Dynamic determination of human glioma invasion in vitro

SVEIN J. T. NYGAARD, M.D., PH.D., HANS K. R. HAUFLAND, M.D.,
OLE DIDRIK LAERUM, M.D., PH.D., MORTEN LUND-JOHANSEN, M.D., PH.D.,
ROLF BJERKVIG, PH.D., AND OLE-BJÖRN TYSNES, M.D., PH.D.

Department of Pathology, The Gade Institute, and Departments of Neurosurgery, Anatomy and Cell Biology, and Neurology, University of Bergen, Haukeland Hospital, Bergen, Norway

Object. The goal of this study was to evaluate whether there is any relationship between survival of patients with brain tumor and tumor proliferation or tumor invasion in vitro.

Methods. Samples of freshly resected brain tumors from 14 patients with glioblastoma multiforme (GBM) were directly grown as three-dimensional multicellular spheroids. The tumor spheroids were cocultured with fetal rat brain cell aggregates (BCAs), used to represent an organotypical normal brain tissue model. Before the coculture, the tumor spheroids and the BCAs were stained with two different carbocyanine dyes, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 3,3'-dioctadecycloxacarbocyanine perchlorate (DiO), respectively. During the coculture, confocal laser scanning microscopy allowed a sequential analysis of tumor cell invasion by visualizing dynamic aspects of the invasive process. Single cocultures were examined at three different time points (24, 48, and 96 hours). During the observation period there was a change in the structural morphology of the cocultures, with a progressive decrease in BCA volume. Furthermore, the scanning confocal micrographs revealed a bidirectional movement of tumor cells and normal cells into brain and tumor tissue, respectively. It is also shown that there is a considerable variation in the rate of BCA destruction in cocultures of glioma spheroids generated directly from biopsy specimens. This variation is seen both between spheroids generated from the same biopsy as well as between spheroids that are grown from different biopsy specimens.

Cell proliferation measured by Ki-67 immunohistochemical analysis of biopsy samples obtained in the same patients revealed a correlation between tumor cell proliferation and tissue destruction of the BCAs, as determined by a reduction in BCA volume (p = 0.0338). No correlation was found when survival was related to the same parameters (p > 0.05).

Conclusions. The present work provides a model for quick and efficient assessment of dynamic interactions between tumor and normal brain tissue shortly after surgery.

Key Words • brain neoplasm • spheroid • confocal laser scanning microscopy

In several reports it has been demonstrated that three-dimensional tumor spheroids can be generated directly from freshly dissected biopsy specimens of human brain tumors. These studies have shown that several biological characteristics of the biopsy specimens, such as labeling index, ploidy, and histological features, also were reflected in the spheroids. Glioma biopsy spheroids invade brain cell aggregates (BCAs), and such cocultures have been used to study specific effects of monoclonal antibodies, growth factors, and cytostatic and other modulating agents.

The fluorescent dyes 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 3,3'-dioctadecycloxacarbocyanine perchlorate (DiO) have been used to visualize single cells within spheroids derived from glioma cell lines and BCAs, respectively. Thus, in cocultures of tumor spheroids and BCAs, single cell interactions can be monitored by using confocal laser scanning microscopy (CLSM).

The median survival of patients with diagnosed glioblastoma multiforme (GBM) is less than 12 months. Prognostic factors that seem to be of importance are the age of the patients, the location and size of the tumor, and the extent of surgical resection. Survival time has been shown to be longer in younger patients in whom the tumor is small and has been macroscopically removed. The amount of proliferating cells in the glioma has also been related to prognosis. However, the extensive variation in histological features, ploidy, and cell proliferation within a tumor may cause errors in the classification of gliomas, as well as in predicting their biological behavior.

In the present study we show that there is considerable variation in the rate of BCA destruction in cocultures of glioma spheroids grown directly from biopsy specimens. This variation was seen both between spheroids generated from the same biopsy as well as between spheroids grown from different biopsy specimens. Furthermore, the amount of BCA destruction was correlated to the amount...
of proliferating cells in the tumor, as determined by the number of Ki-67 cells within the tumors.

