Accumulation of CD133-positive glioma cells after high-dose irradiation by Gamma Knife surgery plus external beam radiation

Clinical article

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Object. Recent evidence suggests that a glioma stem cell subpopulation might contribute to radioresistance in malignant gliomas. To investigate this hypothesis, the authors examined recurrent malignant gliomas for histopathological changes after high-dose irradiation with Gamma Knife surgery (GKS) and external beam radiation therapy (EBRT).

Methods. Thirty-two patients with malignant gliomas (Grade 3 in 8 patients, Grade 4 in 24) underwent GKS in combination with EBRT. Serial MR and L-[methyl-11C] methionine PET images were employed to assess remnant or recurrent tumors after GKS. Twelve patients underwent surgical removal after GKS and EBRT. Histological sections were subjected to immunohistochemistry for MIB-1, factor VIII, and stem cell markers, nestin and CD133.

Results. The site of GKS treatment failure was local in 16 (76.2%) of 21 patients with glioblastomas showing progression; in 9 of these 16 patients, the recurrence clearly arose within the target lesion of GKS. Histopathological examination after GKS and EBRT showed variable mixtures of viable tumor tissues and necrosis. Viable tumor tissues exhibited high MIB-1 indices but reduced numbers of tumor blood vessels. There was marked accumulation of CD133-positive glioma cells, particularly in remnant tumors within the necrotic areas, in sections obtained after GKS plus EBRT, whereas CD133-positive cells appeared very infrequently in primary sections prior to adjuvant treatment.

Conclusions. The results indicate that CD133-positive glioma stemlike cells can survive high-dose irradiation, leading to recurrence, despite prolonged damage to tumor blood vessels. This could be an essential factor limiting the effectiveness of GKS plus EBRT for malignant gliomas. (DOI: 10.3171/2010.2.JNS091607)

KEY WORDS • cancer stem cell • CD133 • Gamma Knife surgery • malignant glioma • radioresistance • tumor blood vessels

MALIGNANT gliomas, especially GBMs, are among the most lethal primary human malignancies, showing rapid growth, high invasiveness and vascularity, and occasionally dissemination into the CSF space.13 Despite multimodal therapy, the prognosis of patients with these tumors remains poor.32,34 Radiation represents the most effective adjuvant therapy for GBM.16 In a prospective randomized trial, overall survival correlated directly with delivery of higher doses of EBRT.3,35 However, even with improved outcomes at higher EBRT doses, patients continued to experience tumor recurrence at the primary site,28 and overall survival times in GBM patients usually averaged less than a year.37 As most tumor progression occurs as a result of local tumor invasion, GKS would appear to be an ideal technique for additional adjuvant treatment.7 Gamma Knife surgery can deliver high-dose radiation to the tumor while avoiding toxicity to normal tissues. Stereotactic radiosurgery (including GKS) not only induces direct injury to tumor cells but also causes vascular damage to tumor blood vessels, resulting in tumor necrosis.24 Earlier institutional studies demonstrated improved median survivals in patients with...
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malignant glioma who were treated with GKS.31,34,38 Recently, the Radiation Therapy Oncology Group concluded their randomized prospective trial and provided level I evidence that “up front” use of stereotactic radiosurgery followed by EBRT and carmustine produced no survival benefit in patients with GBM.31 The lack of survival benefit with the addition of stereotactic radiosurgery in this study is interpreted as being attributable to GBMs being inherently infiltrative neoplasms, although the details of radiosurgical treatment failure for malignant gliomas have not been elucidated.39

