Comparison of bromodeoxyuridine labeling indices obtained from tissue sections and flow cytometry of brain tumors

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Sixteen patients with brain tumors were given a 30- to 60-minute intravenous infusion of bromodeoxyuridine (BUdR), 200 mg/sq m. Grossly viable fragments were taken from the biopsied tumor specimens and divided into two portions. One portion was dissociated into single cells, stained both with fluorescein isothiocyanate (FITC) using anti-BUdR monoclonal antibody as the first antibody and with propidium iodide (for deoxyribonucleic acid), and analyzed by flow cytometry (FCM). The labeling index (LI) was calculated as the number of FITC-labeled cells expressed as a percentage of the total number of cells analyzed. The other portion was fixed in 70% ethanol, embedded in paraffin, sectioned, and stained with immunoperoxidase using anti-BUdR monoclonal antibody as the first antibody. The LI of these tissue sections was calculated in two ways: from selected areas in which the labeled cells were evenly distributed and from the entire tissue section. The LI's obtained by FCM correlated closely with those from the entire tissue sections ($r = 0.99, p < 0.000001$) and were usually lower than LI's from selected areas of tissue sections. The LI's determined by FCM also correlated well with the LI's from selected areas of tissue sections ($r = 0.82, p < 0.00012$), despite the difference in values between them. Thus, the FCM-derived LI and the tissue LI can both provide useful information for predicting the biological malignancy of individual tumors and for designing treatment regimens for individual patients with brain tumors; however, different standards should be used to interpret the LI's obtained by these two methods.

KEY WORDS • brain neoplasm • cell kinetics • bromodeoxyuridine • monoclonal antibody • flow cytometry

PRevious studies have demonstrated the advantage of using bromodeoxyuridine (BUdR) and anti-BUdR monoclonal antibody$^8$ to perform cell kinetics studies on tumors of the central nervous system.$^4,10-13,30-33$ Two methods may be used to obtain the BUdR labeling index (LI), which indicates the fraction of cells undergoing deoxyribonucleic acid (DNA) synthesis: nuclei containing BUdR can be identified in tissue sections by immunoperoxidase staining;$^{19,21}$ alternatively, BUdR-labeled single cells derived from biopsy specimens can be stained with immunofluorescent antibody and detected by flow cytometry (FCM).$^6,22$ In the present study, the LI's of various human brain tumors were determined by those two methods and the correlation between the results was analyzed in individual tumors.

Materials and Methods

Permission to administer BUdR was received from the Human Experimentation Committee at the University of California, San Francisco, and from the National Cancer Institute. Informed consent was obtained from each patient or from a responsible relative. The glial neoplasms were classified according to criteria currently in use at the University of California, San Francisco.$^5$ Astrocytomas were graded as nonanaplastic or as mildly, moderately, or highly anaplastic based on the degree of cellularity, nuclear and cytoplasmic pleomorphism, and vascular proliferation.

Sixteen patients with various brain tumors were entered in this study. All patients received a 30- to 60-minute intravenous infusion of BUdR (200 mg/sq m)
Bromodeoxyuridine labeling indices in brain tumors

at the start of surgery. Each excised tumor specimen was divided into two portions. One portion was immediately placed in 70% ethanol, fixed for at least 12 hours, and embedded in paraffin. From the other portion, small tumor pieces that appeared grossly homogeneous and non-necrotic were selected, minced, and placed in an enzyme cocktail consisting of 0.02% collagenase II (139 U/mg), 0.02% deoxyribonuclease I (7 × 10 dornase U/mg, B grade), and 0.05% pronase (45 PKU/mg, B grade) and stirred with a magnetic stirrer at 37°C for 30 minutes to produce a clean single-cell suspension. The cells were filtered through a 37-μm Nitex mesh filter* and fixed in 70% ethanol for at least 30 minutes.

Immunoperoxidase Histochemistry

The paraffin-embedded tumor specimens were cut into sections 6 μm thick, deparaffinized, and immersed in methanol containing 0.3% H2O2 for 30 minutes to block endogenous peroxidase activity. After denaturation of DNA with 2 N HCl, the sections were reacted for 45 minutes with a 1:5000 dilution of IU-4, a monoclonal antibody against iododeoxyuridine that cross-reacts with BUdR. The slides were then immersed for 45 minutes in a 1:50 dilution of peroxidase-conjugated anti-mouse rabbit immunoglobulin G antibody, developed with diaminobenzidine tetrahydrochloride and H2O2 in Tris buffer, and lightly counterstained with Gill No. 1 hematoxylin.