Materials and Methods

Tissue Samples and Cultures

Tumor tissue samples were obtained in 16 patients undergoing surgery for tumors, histologically classified as GBM, during the period from January 1994 to December 1995. The fragments of tissue were cut into pieces measuring 200 to 500 μm and cultured in an agar overlay culture in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum, four times the prescribed concentration of nonessential amino acids, 2% L-glutamine, penicillin (100 IU/ml), and streptomycin (100 mg/ml). The nonadhesive base coating consisted of 0.75% Noble Agar. After a 2-week culture in a standard tissue culture incubator (5% CO₂/95% air at 37°C), the tissue was fixed in 4% paraformaldehyde in sodium cacodylate buffer (pH 7.4, 25 mOsm). The specimens were washed in the same buffer without glutaraldehyde and postfixed in 0.75% OsO₄ before undergoing serial dehydration in ethanol. Subsequently, the slides were embedded in Epon 812, whichpolymerized after 48 hours at 60°C. Semithin (1.5-μm) sections were cut on a microtome, stained with toluidine blue, and examined by light microscopy.

Fluorescent Dye Staining Procedures

Tissue samples and cultures from 14 cases were collected and optically sectioned from surgical material. Brain cell aggregates were formed in specimens obtained in 14 of the 16 patients.

Brain Cell Aggregates

Brain cell aggregates were formed from brains of fetal BD-IX rats, as previously described.

Fluorescent Dye Staining Procedures

The fluorescent dyes DiI and DiO have maximum absorption at 546 nm and 489 nm and maximum emission at 563 nm and 499 nm, respectively. The difference in fluorescence emission makes the dyes easily separable by specific fluorescence filter optics. Two and one-half milligrams DiI in 1 ml ethanol and 3 mg DiO in 1 ml dimethylsulfoxide (DMSO)/ethanol (1:9) were sonicated for 10 minutes to obtain completely dissolved stock solutions before diluting them in complete DMEM to final concentrations of 0.075 mg/ml and 0.09 mg/ml, respectively. Before the coculture began, equally sized tumor spheroids (300 ± 25 μm), 2 to 3 weeks old, and BCAs (300 ± 25 μm) were incubated for 48 hours in media containing DiI and DiO, respectively. For more details see the article by Nygaard, et al.

Confocal Laser Scanning Microscopy

Cocultures were analyzed by using a CLSM unit. The DiO and Dil fluorescence emissions were detected simultaneously by using fluorescein isothiocyanate (520 nm) and Texas red (620 nm) filter optics.

The Cavalieri principle was applied by interval measurements of serially sectioned specimens. At 24, 48, and 96 hours, five cocultures from 14 cases were collected and optically sectioned from surface to center with 22 μm between each slice; the resulting volume of BCAs was then estimated. Using this method, tumor cell invasion was determined as the amount of BCA tissue destruction, as described previously.

Labeling for Ki-67

Portions of the biopsy material were fixed in 4% paraformaldehyde and embedded in paraffin according to standard histological techniques. Serial 5-μm sections were deparaffinized and rehydrated in decreasing concentrations of ethanol; the Ki-67 antigen was retrieved by applying microwaves (800 W) to 10-nMl citrate-covered slides. Thereafter the slides were immunostained with the MIB-1 monoclonal antibody at a dilution of 1:50 to recognize Ki-67. Antibody binding was visualized using an avidin-biotin complex/horseradish peroxidase reaction. The sections were then counterstained with hematoxylin and the numbers of Ki-67-positive and -negative nuclei were determined. A total number of 1000 cells were counted and the percentage of labeled nuclei was assessed.

Light Microscopy

Cocultures of BCAs and tumor spheroids stained with DiO and DiI, respectively, were fixed in 2% glutaraldehyde in sodium cacodylate buffer (pH 7.4, 300 ± 25 mOsm). The specimens were washed in the same buffer without glutaraldehyde and postfixed in OsO₄ before undergoing serial dehydration in ethanol. Subsequently, the specimens were embedded in Epon 812, which polymerized after 48 hours at 60°C. Semithin (1.5-μm) sections were cut on a microtome, stained with toluidine blue, and examined by light microscopy.

Statistical Analysis

Patient survival time was recorded from data provided by the Norwegian Central Bureau of Statistics. Comparative analyses between the different biological parameters were performed. This involved comparisons between BCA destruction, Ki-67 labeling index, and patient survival data. For this purpose regression analyses were performed.

