Assessment of Alterations in Gene Expression in Recurrent Malignant Glioma after Radiotherapy Using Complementary Deoxyribonucleic Acid Microarrays

Tatsuhiro Joki, M.D., Ph.D., Rona S. Carroll, Ph.D., Ian F. Dunn, B.S., Jianping Zhang, M.S., Toshiaki Abe, M.D., Ph.D., Peter McL. Black, M.D., Ph.D.

OBJECTIVE: We used complementary deoxyribonucleic acid expression microarrays to assess the effects of radiotherapy on gene expression in glioblastoma multiforme. We hypothesized that postradiation recurrent tumors may demonstrate alterations in gene expression from the primary tumor specimen.

METHODS: Patients were diagnosed with glioblastoma multiforme at resection of the initial tumor, and they received 60 Gy of fractionated radiotherapy before recurrence. Ribonucleic acid samples from both the primary and the postradiation recurrent tumor in each patient were screened and compared using complementary deoxyribonucleic acid expression arrays and Northern blot analysis.

RESULTS: Messenger ribonucleic acid levels of growth factors participating in paracrine loops, such as vascular endothelial growth factor and platelet-derived growth factor receptor \( \beta \), were decreased in postradiation recurrent tumors as compared with primary tumors in three of four patients. However, messenger ribonucleic acid levels of growth factors involved in autocrine loops, such as epidermal growth factor receptor, platelet-derived growth factor \( \alpha \), platelet-derived growth factor \( \alpha \), and basic fibroblast growth factor, were decreased in two of four, two of four, three of four, and three of four patients' recurrent tumors, respectively. Microvessel counts demonstrated that blood vessel growth was decreased significantly in postradiation recurrent tumor specimens.

CONCLUSION: After radiotherapy of glioblastoma multiforme, levels of paracrine-acting growth factors are diminished in correspondence with the reduction in vascular density. In contrast, growth factors that participate in autocrine loops demonstrate elevated levels of gene expression. These results suggest that maintenance of autocrine loops may be important in tumor regrowth after radiotherapy. (Neurosurgery 48:195–202, 2001)

Key words: Complementary deoxyribonucleic acid microarray, Gene expression, Glioblastoma, Radiotherapy, Recurrence

Current data indicate that approximately 18,000 primary malignant brain tumors are diagnosed each year and that approximately 13,000 deaths annually are attributable to malignant brain tumors (2, 16). The prognosis for patients with malignant brain cancer is very poor; the recurrence rate is greater than 90%, and the 5-year survival rate is less than 10% (19). Radiotherapy is a vital component of multimodal treatment for malignant brain tumors. Delivered at sufficient doses and in conjunction with surgery, radiation treatment has been demonstrated to prolong survival (21, 22).

Questions remain, however, regarding its effects on recurrent tumor phenotype. For unknown reasons, recurrent high-grade gliomas seem to behave more aggressively after radiotherapy than their primary counterparts, which suggests that radiotherapy may be at least partly responsible for these observed changes. Radiotherapy seems to affect significantly the plasma levels of a number of growth factors measured in patients before and directly after radiotherapy (9).

Although the short-term effects of high- and low-dose irradiation on gene expression have been studied in glioblastoma
multiforme (GBM) cells in vitro and in an animal model (17), the specific effects of long-term radiotherapy on gene expression have not been examined. The purpose of this study was to investigate the effects of radiotherapy on malignant gliomas. We assessed patterns of gene expression in primary tumor tissue and recurrent tumor tissue taken from patients whose treatment consisted solely of surgery and adjuvant radiotherapy. We compared the levels of gene expression in patients’ primary and postradiation recurrent tumor specimens using complementary deoxyribonucleic acid (cDNA) microarray analysis, and we confirmed the differences in expression in a subset of genes by Northern blot analysis.

PATIENTS AND METHODS

Patients

Four patients with recurrent GBM (three men and one woman) were included in the study. Their mean age was 59.25 years, and the mean interval from primary resection to resection of recurrent tumor after radiotherapy was 7.25 months (Table 1). The Brain Tumour Tissue Bank of the National Cancer Institute of Canada, London Regional Cancer Centre, provided the primary tumor and recurrent tumor specimens from each patient. All patients were diagnosed with GBM when the primary tumor was resected, and subsequently they received 2.0 Gy of fractionated radiotherapy per day for 5 consecutive days each week during a 6-week period, for a total dose of 60 Gy. Patients received conventional x-ray photon radiation using a linear accelerator.

