B-Cell Lymphoma-2 Promotes Malignant Progression in Glioma

Tiffany A. Doucette, PhD, and Ganesh Rao, MD

Glioma is the commonest and deadliest primary brain tumor in humans. These diffuse neoplasms are classified as astrocytomas or oligodendrogliomas but can be mixed when displaying features of both lineages. Median survival for oligodendroglioma ranges from 7 to 10 years, but for anaplastic oligodendrogliomas (AOs), median survival is significantly shorter, ranging from 3 to 5 years. Despite aggressive measures such as surgery, radiation, and chemotherapy, these tumors become resistant to treatment and inevitably progress. Upregulation of genes that permit evasion of apoptotic cell death has been observed in recurrent high-grade tumors and is a mechanism by which they develop treatment resistance. However, the contribution of antiapoptotic genes to glioma formation, growth, and grade remains unclear. To parse the consequences of anti-apoptotic signaling on tumorigenesis, we studied overexpression of the antiapoptotic gene, \textit{Bcl-2}, in a platelet-derived growth factor-B (PDGFB)–dependent mouse model of oligodendroglioma.

Both PDGF and its receptor are highly expressed in many cancers, including sarcomas, germ cell tumors, and brain tumors. Successful binding of the PDGF receptor results in activation of proproliferative signaling pathways such as PI3-kinase. In human glioma specimens, upregulation of the beta isoform of the ligand (PDGFB) is observed in low- and high-grade tumors, and increased expression correlates with higher-grade tumors. The relevance of PDGFB in glioma development has been validated in animal models in which its overexpression results in both low- and high-grade oligodendroglioma (AO), although low-grade tumors predominate. This variability in tumor grade has been described as dose dependent in that mice exposed to higher levels of PDGFB in the brain develop more malignant tumors. Although PDGFB can induce tumorigenesis independently, combined expression with other genes produces a more malignant phenotype. Insulin-like growth factor–binding protein 2 (IGFBP2), which promotes tumor cell proliferation, strongly enhances tumor grade in a PDGFB-dependent model of oligodendroglioma. Combined expression of PDGFB with tumor-promoting microRNA (miR-26a) also enhances tumor formation. Expression of PDGFB in an \textit{Ink4a-Arf}–/– background increases tumor incidence and malignancy. Thus, disruptions in other cellular programs may contribute to tumor formation and malignant degeneration.

The ability of a cancerous cell to evade apoptosis is necessary for tumor growth. Members of the B-cell lymphoma-2 (\textit{Bcl-2}) family of genes are potent suppressors of apoptosis, and alterations in their expression contribute to tumorigenesis. Activation of \textit{Bcl-2} has been described in a variety of human malignancies, including hematologic, lung, and head and neck cancers. In high-grade gliomas, members of the \textit{Bcl-2} gene family show increased expression and are upregulated in tumors exhibiting recurrence and progression. The antiapoptotic effect of these genes may shift the tumor to a more malignant phenotype by shifting the tumor cell to a different mechanism of cell death, necrosis, a cardinal feature of high-grade gliomas. Still, the functional consequence of \textit{Bcl-2} expression on glioma development and progression remains unclear, with some studies correlating increased expression with a protective effect on patient survival.

To study the effect of \textit{Bcl-2} on glioma formation in vivo, we modeled ectopic \textit{Bcl-2} expression in mice using the RCAS/Ntv-a system. In this system, a gene of interest is cloned into a modified avian retrovirus (RCAS) that is replication defective in mammalian cells. The vector is introduced into a transgenic mouse line (\textit{Ntv-a}) that expresses TV-A (the receptor for RCAS) under control of the glial-specific \textit{Nestin} promoter. Nestin” cells include glioneuronal precursors that are the presumed cells of origin for glial tumors. The gene is randomly incorporated into the genome of the host cell and expressed by the constitutive retroviral promoter, long terminal repeat. This method of somatic cell transfer can be used to appreciate the functional consequence of ectopic gene expression in vivo. Furthermore, multiple RCAS vectors can be expressed together, permitting the study of cooperative
effects of different genes on tumorigenesis. This system has been used to model various brain tumors, including glioma and medulloblastoma.\textsuperscript{25-28} We modeled Bcl-2 expression independently to examine its tumorigenic effect. We also expressed PDGFB in combination with Bcl-2, hypothesizing that the antiapoptotic effect would enhance tumor formation and grade. Here, we show that Bcl-2 enhances the tumor formation rate, promotes more malignant histological features, and decreases survival in a PDGFB-dependent mouse model of glioma.