Recent cancer development concepts suggest that a small population of cancer stem cells may determine the biological behavior of tumors, including responses to therapy. In malignant brain tumors, CD133 has been suggested to be a cancer stem cell marker, since only CD133-positive cells from brain tumor biopsy material were able to initiate brain cancer in a mouse model.26,27 Recently, Bao et al.1 reported that a glioma stem cell subpopulation expressing CD133 promoted radioresistance in cultured cells and in a rodent model of GBM via preferential activation of the DNA damage response. Several reports have supported this hypothesis using cultured cells and experimental animal models.9,12 However, no direct clinical evidence, based on examination of surgical specimens, has been provided to show that glioma stemlike cells play a role in the mechanism of radiation resistance in malignant gliomas. We hypothesized that glioma stemlike cells might be involved in failure of malignant glioma treatment by GKS and EBRT. To address this question, we investigated patterns of recurrence and histopathological changes in malignant gliomas after GKS plus EBRT. Our results suggest that CD133-positive glioma stemlike cells can survive and cause recurrence despite vascular damage to tumor blood vessels even after high-dose radiation using GKS plus EBRT.

Methods

Patient Population and GKS Treatment

From 2000 to 2006, 51 patients were newly diagnosed as having high-grade gliomas at the Hospital of Tokyo Medical and Dental University. After resection, EBRT was administered using a linear accelerator with 2 Gy as the daily fraction and a median total EBRT dose of 54 Gy. Thirty-two of the 51 patients underwent GKS after initial tumor resection in addition to EBRT. Adjuvant chemotherapy was 4–5 cycles of intravenous cisplatin and etoposide. The criteria used for GKS treatment included: 1) histopathological diagnosis of high-grade glioma at tumor resection, 2) no diffuse gliomatosis, 3) Karnofsky Performance Scale score better than 50, and 4) an expected survival of 3 months or more. The 32 patients who underwent GKS included 24 with Grade 4 gliomas (GBMs) and 8 with Grade 3 gliomas (4 anaplastic astrocytomas and 4 anaplastic oligodendrogliomas). The tumor target inclusion area was determined considering the MET-PET and MR imaging findings. Patients were followed up with MR imaging every 8–12 weeks from GKS treatment until the time of death. When the contrast-enhanced MR images indicated possible tumor progression or recurrence, MET-PET studies were routinely performed to differentiate recurrence from radionecrosis. Surgical removal after GKS plus EBRT was considered after the discovery of progressive changes on MR and/or MET-PET images, regardless of the presence or absence of progression of neurological deficits. For all resections, we used the Stealth Station (Medtronic Sofamor Danek, Inc.) navigation system in order to correlate histopathological findings with the MET-PET and MR images. Informed consent was obtained from patients and/or guardians.

