-tolerated drug, like methotrexate, might potentially be applied clinically for intrathecal chemotherapy of intracranial tumors.

This study was undertaken for examination of the responses of individual glial tumor cells to ara-C in vitro. It was to be determined whether or not the cytotoxic effect of this drug could be enhanced by combination treatment with BCNU, a chemotherapeutic agent used against brain tumors because of its effective penetration through the blood-brain barrier (26, 28).

MATERIALS AND METHODS

Brain Tumor Cell Lines and Culture Medium. All tumor cell strains were derived from biopsied brain tumors that were diagnosed histologically as glioblastomas. The cell strains were maintained in BME plus 10% FCS and were fed every 2 to 3 days. The duration in culture, number of subcultures, and growth rate at the time of experiments are given in Table 1.

These glioma strains were generally pleomorphic, and nuclei varied in size and shape, ranging from oval to round. Cells of both HB8 and HB19 strains showed a spidery appearance with fine, long, cytoplasmic processes. Some were multinucleated, and all nuclei were deeply stained. The HB10 strain showed, for the most part, a loose syncytiat pattern; however, there were some solid areas where the cells were more closely approximated. The morphology of HB25 and HB27 was quite similar to HB10, except that a more fibrillar type of cells was found in the former 2 strains. Two clones have been isolated from HB8 tumor strains; 1 has maintained a plump protoplasmic configuration and the other was of a fusiform type. In primary culture, these glioma cells multiplied more rapidly than cells derived from normal brain tissues. Enzyme histochemical analysis also demonstrated that glioma cells showed much higher activities of acid phosphatase, lactate dehydrogenase, and glucose 6-phosphate dehydrogenase than did cells propagated from normal brain tissue. These enzymatic characteristics may be indicative of the neoplastic nature of glioma cells (13, 20).

Drugs. Stock solution of ara-C was prepared in BME without FCS and filtered through a Millipore membrane. This stock solution was kept frozen at −20°C until use. For each experiment, the frozen solution was thawed and the drug solution was diluted to desired concentrations in BME plus 10% FCS. For each experiment, fresh BCNU was dissolved in dimethyl sulfoxide prior to further dilution with BME. The final concentration of dimethyl sulfoxide in the culture medium was 0.2%, which did not cause any marked effect on the tumor cells, compared to appropriate controls.

Experimental Procedures. Stock cultures of tumor cells were trypsinized, and approximately 10⁶ cells were suspended in 1 ml of BME plus 10% FCS. An 0.02-ml (200 cells) amount of this cell suspension was inoculated into each well of the microtest plates, as described previously (5). After overnight incubation at 37°C in 5% CO₂, 4 different concentrations of the drug were made in BME, and 0.02 ml of each dilution was substituted in the culture wells. Medium with or without drug was changed every 2 days for the duration of the experiment. After different periods of...
exposure to the drug, control and treated cells were fixed with methanol and stained with Giemsa. For recovery experiments, the cultures were treated for 2, 4, or 6 days; the drug was then removed, and the cells were washed. Standard BME medium was added, and the cells were allowed to recover in the same wells for an additional 10 days under standard culture conditions. For drug combination experiments, the concentration of BCNU was kept constant at 8.0 μg/ml, but ara-C was diluted in 4 different concentrations ranging from 0.0005 to 0.5 μg/ml. For sequential treatment, BCNU was 1st added to the cultures for 48 hr; then it was removed and ara-C was added. ara-C was replaced with BCNU 48 hr later, and the cultures were incubated for another 48 hr. With simultaneous treatment, cells were exposed to media containing equal concentrations of both drugs. The medium was changed every 2 days during the 6 days of treatment. The growth inhibition was assessed by direct counting of the number of cells in each well. All determinations herein reported represent average values obtained from cell counts in at least 3 cultures treated at each concentration. The level of significance of these results was analyzed by Dunnett's test (p ≤ 0.05).