**MATERIALS AND METHODS**

**Vector Constructs**

The details of the creation of RCAS–Bcl-2 are described elsewhere.\textsuperscript{29} Briefly, this vector was constructed by ligating a polymerase chain reaction–generated cDNA corresponding to the entire coding sequence of human Bcl-2 into the retroviral vector RCASBP(A). RCAS-PDGFB was constructed with a hemagglutinin epitope tag and is described elsewhere.\textsuperscript{30} Dunlap et al\textsuperscript{2} described the details of the creation of RCAS-IGFBP2. Briefly, the RCAS-IGFBP2 vector was constructed by subcloning the 1.4-kb cDNA fragment of IGFBP2 into a YAP vector, which was then transferred into the RCAS-X vector with NotI and ClaI restriction enzymes.

**Transfection of DF-1 Cells**

DF-1 immortalized chicken fibroblasts were grown in Dulbecco modified Eagle medium with 10% fetal bovine serum (GIBCO, Carlsbad, California) in a 5% CO\textsubscript{2} humidified incubator at 37°C. To produce live virus, we transfected plasmid versions of RCAS vectors into immortalized chicken fibroblasts (DF-1 cells) using FuGene6 (Roche, Nutley, New Jersey) and allowed them to replicate in culture.

**Immunofluorescence**

To verify Bcl-2 expression after infection with RCAS–Bcl-2, we grew untransfected DF-1 cells in culture. At 50% confluence, the cells were exposed to filtered supernatant from DF-1/RCAS–Bcl-2–transfected cells for 48 hours. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline followed by cold MeOH. Immunocytochemical labeling was performed using standard methods. A mouse monoclonal antibody against human Bcl-2 (1:200; Santa Cruz Biotechnology, Santa Cruz, California) and goat anti-mouse Alexa Fluor 594 fluorescent conjugate (1:500; Molecular Probes, Carlsbad, California) were used for detection. Prolong Gold antifade reagent with DAPI (Molecular Probes) was used for labeling cell nuclei and for mounting. Staining was visualized with a Zeiss Axioskop 40 microscope.

**Western Blot Analysis**

Verification of PDGFB and IGFBP2 expression from DF-1 cells after infection was performed by Western blot. We prepared whole-cell lysates from DF-1 cell cultures 48 hours after infection with viral supernatant obtained from DF-1 cells expressing RCAS-PDGFB or RCAS-IGFBP2. Protein samples (10 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10% gels, transferred to polyvinylidene difluoride membrane, and probed with the anti-HA antibody (1:1000; F7, Santa Cruz Biotechnology) to detect PDGFB expression or an antibody for IGFBP2 (1:1000; C-18, Santa Cruz Biotechnology). Secondary antibodies, including goat anti-mouse IgG (1:2500; Pierce, Rockford, Illinois) and donkey anti-goat IgG (1:2500; Santa Cruz Biotechnology), were used for detection. The blots were developed with the ECL Plus Detection Kit (GE Healthcare, Piscataway, New Jersey) following the manufacturer’s protocol.