Histological Study

The pathological specimens were classified according to WHO criteria by staining with H & E. Immunohistochemical detection was carried out using the labeled streptavidin-biotin method with an LSAB Kit (Dako) or the catalyzed signal amplification method with a CSA II kit (Dako). Formalin-fixed and paraffin-embedded sections were pretreated in an autoclave to enhance immunoreactivity.34 The following antibodies were used: rabbit antibody against CD133 (Cell Signaling), mouse monoclonal antibody against Ki 67 antigen (MIB1, Dako), and rabbit antibodies against factor VIII–related antigen (Dako) and nestin (Chemicon). The CSAII system was used for the detection of CD133 antigen and the LSAB system was used for the other antigens. The CSA II system is a biotin-free highly sensitive immunohistochemical staining procedure incorporating a signal amplification method based on the peroxidase-catalyzed deposition of a fluorescein-labeled phenolic compound, followed by a secondary reaction with a peroxidase-conjugated antifluorescein antibody. In comparison with standard immunohistochemical methods, such as LSAB or avidin-biotin complexes, the CSA II system has been reported to be many times more sensitive.33 A nonspecific isotype control instead of the primary antibody was used as a negative control. The sections were developed with diaminobenzidine (Dako) and counterstained with hematoxylin. Masson trichrome staining was performed to examine changes in blood vessels after GKS plus EBRT. We determined MIB-1 staining indices by counting more than 500 nuclei in 3 or more randomly selected high power fields (400 x magnification). Tumor blood vessel density was determined by counting the number of blood vessels per area in sections stained with antibodies against factor VIII–related antigens. The percentage of CD133-positive cells per tumor area was calculated in histological sections immunostained for CD133 antigen according to the method reported previously by Zeppernick et al.29 with some modifications. Because of the variation in CD133-positive cell distributions in histological sections, we selected viable tumor tissues, excluding necrosis and nontumorous brain tissues, and determined the percentage of CD133-positive cells in several small sections of a whole histological section. The percentage of CD133-positive cells in tumor tissues in a whole section was calculated by counting more than 500 cells in 3 or more randomly selected high power fields (400 x magnification) in each small section and by measuring actual areas with ImageJ software for Mac. More than 2 histological sections were examined in tumor tissues obtained from each resection. Immunohis-
Thirty-two patients underwent GKS after initial surgical removal in addition to EBRT. The mean tumor volume treated at the time of GKS was 27.0 cm$^3$ (range 1.3–129.4 cm$^3$) and the median GKS dose was 18 Gy (range 12–25 Gy) with the median prescription isodose line being 50% (range 40–60%). Most tumors initially responded well to GKS but later recurred. Three of the GBM patients died of complications without disease progression. Two patients with Grade 3 tumors have been progression free for 32 and 89 weeks. Twenty-one patients with GBMs and 6 with Grade 3 gliomas showed disease progression. We analyzed the recurrence patterns of these 27 patients (Table 1). The site of GKS treatment failure was local in 16 patients (76.2%) with GBMs, 9 of which had clearly arisen within the GKS target while the others included lesions outside the GKS target (that is, in each of these 7 cases the recurrence was partially within the target and partially adjacent to it). In these cases, methionine uptake on PET was decreased after GKS plus EBRT but was later increased (Fig. 1). Recurrences at remote sites, including 1 GBM with ependymal dissemination, were seen in 5 patients with GBMs, 1 with an anaplastic astrocytoma, and 2 with anaplastic oligodendrogliomas. Complete remission, as assessed by MET-PET, was obtained in 2 patients with GBMs. However, these 2 patients later had recurrences of GBM within the GKS target region. There were 4 patients with anaplastic oligodendrogliomas treated with GKS. Their tumors initially responded well to GKS and EBRT, disappearing completely, but later recurred. The median time to tumor progression in GBM patients was 8.4 months after GKS. The median survival of the 24 GBM patients who underwent EBRT plus GKS was 67 weeks. Survival rates at 1 and 2 years for GBM patients treated with GKS were 75% and 21%, respectively.

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Twelve of the 32 patients underwent second surgeries at the time of tumor recurrence after GKS plus EBRT. Two of these 12 patients underwent craniotomy within 1 week after GKS because of disease progression. Data from the remaining 10 patients were analyzed (Table 2). The site of GKS plus EBRT treatment failure was local in all but 1 patient (Case 10) who had a remote-site recurrence. Histopathological examinations after high-dose irradiation showed a variable mixture of viable tumor tissues and necrosis. The intervals between GKS and the second surgery ranged from 4.6 to 48 months (mean ± SD, 14.6 ± 13.6 months). There were no apparent correlations between the intervals since GKS and the variations in histological characteristics. The mean tumor volume treated at the time of GKS in these 10 patients was 22.9 cm$^3$ (range 1.3–44.4 cm$^3$). The median GKS dose was 18 Gy (range 12–21 Gy) and the median prescription isodose line was 50% (range 40–60%).

We examined glioma tissues for the expressions of stem cell markers, nestin and CD133, by immunohistochemical analysis using paraffin sections and compared between sections obtained during the primary and second surgeries. We found that CD133-positive glioma cells were very infrequent in primary sections but were dramatically increased in sections obtained at the second surgery after GKS plus EBRT (Fig. 2A and B). Positive immunoreactivity for CD133 antigen was clearly identified on the cell membrane but in neither the cytosol nor the nuclei of glioma cells (Fig. 2C). No immunoreactivity was seen in a section treated with a nonspecific isotype control instead of the primary antibody (Fig. 2D). The CD133-positive glioma cells usually appeared in clusters and were often found near the tumor vasculature (Fig. 2E). Immunoreactivity for nestin was diffuse in all glioma cells and did not correspond to CD133 immunopositivity (Fig. 2F and G). No CD133 reactivity was detected in primary sections from 2 patients, while CD133 positivity was less than 1% in 6 others. The 2 patients had values of 2.0 and 7.7%. The mean percentage of CD133-positive tumor cells in sections after GKS plus EBRT was 16.5 ±