The LI was calculated as the number of BUdR-labeled nuclei expressed as a percentage of the total number of nuclei analyzed, excluding the nuclei of vascular components and hematogenous cells. Several viable areas that had a fairly even distribution of labeled cells were selected for analysis. Two hundred to 600 tumor cells from each area and at least 1000 cells (usually approximately 5000 cells) in each specimen were evaluated. The LI was also calculated from the entire tissue section without selecting the well-labeled areas; all of the tumor cells in each section were counted (approximately 15,000 cells), excluding erythrocytes.

Immunofluorescence Staining and FCM Analysis

Ethanol-fixed single cells were incubated with ribonuclease (1 mg/ml) in phosphate-buffered saline for 15 minutes. Histones were then extracted using 0.1 N HCl and 0.7% Triton X-100. After denaturation of DNA in water at 90°C for 10 minutes, the cells were incubated with a 1:5000 dilution of IU-4 monoclonal antibodies for 30 minutes and then with a 1:100 dilution of FITC-conjugated anti-mouse rabbit immunoglobulin G antibodies manufactured by Zymed, South San Francisco, California.

Results

The LI's obtained by FCM analysis and by analysis of peroxidase-stained tissue sections in 16 patients are shown in Table 1. A bivariate DNA/BUdR distribution histogram of Case 3 is shown in Fig. 1. From the DNA distribution of the histogram, aggregated cells and non-neoplastic components (such as red blood cells and fragments of cells) were excluded by computing the number of cells between the diploid and tetraploid cohorts.

In most cases, the FCM-derived LI was considerably lower than the LI of selected areas of tissue section; however, there was a reasonable correlation between these two values (r = 0.82, p < 0.00012). The LI's of entire sections showed very high standard deviations.

* Nitex mesh filter manufactured by Tetco, Inc., Elmsford, New York.
† Anti-iododeoxyuridine monoclonal antibody IU-4 supplied by Martin Vanderlaan, Ph.D., Biomedical Science Division, Lawrence Livermore Laboratory, University of California, Livermore, California.
‡ Peroxidase-conjugated anti-mouse rabbit immunoglobulin G antibody manufactured by Zymed, South San Francisco, California.
§ FITC-conjugated anti-mouse rabbit immunoglobulin G antibodies manufactured by DACO, Santa Barbara, California.
TABLE 1
Labeling indices measured by immunoperoxidase staining of tissue sections and by flow cytometry*

<table>
<thead>
<tr>
<th>Case No</th>
<th>Age (yrs), Sex</th>
<th>Type of Tumor</th>
<th>BUdR Dose (mg/sq m)</th>
<th>FCM LI (%)</th>
<th>Tissue LI (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71, M glioblastoma multiforme</td>
<td>200</td>
<td>1.7</td>
<td>1.3 ± 1.4</td>
<td>11.9 ± 3.4</td>
</tr>
<tr>
<td>2</td>
<td>40, M glioblastoma multiforme</td>
<td>200</td>
<td>0.2</td>
<td>1.6 ± 3.1</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td>3</td>
<td>40, M glioblastoma multiforme</td>
<td>200</td>
<td>3.2</td>
<td>3.6 ± 2.8</td>
<td>6.9 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>54, M glioblastoma multiforme</td>
<td>200</td>
<td>3.5</td>
<td>3.3 ± 2.0</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>43, F highly anaplastic astrocytoma</td>
<td>200</td>
<td>4.5</td>
<td>3.0 ± 2.6</td>
<td>10.8 ± 3.4</td>
</tr>
<tr>
<td>6</td>
<td>39, F highly anaplastic astrocytoma</td>
<td>200</td>
<td>0.8</td>
<td>0.9 ± 0.7</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>7</td>
<td>26, F highly anaplastic astrocytoma</td>
<td>200</td>
<td>0.4</td>
<td>0.2 ± 0.3</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>23, M moderately anaplastic astrocytoma</td>
<td>200</td>
<td>1.5</td>
<td>0.4 ± 0.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>33, M moderately anaplastic astrocytoma</td>
<td>200</td>
<td>0.6</td>
<td>0.2 ± 0.7</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>21, F moderately anaplastic astrocytoma</td>
<td>200</td>
<td>1.7</td>
<td>0.04 ± 0.2</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>11</td>
<td>2, F ependymoma</td>
<td>200</td>
<td>1.0</td>
<td>0.3 ± 0.4</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>12</td>
<td>43, F medulloblastoma</td>
<td>200</td>
<td>1.3</td>
<td>0.2 ± 0.7</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>13</td>
<td>59, F malignant meningioma</td>
<td>200</td>
<td>1.3</td>
<td>0.8 ± 0.7</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>14</td>
<td>55, F meningioma</td>
<td>200</td>
<td>1.5</td>
<td>1.2 ± 0.9</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>15</td>
<td>60, F meningioma</td>
<td>200</td>
<td>0.2</td>
<td>0.2 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>16</td>
<td>66, M metastatic</td>
<td>200</td>
<td>22.9</td>
<td>17.7 ± 1.2</td>
<td>20.9 ± 2.6</td>
</tr>
</tbody>
</table>

* BUdR = bromodeoxyuridine; FCM = flow cytometry; LI = labeling index.
† Mean ± standard deviation.

which implies heterogeneous labeling patterns, but correlated more closely with the FCM LI's (r = 0.99, p < 0.000001) than the LI's computed from selected areas of tissue sections.