Sources of Supplies and Equipment

The DMEM, 10% heat-inactivated newborn calf serum, nonessential amino acids, L-glutamine, penicillin, and streptomycin were purchased from BioWhittaker (Walkersville, MD) and the Noble Agar from Difco Laboratories (Detroit, MI). Tissue-culture plastic articles were obtained from Nunc (Roskilde, Denmark) and the DiI/DiO (3, 1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate) and DiI/DiOC18(3) (3,3'-dioctadecyloxycarbocyanine perchlorate) from Molecular Probes, Inc. (Eugene, OR).

The instrument used to sonicate the DiI ethanol and the DiO in DMSO/ethanol was manufactured by Sonorex, Bandelin (Berlin, Germany). The cocultures were analyzed using the CLSM unit (model MRC 1000) obtained from BioRad (Hertfordshire, England). For the purposes of Ki-67 labeling and visualization of antibody binding, the MIB-1 monoclonal antibody and the avidin-biotin complex/horseradish peroxidase were purchased from Dako (Glostrup, Denmark). The semithin sections of specimens obtained from the cocultures of BCA and tumor to be examined by light microscopy were cut on a microtome (model 2040) obtained from Reichert-Jung (Vienna, Austria).

Results

Clinical Data

Glioblastoma multiforme was identified in 14 consecu-
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Table 2: Confrontations between tumor spheroids and BCA in 14 cocultures

<table>
<thead>
<tr>
<th>Case No.</th>
<th>24 Hrs</th>
<th>48 Hrs</th>
<th>96 Hrs</th>
<th>m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69.8 ± 11.5</td>
<td>55.8 ± 12.9</td>
<td>28.1 ± 9.2</td>
<td>-0.715</td>
</tr>
<tr>
<td>2</td>
<td>85.6 ± 7.8</td>
<td>71.1 ± 12.1</td>
<td>38.3 ± 4.5</td>
<td>-0.644</td>
</tr>
<tr>
<td>3</td>
<td>79.9 ± 14.0</td>
<td>74.7 ± 10.0</td>
<td>37.9 ± 13.1</td>
<td>-0.623</td>
</tr>
<tr>
<td>4</td>
<td>70.8 ± 21.7</td>
<td>49.8 ± 7.3</td>
<td>50.7 ± 6.3</td>
<td>-0.484</td>
</tr>
<tr>
<td>5</td>
<td>63.4 ± 7.4</td>
<td>49.7 ± 8.9</td>
<td>31.7 ± 7.2</td>
<td>-0.679</td>
</tr>
<tr>
<td>6</td>
<td>83.2 ± 13.6</td>
<td>60.7 ± 18.4</td>
<td>41.2 ± 15.1</td>
<td>-0.617</td>
</tr>
<tr>
<td>7</td>
<td>76.6 ± 12.6</td>
<td>60.0 ± 16.6</td>
<td>31.5 ± 15.5</td>
<td>-0.699</td>
</tr>
<tr>
<td>8</td>
<td>78.7 ± 7.9</td>
<td>65.3 ± 6.1</td>
<td>62.3 ± 14.3</td>
<td>-0.369</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td>57.9 ± 5.3</td>
<td>47.8 ± 7.3</td>
<td>-0.544</td>
</tr>
<tr>
<td>10</td>
<td>75.9 ± 21.7</td>
<td>ND</td>
<td>43.2 ± 2.8</td>
<td>-0.592</td>
</tr>
<tr>
<td>11</td>
<td>ND</td>
<td>80.3 ± 26.4</td>
<td>31.5 ± 15.3</td>
<td>-0.706</td>
</tr>
<tr>
<td>12</td>
<td>ND</td>
<td>59.6 ± 14.4</td>
<td>33.1 ± 7.2</td>
<td>-0.697</td>
</tr>
<tr>
<td>13</td>
<td>71.3 ± 28.5</td>
<td>ND</td>
<td>60.7 ± 18.6</td>
<td>-0.349</td>
</tr>
<tr>
<td>14</td>
<td>80.8 ± 16.0</td>
<td>78.3 ± 5.1</td>
<td>27.2 ± 11.5</td>
<td>-0.743</td>
</tr>
<tr>
<td>mean</td>
<td>76.0 ± 13.5</td>
<td>65.1 ± 11.4</td>
<td>40.4 ± 10.6</td>
<td>-0.604</td>
</tr>
</tbody>
</table>

* Data represent means ± standard deviations of five confrontations from each case. The slope of the curve, m, indicates the rate of destruction of BCA. Abbreviation: ND = not determined.