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**Table 1: Patterns of treatment failure in malignant gliomas treated with GKS plus EBRT**

<table>
<thead>
<tr>
<th>Failure Site</th>
<th>Grade 4 (21 cases)</th>
<th>Mean Time to Failure (mos)</th>
<th>Grade 3 (6 cases)</th>
<th>Mean Time to Failure (mos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>local failure</td>
<td>16</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>w/in GKS target</td>
<td>9</td>
<td>11.0 ± 7.2</td>
<td>2</td>
<td>4.5</td>
</tr>
<tr>
<td>including lesions adjacent to GKS target</td>
<td>7</td>
<td>5.7 ± 1.5</td>
<td>1</td>
<td>6.0</td>
</tr>
<tr>
<td>remote failure</td>
<td>4</td>
<td>3.5 ± 1.3</td>
<td>3</td>
<td>17.0 ± 21.0</td>
</tr>
<tr>
<td>ependymal failure</td>
<td>1</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data represent numbers of cases unless otherwise indicated. Means are presented with SDs.
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**TABLE 2:** Histopathological changes after GKS plus EBRT in malignant gliomas

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs), Sex</th>
<th>Malignancy Grade</th>
<th>GKS Dose (Gy)†</th>
<th>Vol (cm³)</th>
<th>Interval From GKS (mos)</th>
<th>Failure Site</th>
<th>Histopathology After GKS + EBRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28, M</td>
<td>4</td>
<td>36/18</td>
<td>26.5</td>
<td>6.3</td>
<td>local</td>
<td>4 mostly necrosis</td>
</tr>
<tr>
<td>2</td>
<td>56, F</td>
<td>4</td>
<td>30/15</td>
<td>31.6</td>
<td>9.2</td>
<td>local</td>
<td>4 mostly necrosis</td>
</tr>
<tr>
<td>3</td>
<td>66, F</td>
<td>4</td>
<td>35/21</td>
<td>3.0</td>
<td>22.7</td>
<td>local</td>
<td>4 mostly necrosis</td>
</tr>
<tr>
<td>4</td>
<td>65, F</td>
<td>4</td>
<td>24/12</td>
<td>44.4</td>
<td>8.9</td>
<td>local</td>
<td>4 mostly necrosis</td>
</tr>
<tr>
<td>5</td>
<td>40, M</td>
<td>4</td>
<td>30/18</td>
<td>32.4</td>
<td>6.7</td>
<td>local</td>
<td>4 necrosis plus viable tumor</td>
</tr>
<tr>
<td>6</td>
<td>53, M</td>
<td>4</td>
<td>30/18</td>
<td>9.0</td>
<td>26.2</td>
<td>local</td>
<td>4 necrosis plus viable tumor</td>
</tr>
<tr>
<td>7</td>
<td>57, M</td>
<td>4</td>
<td>25/15</td>
<td>38.6</td>
<td>4.6</td>
<td>local</td>
<td>4 necrosis plus viable tumor</td>
</tr>
<tr>
<td>8</td>
<td>54, F</td>
<td>4</td>
<td>30/18</td>
<td>16.9</td>
<td>7.8</td>
<td>local</td>
<td>4 necrosis plus viable tumor</td>
</tr>
<tr>
<td>9</td>
<td>23, M</td>
<td>3 (AA)</td>
<td>30/15</td>
<td>25.7</td>
<td>5.1</td>
<td>local</td>
<td>4 mostly necrosis</td>
</tr>
<tr>
<td>10</td>
<td>33, F</td>
<td>3 (AA)</td>
<td>40/20</td>
<td>1.3</td>
<td>48.0</td>
<td>remote</td>
<td>4 mostly viable tumor</td>
</tr>
</tbody>
</table>