Discussion

Studies of BUdR labeling have provided a considerable amount of information on the growth and biology of various brain tumors.4,10–13,20,24 These data supplement the histopathological findings and thereby allow more accurate prediction of prognosis and aid in the design of treatment regimens for individual patients. For more than 20 years, BUdR has been used as a radiosensitizing agent without causing serious side effects.21–26 Although teratogenic and mutagenic effects could be induced by high doses or prolonged administration of BUdR,27,28 the doses required for in vivo labeling studies (150 to 200 mg/sq m) were small compared with the doses used therapeutically29 and resulted in a low serum BUdR concentration that did not induce mutation experimentally19–27,30 and did not increase the sister chromatid exchange rate.32 Thus, BUdR labeling studies can be applied with fewer restrictions than in therapeutic protocols to elucidate the complex proliferation kinetics of human tumors in situ and to better understand their malignant behavior.

In calculating the LI from tissue sections, viable areas in which the labeled cells are evenly distributed were usually selected for analysis, as these areas represent the greatest proliferative potential of each tumor. The areas in which labeling is minimal or absent, possibly as a result of necrosis, biocarcinosis, or restricted delivery of BUdR17,23 are excluded. In this study, however, the LI of entire sections was also calculated without consideration of the labeling pattern. As expected, this LI value was considerably lower than the LI of selected areas of tissue sections and had a very high standard deviation, indicating very heterogeneous labeling.

The single-cell suspension for FCM analysis was obtained from the whole tumor specimen and therefore contained cells from the areas in which the labeling was limited or absent as well as an unpredictable number of normal cells (such as hematogenous cells and fibroblasts). In most cases, the presence of such cells appeared to make the FCM-derived LI much lower than the LI of selected areas of tissue sections, from which obviously non-neoplastic cells were excluded. Nevertheless, these two values correlated closely. In previous studies, the tissue LI of selected areas was fairly consistent with the histopathological diagnosis and the clinical behavior of individual tumors4,13–20 and had a prognostic value in individual patients.4,15 Because the FCM-derived LI correlates with the LI of selected areas of tissue sections, it can serve as an index to predict the biological and clinical behavior of each tumor. In this study, five of six tumors that had high tissue LI's in selected areas (6.2% to 20.9%) had FCM-derived LI's of more than 2%, and all 10 tumors that had tissue LI's of less than 5% in selected areas had LI's of less than 2% based on FCM. Thus, the FCM-derived LI may also predict the proliferative potential of individual tumors, but different standards must be used to interpret the FCM and tissue LI's.

Bookwalter, et al.,3 investigated the correlation between the patient's survival time and the LI obtained by analysis of single-cell suspensions of 38 glioblastomas multiforme labeled in vitro with tritiated thymidine. The tumors were classified into two groups based on whether the LI's were above or below 5%. Their
results did not confirm the findings of an early study from our laboratory, in which LI's obtained by analysis of selected areas of tissue sections of tumors labeled with tritiated thymidine in vivo and in which an LI of 5% was critical in predicting the prognosis. The discrepancies between these two studies can be explained by the fact that in this current study the LI's obtained from a single-cell suspension of an entire tumor specimen were much lower than those from selected areas of tissue sections. Therefore, it is not surprising that Bookwalter, et al., found LI's below 5% in more than 50% of the glioblastomas in their study, whereas in our previous studies most such tumors had LI's greater than 5%. We believe that the LI's measured by different methods cannot be compared directly using the same criteria for interpretation. The conclusion of Bookwalter, et al., that cell kinetics studies are useless for predicting prognosis is, at the very least, premature.

The good correlation between survival time and LI obtained from tissue sections by in situ labeling studies has been confirmed in other kinds of solid tumors. The results of the current study indicate that the LI obtained by FCM analysis of single-cell suspensions from biopsied tumor specimens may also have a prognostic value in individual patients. The importance of this line of research is to elucidate the significance of LI's obtained by various methods in relation to the actual rate of tumor growth, response to treatment, and patients' survival time so that the benefit of cell kinetics studies are validated for routine clinical practice.

Acknowledgments

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References


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