The amount of remaining brain tissue in the cocultures was determined by making morphometric measurements of serial sections. The “rate” of tissue destruction during the 96-hour coculture period is illustrated in Fig. 1. The aggressiveness of the tumor was defined as: the difference in remaining BCA volume (Δy) the difference in time (Δx) = m. This was assessed in all cases (Table 2, right column) and showed that the tumor obtained in Case 14 was the most destructive tumor (m = -0.743) and that the one in Case 8 was the least destructive tumor (m = -0.369) as determined by the actual measured volume at 96 hours. The range of aggressiveness between the cases is illustrated by the slopes of the curves for Case 14 (fastest) and Case 8 (slowest) (Fig. 2) with the stipulation of end points at 100% destruction. Figure 3 shows a coculture including tumor from Case 2 that lasted for 192 hours with a complete fusion between BCA and tumor tissue, with green and red fluorescence, respectively.

The remaining BCA volume determined in each case at 24, 48, and 96 hours and the calculated destruction rates (m) are given in Table 2. The mean remaining BCA volumes at these time-points were 76%, 65.1%, and 40.4% of the remaining BCA volume during the coculture period. Figure 4 shows the results of an optical scan obtained 88 μm from the coculture surface (tumor from Case 14) at 24, 48, and 96 hours. The photomicrographs illustrate the gradual decrease in solid BCA volume, but also indicate single tumor cell infiltration into the BCA. In these specimens the movement of normal cells was also observed.
**FIG. 3.** A CLSM photomicrograph showing a confrontation between a tumor spheroid from Case 2 and BCA after 192 hours. A complete fusion between normal (green) and tumor (red) tissue is seen. Original magnification X 400.

**Intra- and Intertumor Variations**

As indicated in Fig. 2 and Table 2, considerable variation in BCA destruction was observed between the different tumor specimens. The tumor spheroid grown from Case 14 destroyed BCA at the highest rate and those grown from Cases 8 and 13 to the least extent (Table 2). However, not surprisingly, a pronounced intratumor variation was also observed between different organotypical spheroids; this was most pronounced in Case 13 (−0.567 < m < −0.319, ∆m = 0.248 intratumor variation). This variation was less than the variation between the different tumors.

The optical sections were also compared with standard semithin toluidine-stained light microscopic sections. Figure 5 shows the remaining BCA 66 μm from the surface as it appears in unmerged and merged scans. As indicated by the confocal photomicrographs, a severe infiltration of tumor cells into normal brain tissue is seen, which is not recognized to the same extent in ordinary histological sections.

**Comparative Analyses Between Biological Parameters**

Because the biopsy spheroids show several biological and histological features of the original tumors in vivo, the extent of BCA destruction was plotted against patient survival data (Fig. 6). No significant relationship between the two data sets was observed (p = 0.8007). The Ki-67 staining was used as an indicator of proliferation. The labeling index ranged from 3.6 to 30% of the cells with a mean value of 11.7% (Table 1). When Ki-67 data were plotted against BCA destruction after 96 hours (Fig. 7), a positive relationship appeared between BCA destruction and cell proliferation in the tumor specimens studied (p = 0.0338).

**Discussion**

The present study shows that glioma cell invasion can be assessed by using the CLSM technique in vitro to examine cocultures of BCAs and tumor spheroids grown directly from biopsy specimens. Our group earlier quantified tumor cell invasion in serial histological sections by calculating the amount or volume of remaining brain tissue. However, the confocal photomicrographs obtained in the present study by using vital dyes to identify single tumor cell invasion show that the invasive process is far more complex than mere tissue destruction. We used the vital dyes DiI and DiO to discriminate tumor cells from normal cells. These dyes have been regarded as being nontoxic; however, a recent report has shown that the dyes may affect adhesion and migration of tumor cells. In this study highly variable effects of DiI and DiO were observed, indicating that the dyes may exert both inhibitory and stimulatory effects on glioma cell lines. In the present work, the dyes used seemed to have no effects on the invasive properties of the cells. This has also been indicated in previous studies from our group using cell lines.