* AA = anaplastic astrocytoma.
† Values represent maximum dose/peripheral dose.
12.2%, which was significantly higher than the percentage of CD133-positive cells in sections obtained from the primary surgery (1.23 ± 2.36%, p < 0.002, paired t-test) (Fig. 3). In cases showing local recurrence (Cases 1–9), the number of factor VIII-positive tumor blood vessels was significantly decreased in tissue sections obtained from the second surgery after GKS plus EBRT (p < 0.025, paired t-test), while MIB-1 staining indices did not differ significantly between sections from the primary and second surgeries (Fig. 4). Masson trichrome staining showed numerous small blood vessels containing abundant collagen fibers but lacking cells in the vascular wall, possibly due to damage from high-dose irradiation. Our results indicate that CD133-positive stemlike glioma cells can survive high-dose irradiation, even though GKS and EBRT can effectively induce tumor tissue necrosis and vascular damage in malignant gliomas.

Discussion

Malignant gliomas have the unique biological characteristic of being highly invasive into surrounding brain tissue.20,25 Tumor cells invading the surrounding brain may have escaped the stereotactic radiosurgery target. This has been considered to be the major limitation of GKS in the treatment of malignant gliomas. In the present study, most tumors initially responded well to GKS but later recurred. We observed, in more than two-thirds of cases, that ma-
cells after irradiation of malignant gliomas. Our findings indicate that CD133-positive glioma stemlike cells can survive and serve as a potential source of tumor recurrence after high-dose irradiation by GKS plus EBRT.

Tumor growth has been considered to be angiogenesis-dependent, and microvascular endothelial cells recruited by the tumor have been regarded as an important second target in cancer therapy. Malignant gliomas are highly vascular brain tumors showing elevated expressions of vascular endothelial growth factors, which promote blood vessel formation from endothelial precursors. Calabrese et al. demonstrated that CD133-positive/nestin-positive GBM cancer stem cells are located close to tumor capillaries and are maintained within vascular niches that mimic the neural stem cell niche. In our study, CD133-positive glioma cells were often found to be in intimate contact with the tumor vasculature. After high-dose irradiation with GKS plus EBRT, tumor cells immunopositive for CD133 accumulated even within the areas of necrosis induced by irradiation. Immunohistochemistry for factor VIII–related antigen confirmed markedly decreased numbers of tumor blood vessels even in viable tumor areas after GKS plus EBRT. These results indicate that CD133-positive glioma cells show marked accumulation after GKS plus EBRT despite prolonged damage to tumor blood vessels.

The glycoprotein CD133 has been suggested to be a cancer stem cell marker for malignant brain tumors, since only CD133-positive cells from brain tumor biopsy material were able to initiate brain cancer in a mouse model. However, several groups have recently reported that CD133-negative glioma cells also possess capacities for self-renewal and tumor initiation, raising the possibility that CD133 may not be a universal enrichment marker for cancer stem cells in malignant gliomas. Very recently, Read et al. and Son et al. reported that CD15/SSEA1 could be an alternative and/or more general enrichment marker for tumor-initiating cells in medulloblastoma or GBM. It remains to be determined whether CD15/SSEA1–positive glioma stemlike cells also play a critical role in radioresistance in GBMs. In addition, recent observations indicate that CD133 expression may be modulated by environmental factors such as hypoxia and bioenergetic stresses affecting mitochondrial functions. Although increased CD133-positive cell fractions after high-dose irradiation appear to result from expansion of CD133-positive subpopulations, the possibility remains that radiation has the potential to modulate these environmental factors and thereby increase the level of CD133 expression in glioma cells.