Isolation of RNA

Total ribonucleic acid (RNA) was isolated from tumor specimens as described by Chirgwin et al. (4). Tissue samples were placed in 4 mol/L guanidinium isothiocyanate and were homogenized with a polytron. Samples were then centrifuged for 10 minutes at 20°C and 3000 rpm to remove debris, and the supernatant was layered over 5.7 mol/L cesium chloride. The resulting RNA pellet was dissolved in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Resulting RNA pellet was dissolved in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA. Then the RNA pellet was dissolve in 0.3 mol/L sterile sodium acetate, followed by ethanol precipitation of the RNA.

Northern blot analysis

Northern blot analysis was performed to confirm differences in gene expression between primary and postradiation

TABLE 1. Patient Profiles

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Diagnosis</th>
<th>Interval (mo)a</th>
<th>Radiation Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46/M</td>
<td>GBM</td>
<td>6</td>
<td>60 Gy</td>
</tr>
<tr>
<td>2</td>
<td>60/M</td>
<td>GBM</td>
<td>13</td>
<td>60 Gy</td>
</tr>
<tr>
<td>3</td>
<td>69/M</td>
<td>GBM</td>
<td>5</td>
<td>60 Gy</td>
</tr>
<tr>
<td>4</td>
<td>62/F</td>
<td>GBM</td>
<td>5</td>
<td>60 Gy</td>
</tr>
</tbody>
</table>

a GBM, glioblastoma multiforme.
b The elapsed period of time between initial resection of the primary tumor and resection of the recurrent tumor.

32P-labeled cDNA was synthesized from total RNA isolated from both primary tumor specimens and postradiation recurrent tumor tissue by reverse transcriptase in the presence of [α-32P]deoxyadenosine triphosphate. In brief, 10 µg of total RNA was denatured at 75°C for 10 minutes in the presence of 8 pmol of deoxythymidine mixture. After the denaturation step, cDNAs were synthesized by incubation at 42°C for 45 minutes in a master mixture containing 3 µl of deoxynucleoside triphosphate, 5 µl of [α-32P]deoxyadenosine triphosphate (3000 Ci/mmol; NEN Life Science Products, Boston, MA), and 1600 U of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL, Rockville, MD). The reaction was terminated by heating for 15 minutes at 70°C, and unincorporated nucleotides were removed by spin column purification (Chroma Spin-200; Clontech). For each reaction, 2 × 107 cpm were incorporated into the final product. After purification, labeled cDNAs were denatured by boiling for 5 minutes, and they were then hybridized to Atlas human cDNA array blots (Clontech) in 10 ml ExpressHyb hybridization solution (2 × 107 cpm/ml) (Clontech). Membranes were prehybridized at 68°C for at least 1 hour before probe addition. Hybridization was performed at 68°C in a rolling bottle overnight. After two washes with 2× standard sodium citrate (SSC) (0.30 mol/L NaCl, 30 mmol/L sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate (SDS) at 68°C for 20 minutes, the membranes were washed twice in 0.1× SSC (0.15 mol/L NaCl, 0.015 mol/L sodium citrate, pH 7) and 0.5% SDS for 30 minutes per wash at 68°C. Membranes were then exposed to BioMax x-ray film (Kodak, Rochester, NY) for 1 or 3 days at −70°C. The density of each signal was determined using an ImageQuant PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Northern blot analysis

Northern blot analysis was performed to confirm differences in gene expression between primary and postradiation...
Microvessel count

Microvessel counts were performed as described previously (23). In brief, the blood vessels were counted in three areas in each tumor section in 1.0 mm² 200× microscope fields using an Olympus BH2 microscope (Olympus Optical Co., Tokyo, Japan) on vWF-stained tissue sections. Vascular density was determined by calculating an average number of vessels in the three most vascular areas. A neuropathologist performed all microvessel counts.