RESULTS

Effect on the Growth of 5 Glial Tumor Cell Cultures. The growth inhibition determined simultaneously in 5 glial tumor cell strains after a 4-day exposure to a wide dose range of ara-C is shown in Table 2. Greater than 50% growth inhibition was found in most of the tumor cell strains with doses between 0.05 and 0.5 μg/ml. The dose response was not parallel to the increase in dose level but did reach a plateau at higher doses.

Growth Inhibition with Various Concentrations and Different Durations of Treatment. The kinetics of growth inhibition in the presence of 4 different doses of ara-C on 2 glial tumor cell strains are shown in Chart 1. Average cell numbers from 3 wells were plotted against 4 different concentrations. The drug was added to the cultures on the 1st day, and cells were exposed to the drugs for 2 or 4 days with 2 changes of medium (2-day interval). In this particular experiment, approximately 500 cells for HB10 and 200 cells for HB8 were plated initially in each well.

The results illustrate that growth inhibition, measured by the decrease in cell numbers, was closely related to duration of treatment and drug concentrations. The growth of both tumor cells was more inhibited with 4-day than with 2-day treatments in the same concentrations (0.5 to 50 μg/ml). The inhibition was also closely related to the dose between 0.05 to 5.0 μg/ml for HB10 and 0.05 to 0.5 μg/ml for HB8 cells, but not at higher doses.

Recovery Experiments. The degree of recovery in 2 tumor strains (HB8 and HB10) was determined after tumor cells were exposed to different concentrations of ara-C for various periods of time. The results of these experiments, depicted in Charts 2 and 3, show that growth inhibition was again more profound with prolongation of treatment. After a 6-day exposure to the drug at the lowest concentration (0.05 μg/ml), growth inhibition was nearly the same as with the highest concentration (50 μg/ml) in both tumor cell strains. After 13 days of incubation in the control group, cell numbers exceeded 1200 relatively small cells/well for HB8 strain and 480 larger cells/well for HB10 strain. Significant recoveries of HB10 cells were found, however, in cultures exposed to the lowest dose (0.05 μg/ml) for 2, 4, and 6 days and to a dose of 0.5 μg/ml for 2 days only. In HB8 cells, meaningful recovery was found only in the culture exposed to 0.05 μg/ml for 2 days. No significant recovery was found in treatments with concentrations higher than those previously mentioned. The morphological alterations after treatment for 6 days at 0.5 μg/ml, and following recovery of 10 days after removal of the drug, are given in Figs. 1 to 8. No essential changes were observed after the 6-day treatment with the drug, except for a decrease in cell numbers (Figs. 3 and 7). After drug removal for recovery, the cells tended to become enlarged, and some multinucleated cells were found (Figs. 6 and 8). In a separate experiment, cloned HB8 cells were exposed to the same concentrations of drug for 6 days and allowed to remain in the well for 20 days after the drug was removed; similar results were obtained.

Table 1

<table>
<thead>
<tr>
<th>Tumor strains</th>
<th>No. of subcultures</th>
<th>Days in culture</th>
<th>Doubling time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB8</td>
<td>18</td>
<td>369</td>
<td>48</td>
</tr>
<tr>
<td>HB10</td>
<td>11</td>
<td>345</td>
<td>60</td>
</tr>
<tr>
<td>HB19</td>
<td>10</td>
<td>210</td>
<td>53</td>
</tr>
<tr>
<td>HB25</td>
<td>3</td>
<td>96</td>
<td>120</td>
</tr>
<tr>
<td>HB27</td>
<td>2</td>
<td>43</td>
<td>96</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Concentration of ara-C (μg/ml)</th>
<th>HB8</th>
<th>HB10</th>
<th>HB19</th>
<th>HB25</th>
<th>HB27</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>241 ± 33b</td>
<td>121 ± 26</td>
<td>134 ± 54</td>
<td>21 ± 1.0b</td>
<td>31 ± 4.0b</td>
</tr>
<tr>
<td>5.0</td>
<td>60 ± 5.0b</td>
<td>52 ± 10b</td>
<td>67 ± 7.5b</td>
<td>17 ± 2.0b</td>
<td>18 ± 4.0b</td>
</tr>
<tr>
<td>50</td>
<td>61 ± 11b</td>
<td>49 ± 6.0b</td>
<td>48 ± 1.4b</td>
<td>15 ± 5.0b</td>
<td>19 ± 4.1b</td>
</tr>
</tbody>
</table>