Since the glioma stem cell concept provides a possible explanation for resistance to irradiation or chemotherapy in malignant gliomas, targeting these cancer stem cells could prove a highly effective treatment of malignant gliomas. Hambardzumyan et al. recently showed that medulloblastoma stem cells residing in the perivascular niche survive radiation via activation of the PI3K/Akt pathway. If the brain tumor microvasculature forms a niche that is critical for the maintenance of cancer stem cells, targeting the tumor microvasculature to disrupt stem cell maintenance could be a promising approach to the treatment of malignant gliomas.

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![Graph showing percentages of CD133-positive cells in histological sections obtained after GKS plus EBRT. The percentage of CD133-positive glioma cells in sections obtained after GKS plus EBRT was significantly greater than in those obtained from the primary surgery (p < 0.002, paired t-test). Closed squares and the error bars represent the mean and SD of each group.]

Fig. 3. Graph showing percentages of CD133-positive cells in histological sections obtained after GKS plus EBRT. The percentage of CD133-positive glioma cells in sections obtained after GKS plus EBRT was significantly greater than in those obtained from the primary surgery (p < 0.002, paired t-test). Closed squares and the error bars represent the mean and SD of each group.
nant gliomas. However, the present clinical investigation indicates that, despite prolonged tumor vasculature damage after GKS plus EBRT, CD133-positive glioma stemlike cells can survive and cause recurrence of malignant gliomas. Sakariassen et al. reported that highly infiltrative gliomas with a stem cell–like phenotype showed angiogenesis-independent growth in an early passage after xenotransplantation, although serial passages in animals gradually transformed the tumors into an angiogenesis-dependent phenotype. This uncoupling of invasion and angiogenesis, represented by stemlike cancer cells, may be characteristic of tumor progression in gliomas and could explain recurrent growth of gliomas after GKS and EBRT. Alternatively, as a result of tumor vascular damage within the target lesion of high-dose irradiation, surviving glioma stemlike cells might be apt to invade surrounding brain tissues by co-opting normal blood vessels. Further laboratory as well as clinical investigations, focusing on glioma stemlike cells and their microenvironment, are anticipated to elucidate the mechanism underlying radioresistance in malignant gliomas, possibly allowing the treatment limitations of malignant gliomas to be overcome.

Conclusions

The number of CD133-positive glioma stemlike cells was dramatically increased in recurrent malignant gliomas after high-dose irradiation with GKS plus EBRT. Our results indicate that glioma stemlike cells can survive, leading to recurrence, despite prolonged damage to tumor blood vessels. This could be an essential factor limiting the effectiveness of GKS and EBRT for malignant gliomas.

**Fig. 4.** Proliferative potentials and tumor vascular density before and after GKS plus EBRT. A–D: Immunostaining for factor VIII (A and B) and MIB-1 (C and D) in sections obtained during the initial surgery (A and C) and the second surgery 6 months after GKS (B and D) in a GBM case (Case 1). Bars = 100 µm. E and F: Graphs showing tumor blood vessel density (E) and MIB-1 staining indices (F) before and after GKS plus EBRT in 10 patients with malignant glioma. In those cases showing local recurrence (Cases 1–9), tumor blood vessel density was significantly decreased in sections obtained after GKS plus EBRT (p < 0.025, paired t-test), while MIB-1 staining indices did not differ significantly.
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Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: K Tamura, M Aoyagi, K Ohno. Acquisition of data: K Tamura, M Aoyagi, H Wakimoto, N Ando, T Nariai, M Yamamoto, K Ohno. Analysis and interpretation of data: K Tamura, M Aoyagi, H Wakimoto, N Ando, T Nariai, M Yamamoto, K Ohno. Drafting the article: K Tamura, M Aoyagi. Critically revising the article: K Tamura, M Aoyagi, H Wakimoto, T Nariai, M Yamamoto, K Ohno. Reviewed final version of the manuscript and approved it for submission: K Tamura, M Aoyagi, T Nariai, M Yamamoto, K Ohno. Statistical analysis: K Tamura, M Aoyagi. Administrative/technical/material support: N Ando, M Yamamoto. Study supervision: M Aoyagi, K Ohno.

References