**In Vivo Somatic Cell Transfer in Transgenic Mice**

Creation of the transgenic Ntv-a mouse has been described previously.\textsuperscript{30} The mice are mixtures of the following strains: C57BL/6, BALB/C, FVB/N, and CD1. To transfer genes via RCAS vectors, we injected DF-1 producer cells transfected with a particular RCAS vector (1 × 10\textsuperscript{5} cells in 1-2 μL phosphate-buffered saline) into the right frontal lobe of Ntv-a mice from an entry point just anterior to the coronal suture of the skull using a 10-μL gas-tight Hamilton syringe. We injected mice within 24 to 72 hours after birth because the population of Nestin+ cells producing TVA receptors diminishes progressively with time. In the injection sets consisting of 2 vectors, equal numbers of DF-1 cells were injected. The mice were killed 90 days after injection or sooner if they demonstrated morbidity related to tumor burden, including hydrocephalus or debility. The brains were fixed in formalin, embedded in paraffin, sectioned for immunohistochemical analysis, and analyzed for tumor formation. Histological verification of tumor formation and determination of low- or high-grade type were performed by a neuropathologist. High-grade tumors were differentiated by the presence of microvascular proliferation, brisk mitotic activity, and foci of necrosis.

**Immunohistochemistry**

Mouse brains were paraffin embedded and sectioned into 4-μm sections for immunohistochemical analysis. The ThermoScientific PTModule (Thermo Fisher Scientific, Fremont, California) with citrate buffer was used for antigen retrieval. Staining was performed using the Lab Vision Immunohistochemical Autostainer 360 (Thermo Fisher Scientific). Immunohistochemical staining was visualized with an avidin-biotin complex technique with diaminobenzidine (Invitrogen, Carlsbad, California) as the chromogenic substrate and hematoxylin as the counterstain. To detect expression of the human Bcl-2 expressed by RCAS in tumor sections, we used a primary monoclonal antibody that
specifically detects human Bcl-2 and does not cross-react with endogenous mouse Bcl-2 (1:100; Santa Cruz Biotechnology). To detect apoptosis, we used a primary antibody for cleaved caspase 3 (1:1000, Cell Signaling Technology, Beverly, Massachusetts). This antibody specifically detects the large fragment of activated caspase-3 and is an indicator of both mitochondria-mediated (intrinsic) and death receptor-mediated (extrinsic) apoptosis. To analyze the extent of mitotic activity as an indicator of cellular proliferation, we used an antibody to phosphohistone H3 (pHH3; 1:1000, Millipore, Temecula, California).

Mitotic Index
To detect and quantify mitotic activity in the different injection sets, we immunostained formalin-fixed, paraffin-embedded, tumor-bearing tissue sections with an antibody against pHH3. We counted the number of positively stained cells in the area of highest tumor cell density and mitotic activity in 10 nonoverlapping high-power microscopic fields (at × 400 magnification) from 5 different tumor-bearing brains from each injection set. The mitotic index was calculated as the number of positive cells divided by the number of total cells in each field.

Apoptotic Assay
We detected and quantified apoptosis in the different injection sets by immunostaining formalin-fixed, paraffin-embedded, tumor-bearing tissue sections with an antibody against cleaved caspase-3. We counted the number of positively stained cells in the area of highest tumor cell density and apoptotic activity in 10 nonoverlapping high-power microscopic fields (at × 400 magnification) from 5 different tumor-bearing brains from each injection set. The apoptotic index was calculated as the number of positive cells divided by the number of total cells in each field.

Statistical Analysis
To compare tumor formation rates between different groups, we used the χ² test, and a value of P < .05 was considered significant. To compare the time to death for tumor-related neurological morbidity between groups, the Kaplan-Meier method was used. This time period was defined as the number of days from RCAS vector injection to the death of the mouse. The log rank test was used to compare the distributions of samples. A value of P < .05 was considered significant. Statistical analysis was performed with SPSS 17.0 (SPSS Inc, Chicago, Illinois).