RESULTS

cDNA microarrays

We assessed levels of gene expression in primary tumor and postradiation recurrent tumor specimens from patients who had not received chemotherapy. Of the 588 cDNA fragments spotted on the Atlas human expression microarray, 172 were detectable (Fig. 1); the remaining 416 were undetectable even after exposure to BioMax film for 72 hours. We narrowed our analysis of the 172 detectable genes to the 13 genes for which the levels of expression varied in at least two of four patients (Table 2). As indicated in Table 2, the levels of expression in 12 of these 13 genes were decreased in the postradiation recurrent tumors as compared with the primary tumors. Of these 12 genes, two are oncogenes (angiopoietic 1 receptor precursor, proto-oncogene p-A multidrug resistance protein), one is a signal transduction modulator (ras-related protein RAB-5A), and three are transcription factors (SNF2L1, DB1, FUSE-binding protein). The remaining six genes in which levels of expression were decreased in recurrent tumors were either growth factors or growth factor receptors: PDGFRα, pro-PDGFRβ, VEGF, pleiotrophin precursor/osteoblast-specific factor 1, and migration-inhibitory factor-related protein 8. Only 2 of 13 genes demonstrated higher levels of expression in recurrent tumors as compared with primary tumors: PDGFRα and epidermal growth factor receptor. Additional studies using Northern blot analysis were performed on genes that have been demonstrated to be involved in glioma etiology and biology.

Northern blot analysis

Northern blot analysis was performed to confirm differences in gene expression between primary and postradiation recurrent tumors demonstrated by cDNA microarray for VEGF, PDGFRβ, PDGFRα, and PDGF A. bFGF has been strongly implicated in glioma biology, but it is not represented on the cDNA array membrane. Northern blot analysis also was performed to assess the levels of expression of this growth factor (Fig. 2). A marked decrease in VEGF and PDGFRβ expression in postradiation recurrent tumors as compared with primary tumors was observed in three of four patients and in two of four patients, respectively. In the same patients, similar results in the expression of these genes were demonstrated by cDNA microarray analysis. Differences in expression of PDGFRα in primary and postradiation recurrent tumors as demonstrated by Northern blot analysis also mirrored the results from microarray analysis; mRNA levels were decreased in postradiation recurrent tumors in Patients 1 and 2, and they were increased from primary to recurrent tumors in Patients 3 and 4. Levels of PDGF A, as determined by Northern blot analysis,
were increased in recurrent tumors as compared with primary tumors in three of four patients; no difference was noted in one patient. A significant increase in bFGF expression in postradiation specimens was observed on Northern blot analysis in three of four patients.

**DISCUSSION**

In this study, we used cDNA microarray technology to investigate the biological differences between primary tumors and postradiation recurrent tumors after radiotherapy. This technique permitted the screening of a large number of genes simultaneously. The ability to assess global levels of gene expression in primary and postradiation recurrent tumors in parallel fashion served as a powerful comparative tool. Although this analytical approach is useful in the number of cDNAs that may be screened, the method requires more sensitivity to lower levels of tissue mRNA. In addition, our analysis was limited by the low quantities of mRNA available for these studies; very few patients fit the inclusion criteria for this study, because the overwhelming majority of brain tumor patients receive chemotherapy as well as radiotherapy.

A variety of growth factors and their receptors that are important for GBM development have been identified (1, 5, 11, 13, 15, 24, 25). These include proteins such as endothelial growth factor, bFGF, and VEGF, which not only can promote neoplastic growth (20) but also may play significant roles in...
the outcome of radiotherapy. It has been demonstrated that ionizing radiation can activate genes encoding certain cytokines such as tumor necrosis factor \( \alpha \), transforming growth factor \( \beta \), interleukin-1, and VEGF (6, 10). Secreted proteins also may modify cells’ responsiveness to radiation therapy. For example, interleukin-1 has been demonstrated to be radioprotective, whereas tumor necrosis factor \( \alpha \) seems to protect normal tissues and enhance the effects of radiation on neoplastic cells (7, 8, 10, 14). In animal models, a cascade of acute changes in cytokine gene expression has been reported after irradiation of the lung, brain, and other organs (3, 12, 18).

Although the data support cytokine involvement in normal and tumor tissue acutely after radiation, the long-term effects of radiotherapy on the specific profiles of these factors and their receptors in recurrent tumors after radiotherapy, as well as their particular roles in radioreponsiveness, are poorly understood. We focused our attention on a particular subset of molecules implicated in glioma angiogenesis and/or proliferation, and we investigated their gene expression in primary and postradiation recurrent tumors after radiotherapy. Growth factors secreted by GBMs may exert their effects on tumor growth by interacting with receptors on the same cell in an autocrine fashion or by binding receptors on adjacent cells of the same or a different cell type in a paracrine loop.