a Mean ± S.D.
b Significantly different from controls (p ≤ 0.05).
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Combination Chemotherapy with BCNU. The effect of ara-C alone and in combination with BCNU was studied in the HB8 cell strain. The cells were exposed to the drugs either sequentially or simultaneously for a period of 6 days with 2 changes of drug medium (total of 3 doses). The results of these treatments are shown in Chart 4. Irrespective of treatment, the growth inhibition was closely related to the concentration of ara-C, ranging from 0.0005 to 0.05 µg/ml, but the slope of the curve became flat as the dose increased from 0.05 to 0.5 µg/ml. There was no significant difference between the treatments with a single drug (ara-C) and with the 2 drugs simultaneously. After exposure to BCNU for 6 days, the number of surviving cells was approximately 30% of the controls. However, these surviving cells could eventually recover from growth inhibition if no further treatment of BCNU were made. The maximum growth inhibition was obtained when ara-C was combined with BCNU in sequential treatments (2 doses of BCNU and 1 dose of ara-C). At doses of 0.0005 µg/ml of ara-C, the growth inhibition by sequential treatment increased approximately 2- to 3-fold over ara-C treatment but only slightly over BCNU treatment. At a dose of 0.005 µg/ml, the inhibition by the sequential combination was 2-fold higher than with BCNU alone and 2.5-fold greater than with ara-C alone. These differences between treatments were statistically significant. When doses of ara-C reached 0.05 to 0.5 µg/ml, however, no significant increase in additive inhibition was found.

Chart 1. The effects of different concentrations and treatments of ara-C, added 1 day after plating, on the growth of glioma cell strains HB8 and HB10.

Chart 2. The effects of different concentrations and treatments of ara-C, added 1 day after plating, on the growth of glioma cell strain (HB10), and its recovery after 10-day incubation.

Chart 3. The effects of different concentrations and treatments of ara-C, added 1 day after plating, on the growth of glioma cell strain, and its recovery after 10-day incubation.
Responses of Gliarial Tumor Cells to ara-C and BCNU

The present studies demonstrate that ara-C effectively inhibits the growth of 5 cell strains of human glioblastoma in concentrations between 0.05 and 5.0 μg/ml. The response to ara-C paralleled increases in dose over a considerable range; but eventually, further increases in the dose produced no significant additional growth inhibition (Table 2; Chart 1). The survival of cells decreased as the repeated treatments were prolonged. These responses have been interpreted as typical responses of a cycle-active agent that affects cells only during the DNA-synthesis phase of the mitotic cycle (4, 6, 15, 16).

Surviving cells reentering the proliferating pool would be more susceptible to ara-C, chemotherapy may prove of greater value early in the postoperative period.

ACKNOWLEDGMENTS

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REFERENCES


2. Cytogenetic and Morphologic Abnormalities in Human Marrow
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Figs. 1 to 4. Cytomorphology of uncloned HB8 glioma cultures after 6-day exposure to ara-C (0.5 μg/ml) and after 10 days without drug. x 170.

Fig. 1. A 7-day-old control culture.
Fig. 2. A 17-day-old control culture.
Fig. 3. A 7-day-old culture after 6-day exposure to ara-C.
Fig. 4. A 17-day-old culture after 6-day exposure to ara-C and 10 days with the drug removed.
Figs. 5 to 8. Cytomorphology of HB10 glioma cultures after 6-day exposure to ara-C (0.5 μg/ml) and after 10 days without the drug. X 170.

Fig. 5. A 7-day-old control culture.

Fig. 6. A 17-day-old control culture.

Fig. 7. A 7-day-old culture after 6-day exposure to ara-C.

Fig. 8. A 17-day-old culture after 6-day exposure to ara-C and 10 days after drug removal.
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