RESULTS
Combined expression of PDGFB and Bcl-2 in Ntv-a mice resulted in a significantly higher incidence of tumors compared with independent expression of PDGFB. Tumors were identified in 27 of 33 mice (82%) coinjected with RCAS-PDGFB and RCAS–Bcl-2, whereas injection of RCAS-PDGFB alone resulted in 11 tumors in 26 mice (42%; χ² test, P = .002; Table). In the mice injected with RCAS-PDGFB and RCAS–Bcl-2, high-grade tumors, consistent with AO, were observed in 20 mice (61%). Necrosis was identified in 14 mice (42%) injected with RCAS-PDGFB and RCAS–Bcl-2. Low-grade tumors (lacking necrosis, mitotic figures, or microvascular proliferation), consistent with oligodendroglioma, were seen in 7 mice (21%). Bcl-2 expression induced by RCAS–Bcl-2 was verified in tumor-bearing sections. In the 26 mice injected with RCAS-PDGFB alone, we observed high-grade tumors in 5 (19%) and low-grade tumors in 6 (23%). Necrosis was identified in 3 mice (12%) injected with RCAS-PDGFB alone. We injected 19 mice with RCAS–Bcl-2 alone. None of these mice developed any sign or symptom of neurological morbidity during the 90-day observation period, and no tumors were detected in any of the mice at necropsy. The time to symptomatic tumor development was significantly shorter in mice injected with RCAS-PDGFB and RCAS–Bcl-2 than in mice injected with PDGFB alone. The median time to development of symptoms for mice injected with RCAS-PDGFB and RCAS–Bcl-2 was 57 days (range, 21-90 days). In contrast, mice injected with RCAS-PDGFB alone had a median time to development of symptoms of 90 days (range, 25-90 days), and the majority of these mice did not develop any sign or symptom of neurological morbidity during the 90-day observation period. The difference in tumor latency between these groups was significant (log rank test, P = .003).

Tumor cell proliferation was detected and quantified by immunostaining tumor-bearing brains for mitotic figures with an antibody against pHH3. We compared the number of pHH3-positive cells in low-grade tumors generated by RCAS-PDGFB with high-grade tumors generated by RCAS-PDGFB independently and in combination with RCAS–Bcl-2. We also compared this mitotic index with high-grade tumors induced by combined expression of RCAS-PDGFB and RCAS-PDGFB and RCAS–Bcl-2.

<table>
<thead>
<tr>
<th>RCAS Vector</th>
<th>Overall Tumor Incidence, n/N (%)</th>
<th>AO Incidence, n/N (%)</th>
</tr>
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<tbody>
<tr>
<td>PDGFB</td>
<td>11/26 (42)</td>
<td>5/26 (19)</td>
</tr>
<tr>
<td>PDGFB + Bcl-2</td>
<td>27/33 (82)</td>
<td>20/33 (61)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0/19 (0)</td>
<td>0/19 (0)</td>
</tr>
</tbody>
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AO, anaplastic oligodendroglia; Bcl-2, B-cell lymphoma-2; PDGFB, platelet-derived growth factor-B. The difference in overall tumor formation was significantly different between mice injected with RCAS-PDGFB alone and RCAS-PDGFB plus RCAS–Bcl-2 (χ², P = .002). The difference in high-grade tumor formation (AO) was also significantly different between these injection sets (χ², P = .001).
IGFBP2. The AOs formed by RCAS-PDGFβ and RCAS-IGFBP2 are phenotypically indistinguishable from those formed from overexpression of RCAS-PDGFβ and RCAS-Bcl-2. Consistent with its stimulation of more malignant tumors, IGFBP2 increases mitotic activity and thus served as an additional control for tumor cell proliferation. We injected a cohort of mice (n = 30) with RCAS-PDGFβ and RCAS-IGFBP2 and found that overall survival was similar to that of the group injected with RCAS-PDGFβ plus RCAS–Bcl-2. In this way, we were able to compare tumor cell proliferation in another PDGFβ-dependent model of AO. The median mitotic index in tumors generated by RCAS-PDGFβ and RCAS–Bcl-2 was 11% (range, 1.4%-44.2%). For low-grade and high-grade tumors induced by RCAS-PDGFβ alone, it was 0.85% (range, 0.3%-13%) and 1.8% (range, 0%-9.8%), respectively. For high-grade tumors formed by RCAS-PDGFβ and RCAS-IGFBP2, it was 2.1% (range, 0%-8.8%). Tumors formed by RCAS-PDGFβ and RCAS–Bcl-2 had a significantly higher mitotic index than tumors formed by all other injection sets (P < .001).