As shown in Fig. 4, the invasive process involves both single cell infiltration of tumor cells into the BCAs (with no apparent destruction of brain tissue) as well as infiltration of cells from the BCAs into the tumor tissue. We scored the aggressiveness of the tumors by measuring the remaining BCA volume. Using this as a parameter, it was shown that the tumor spheroids progressively destroyed the BCAs in a linear fashion and, from the results provided, it is most likely that the disintegration of normal brain tissue is complete after approximately 200 hours of cocult-
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Fig. 5. Photomicrographs showing elements of coculture with tumor spheroid from Case 13 at 96 hours. A: Merged scan obtained at the level of 66 μm from the surface. B: Unmerged scan with visualization of the tumor on the right and BCA on the left, which is the area that is measured for determination of the invasional degree of the cancer cells. C: Epon-embedded section of a parallel coculture. Original magnification × 400.

ture, ranging from 140 to 270 hours as calculated from the present data. This variation probably reflects the extensive cellular heterogeneity that exists within as well as between different tumors. As seen in Table 2, the BCA destruction ranged from an m of −0.743 (Case 14; most destructive tumor) to an m of −0.369 and an m of −0.349 (Cases 8 and 13, the least destructive tumors). The variation between spheroids obtained from the same tumor was considerably less than that observed between tumors (Δm = 0.248 and Δm = 0.399, respectively).

Numerous studies have shown that glioma cells in vitro as well as in vivo secrete a wide variety of proteolytic enzymes and corresponding inhibitors. Several cysteine, serine, and metalloproteinases have been identified in brain tumors, and it is likely that the destruction of BCAs, as shown in the present study, represents a reflection of an imbalance between several proteases and their inhibitors. The CLSM technique that is described may provide an experimental tool for studying specific effects of certain proteases and their inhibitors in a complex three-dimensional system consisting of brain tumor biopsy specimens and brain tissue. The tumor spheroids generated directly from biopsy specimens reflect several of the biological features found in the patients. Opposed to monolayer cultures, the clonal selection is considerably reduced and the tissues express the same ploidy for prolonged periods in vitro as found in the original biopsy specimens. Furthermore, in these spheroids, the histolog-

Fig. 6. Scatterplot graph showing patient survival time plotted against BCA destruction. No correlation was found between the two parameters (p = 0.8007).

Fig. 7. Graph showing tumor proliferation rate plotted against BCA destruction. The number of Ki-67 positive cells correlated positively to the rate of BCA destruction (p = 0.0338).
tical features and labeling indices are also similar to those of the corresponding biopsy specimens.24,25

It has been shown that there may be an inverse correlation between cell migration and proliferation and several groups have focused on these two different but important aspects of malignant cell behavior.217,34,62 Our data revealed a correlation between tumor cell proliferation and tissue destruction of the BCAs as determined by the reduction in BCA volume (p = 0.0338). This may imply that the destruction of brain tissue involves independent parameters that are not necessarily important during tumor cell migration. This notion is supported by histopathological observations in which single tumor cells frequently are observed within the brain parenchyma without any apparent tissue destruction.23 Thus, the single tumor cell infiltration into the BCA that was observed may reflect a cell migratory process that is not necessarily related to the reduction in BCA volume. Moreover, it is well known that there is a close link between protease production and several growth factors that affect tumor cell proliferation.7,40 This correlates well with the presently observed connection between the Ki-67 labeling index and BCA destruction.

Tumor cell proliferation, as measured by the amount of Ki-67–positive cells, has been proposed as a predictor of survival times in glioma patients.41 This notion may be controversial.18 In the present study we were unable to demonstrate a correlation between the Ki-67 labeling index and patient survival. We also were not able to find any correlation between BCA destruction and patient survival data. This can be explained by the restricted number of patients observed in the present study. Furthermore, correlative studies involving patient data and biological parameters in vitro may be difficult merely because of the location and inherent complexity of these tumors in vivo.

From the present study it is concluded that BCA destruction in vitro varies considerably within as well as between glioma biopsy specimens. In the biopsies studied the amount of BCA destruction correlated positively with the Ki-67 labeling index within the tumors, but not to patient survival data. Furthermore, the present work provides a model for quick and efficient studies of dynamic interactions between tumor and normal brain tissue shortly after surgery. The model may provide important information to be used when evaluating therapy regimens directed against normal brain destruction as well as against biological parameters governing cell migration.

References