**FIGURE 2.** A, Northern blot analysis of growth factors. To assess primary (P) and recurrent (R) GBM-specific gene expression patterns, RNA was isolated from primary and recurrent GBM. For loading control, the same membranes were rehybridized with \( \beta \)-actin cDNA. B, the expression of specific genes in postradiation recurrent tumor as compared with primary tumor and as measured by densitometry. \( BFGF \), basic fibroblast growth factor; \( PDGFR \alpha \), platelet-derived growth factor receptor \( \alpha \); \( PDGF \) A; platelet-derived growth factor \( A \); \( VEGF \), vascular endothelial growth factor; \( PDGFR \beta \), platelet-derived growth factor receptor \( \beta \); \( kb \), kilobases.

**TABLE 3.** Microvessel Counts (Standard Errors)

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor</td>
<td>196.0 ± 12.1</td>
<td>60.7 ± 1.2</td>
<td>80.7 ± 2.3</td>
<td>87.0 ± 4.7</td>
</tr>
<tr>
<td>Recurrent tumor</td>
<td>66.0 ± 11.6 ( ^a )</td>
<td>22.0 ± 4.9 ( ^b )</td>
<td>15.0 ± 2.6 ( ^a )</td>
<td>70.3 ± 3.9</td>
</tr>
</tbody>
</table>

\( ^a P \leq 0.001 \).

\( ^b P \leq 0.005 \).

Autocrine loops represent a method of directly stimulating tumor cell growth and producing growth factor receptors that are activated constitutively in the absence of a ligand. Paracrine loops may mediate the recruitment of new blood vessels or immune responses. Receptors for this class of ligand have been identified on endothelial cells contained within the tumor parenchyma (1, 5, 11, 15). The binding of tumor cell-derived paracrine growth factors on endothelial cells has been demonstrated to induce endothelial cells within the tumor to secrete growth factors, which provides an additional method to stimulate tumor growth.
Among the four patients studied, the average time of recurrence after radiotherapy was 7.25 months, which allowed us to investigate long-term rather than acute effects of radiation on the expression of particular genes in malignant gliomas. Of interest, growth factors and/or receptors that exert their biological effects in a paracrine fashion were associated with decreased levels of expression in recurrent postradiation tumors as compared with primary tumors. In three of four patients, VEGF levels as determined by cDNA microarray and Northern blot analysis were decreased in recurrent tumors as compared with primary tumors. In two of four patients, cDNA microarray analysis demonstrated that the expression of PDGFRβ decreased from primary to recurrent tumors and was unchanged in the remaining patients. Northern blot analysis revealed that PDGFRβ levels decreased in three of four patients, and the levels of expression remained the same in primary and postradiation recurrent tumors in one patient.

Although paracrine factors such as VEGF and PDGFRβ demonstrated decreased levels of expression in tumor specimens after radiotherapy, proteins that participate in autocrine loops were associated with increased levels of expression in postradiation recurrent tumors as compared with primary tumors. Northern blot analysis revealed that PDGF A, which was undetectable by cDNA microarray analysis, was up-regulated in postradiation recurrent tumors in three of four patients, as was bFGF. PDGF receptors also were up-regulated in two of four patients as demonstrated by Northern blot and cDNA microarray analysis. Furthermore, epidermal growth factor receptor, which has been strongly implicated in GBM pathogenesis, was expressed more strongly in postradiation recurrent tumors, with levels of expression that were increased in two out of four patients and unchanged in the remaining two patients.

CONCLUSION

The differences in microvessel density between primary and postradiation recurrent tumors mirrored the decreased expression of paracrine-acting growth factors. In all patients, microvessel count was decreased. Taken together, these data suggest that radiotherapy of primary GBMs is capable of inducing long-term changes in the expression of particular genes. Furthermore, our results suggest that although autocrine- and paracrine-acting growth factors are intact in the primary tumor, they are differentially affected by radiotherapy. In recurrent tumors after radiotherapy, proteins participating in autocrine loops were up-regulated, indicating growth and proliferation of radioresistant tumor cells. However, paracrine factors were down-regulated or unchanged, which may be explained by a concomitant decrease in a sizable fraction of tumor along with vulnerable endothelial cells. Consistent with a down-regulation of paracrine factors such as VEGF and PDGFRβ in recurrent tumors, microvessel growth, a process fueled by intratumoral paracrine action, also was significantly reduced. Therefore, recurrence and the clinical response to radiation in GBMs may be determined, at least in part, by tumor-derived cytokines that enhance growth in an autocrine manner, as well as by intrinsic tumor radiosensitivity.