To determine whether a suppressive effect on apoptosis was induced by and specific to Bcl-2 overexpression, we compared the apoptotic indexes from high-grade tumor-bearing brains generated by RCAS-PDGFβ and RCAS–Bcl-2 with those generated by RCAS-PDGFβ expression alone and RCAS-PDGFβ and RCAS-IGFBP2. The median apoptotic index of high-grade tumors formed by RCAS-PDGFβ and RCAS–Bcl-2 was 0.4% (range, 0%-10.9%). The apoptotic index of high-grade tumors formed by PDGFβ alone was 6.7% (range, 0.8%-22%). The apoptotic index of high-grade tumors formed by RCAS-PDGFβ and RCAS-IGFBP2 was 4.8% (range, 0.2%-25%). We found that the apoptotic index was significantly lower in the high-grade tumors formed by RCAS-PDGFβ and RCAS–Bcl-2 compared with those formed by either RCAS-PDGFβ alone or the combination of RCAS-PDGFβ and RCAS-IGFBP2 (P < .001 in both cases). The apoptotic index was not significantly different between high-grade tumors formed by RCAS-PDGFβ alone and RCAS-PDGFβ plus RCAS-IGFBP2 (P < .11).

DISCUSSION

The initiation, maintenance, and progression of cancer require the combination of signaling programs that promote cellular proliferation and those that suppress apoptosis (reviewed by Green and Evan31). The first antiapoptotic gene discovered, Bcl-2, has been implicated in a variety of cancers, including gliomas.15 Although several studies have found increased expression or dysregulation of Bcl-2 (and its related family members) in different types of glioma, an inverse relationship between its expression and patient survival has been reported in some studies32,33 but not in others.24,34-36 Furthermore, a correlation between Bcl-2 expression and histological grade in human tumor specimens has not been consistently found.37-39 We used a murine model to determine the functional impact of Bcl-2 on the initiation and progression of glioma in vivo. Here, we show that expression of Bcl-2 significantly enhances the tumor formation rate in a PDGFβ-dependent mouse model. We also show that Bcl-2 increases malignancy when coexpressed with PDGFβ.

Our results lend support to clinical studies showing that Bcl-2 expression correlates with poorer prognosis and higher tumor grade,22,39 as well as in vitro studies showing that ectopic Bcl-2 expression in glioma cell lines promotes a more malignant phenotype.40 The exact mechanism by which Bcl-2 exerts its tumor-enhancing effect in our study is difficult to surmise because of the complexity of the functions (which affect apoptosis, autophagy, and necrosis) and structure of Bcl-2.41 Nonetheless, we were able to validate the effect of apoptotic suppression mediated through Bcl-2 on tumor formation by quantifying the expression of cleaved caspase-3 in tumor-bearing sections. This is a sensitive method for assessing apoptosis because expression of cleaved caspase-3 is an early and likely universal signaling event in apoptosis.42 Cleaved caspase-3 expression was significantly lower in high-grade tumors induced by PDGFβ and Bcl-2 (compared with high-grade tumors induced by PDGFβ alone or in combination with IGFBP2), confirming the antiapoptotic effect of Bcl-2 expression. Cellular proliferation (as measured by pHH3 expression) was significantly higher in tumors generated by PDGFβ and Bcl-2 compared with high-grade tumors induced by PDGFβ alone or PDGFβ and IGFBP2 (not surprisingly, mitotic activity was also significantly higher in high-grade tumors than low-grade tumors). Thus, Bcl-2 supplies a proliferative effect in addition to a suppressive effect on apoptosis. Taken together, these results suggest that the increased tumor formation rate and higher incidence of the AO phenotype observed in our study are due to the combination of proproliferative and antiapoptotic effects of Bcl-2 expression.