ACKNOWLEDGMENTS

We thank the Brain Tumour Tissue Bank of the National Cancer Institute of Canada, London Regional Cancer Centre, for supplying glioblastoma tissue and Dr. Rebecca Folkther for serving as the independent neuropathologist on this project. We thank Dr. Judith Abraham, Dr. Tucker Collins, Dr. Daniel Pope-Bowen, and Dr. N. Ferrara for providing cDNA probes. This work was supported by a grant from the Boston Neurosurgical Foundation. TJ is a recipient of a fellowship from Jikei University School of Medicine.

Received, March 15, 2000.
Accepted, September 11, 2000.
Reprint requests: Rona S. Carroll, Ph.D., Brigham and Women’s Hospital, Division of Neurosurgery, Harvard Medical School, 221 Longwood Avenue, LMRC Room 121, Boston, MA 02115. Email: RCarroll@rics.bwh.harvard.edu

REFERENCES

Radiotherapy Effects on Recurrent Glioblastoma

In this article, gene expression in glioblastoma was examined using microarray technology. Four tumors were examined, and each tumor was examined twice after treatment with radiation therapy (once at presentation and once at recurrence). Although it is rather impressive to use arrays containing 588 complementary deoxyribonucleic acids (cDNAs), these arrays are actually somewhat outdated, as 10,000 and even 20,000 cDNAs are now available. The stated goal of this technology is to develop an array containing all of the genes in the human genome.

The authors have demonstrated an alteration in gene expression in the tumor samples before and after radiation. These results occurred in two or three of the tumors, but never in all four. The authors suggest that these changes are caused by radiation therapy. Unfortunately, this conclusion is not entirely warranted. Because gene expression by individuation cells in a glioblastoma is heterogeneous, it might be thought that the radiotherapy-resistant cells that survive radiation therapy are simply a subpopulation of cells with different gene expression from the tumor as a whole. Therefore, radiation therapy would not cause alterations in gene expression; rather, it would select for cells that have a specific pattern of gene expression.

Instead, the ribonucleic acid isolated for analysis comes from all the cells present in a sample. Clearly, the cells in the initial tumor and the cells in the postradiation tumor will be different because the stroma cells will have changed. The authors have demonstrated a decrease in the vascular endothelium in the recurrent tumors. If some of the genes detected were expressed by the vascular endothelium but not by the tumor cells, then one certainly would expect a decrease in the expression of these genes simply on the basis of the decrease in vascular endothelium in the sample.

The authors have used cutting-edge technology to ask interesting questions regarding glioblastoma and the effects of radiation therapy. A larger number of tumors must be investigated before firm conclusions can be drawn.

Corey Raffel
Rochester, Minnesota

The authors have used cDNA expression microarrays to assess the effects of radiotherapy on gene expression in glioblastoma multiforme. In the four patients studied, they found that radiotherapy acts differently on paracrine- and autocrine-acting growth factor loops. We are just beginning to realize the power of testing tissue samples and control patients with cDNA expression microarrays. The importance of control selection for the tumor or treatment groups analyzed is becoming very clear. In this study, the authors used tissue obtained before and after radiation therapy. Understandably, there was no preestablished protocol. All patients received the same dose of radiation therapy, but the interval until resection of the recurrent tumors differed. It would be interesting to know whether the length of time from radiation therapy influences the results of the profile from the cDNA expression microarrays. During the next 12 months, a large number of studies in the neurosurgical literature will use this technology.

James T. Rutka
Toronto, Ontario, Canada

Joki et al. studied alteration of gene expression in glioblastoma after irradiation using cDNA microarrays and Northern blot analysis. They found that after radiotherapy for glioblastoma, levels of expression of paracrine-acting growth factors such as vascular endothelial growth factor and platelet-derived growth factor receptor $\beta$ are decreased with the reduction in vascular density. However, levels of expression for growth factors participating in autocrine loops, such as platelet-derived growth factor receptor $\alpha$, platelet-derived...
growth factor receptor A, and basic fibroblast growth factor, were increased in postradiation recurrent tumors as compared with the primary tumor.

It is our clinical experience, as well as that of others, that recurrent glioblastomas are different from primary tumors with regard to growth behavior and response to therapy. It has been speculated that postoperative radiotherapy and chemotherapy induce genetic alterations in the tumor. It also has been suggested that radiotherapy facilitates malignant transformation of low-grade gliomas. This study beautifully demonstrates that radiotherapy is responsible for the biological alteration of glioblastomas. Although difficult to carry out, this type of study is important to understanding the pathophysiology of recurrent glioblastoma and improving the treatment of this malignant tumor.

Yukitaka Ushio
Kumamoto, Japan