The finding that Bcl-2 overexpression promotes the histological features of high-grade glioma is consistent with the observation that other members of the Bcl-2 family stimulate formation of necrotic foci in glioma while suppressing apoptosis.43,44 Bcl-2–like12, a member of the Bcl-2 family, is a potent inhibitor of apoptosis. Its downstream effector, αB-crystallin, appears to possess oncogenic properties, which include inducing cell migration and invasion through activation of the mitogen-activated protein kinase kinase/extracellular receptor kinase pathway.43,45 Bcl-2–like12 exhibits unique mechanisms to inhibit caspases 3 and 7, which shift the tumor cell from an apoptotic death to a necrotic one. The necrotic phenotype is relevant in glioma because it is associated with poor survival and is a hallmark of high-grade lesions. In our study, necrosis was much more common in tumors formed by coexpression of Bcl-2 and PDGFβ than...
PDGFB alone, correlating with the increased incidence of high-grade tumors. The PDGFB-dependent RCAS/Ntv-a–based model of glioma has been used by several investigators to evaluate the functional consequences of gene overexpression on tumor formation. In a study using the RCAS/Ntv-a system, IGFBP2 was overexpressed in combination with PDGFB and was shown to enhance progression to AO in Ntv-a mice. Similar to our result with Bcl-2, IGFBP2 was not tumorgenic independently. Although the overall tumor formation rate was higher in that study (96.6%) than in ours (82%), high-grade tumors formed more frequently in our study (61% compared with 38%), although overall survival between these 2 groups was similar. The comparative decrease in apoptotic activity in high-grade tumors induced by PDGFB plus Bcl-2 compared with PDGFB plus IGFBP2 may explain this difference in high-grade tumor formation rates. Our tumor formation rate with PDGFB is somewhat lower than what has been reported previously, although this rate has varied from 50% to 97%. Tumor formation by PDGFB is known to be dose dependent, which may also account for the different rates of tumor formation. Furthermore, in these same studies, the rate of high-grade tumors generated by independent PDGFB overexpression has also been variable, ranging from 0% to 29%. A possible explanation for these differences includes the genetic variability in the strains of mice used or different injection techniques among investigators. We controlled for these issues by injecting a consistent number of our vectors in the same colony of mice by only a few personnel. The PDGFB injections and the combined PDGFB and Bcl-2 injections were performed contemporaneously so that the experimental cohorts were studied in a few generations of mice.

Independent expression of Bcl-2 did not result in tumor formation. Similarly, a study evaluating the effect of Bcl-2 expression in a sonic hedgehog–dependent model of medulloblastoma showed that coexpression with sonic hedgehog resulted in a higher tumor formation rate but was not sufficient to form tumors independently. In that study, independent expression of Bcl-2 resulted in noncancerous but definite histological changes in the cerebellum. We were unable to identify any histological abnormalities after Bcl-2 expression in the forebrains of Ntv-a mice. This may be a consequence of the differences in the microenvironment of the cerebellum and forebrain. The granule neuron precursor, a progenitor cell unique to the cerebellum considered the putative cell of origin of medulloblastoma, may be more vulnerable to ectopic Bcl-2 overexpression than the glioneuronal precursors in the forebrain.

Targeting Bcl-2 may provide a therapeutic benefit by mitigating its antiapoptotic effects. The inhibition of Bcl-2 with antisense constructs results in tumor cell death in vitro. Inhibition of Bcl-2 with chemical inhibitors has also been shown to reactivate tumor necrosis factor–related apoptosis inducing ligand. Cell death induced by tumor necrosis factor–related apoptosis inducing ligand may be an effective approach against malignant gliomas, which are otherwise resistant to apoptosis. Another small-molecule inhibitor of several genes in the Bcl-2 family, ABT-737, was recently shown to induce apoptosis in glioma cell lines and to increase survival in mice implanted intracranially with glioma cells. Our results indicate that Bcl-2 strongly inhibits apoptotic cell death in glioma, supporting the strategy of suppressing Bcl-2 signaling. The profound negative effect of Bcl-2 signaling on survival in our study also increases its appeal as a therapeutic target.

**CONCLUSION**

We have shown that the antiapoptotic gene Bcl-2 enhances tumor formation in a PDGFB-dependent model of glioma. We have found that Bcl-2 possesses both antiapoptotic and proliferative qualities that promote a more malignant phenotype. Our results confirm and extend observations in clinical studies that increased Bcl-2 expression correlates with poorer survival.

**Disclosure**

The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

**REFERENCES**


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