Oncoprotein Bmi-1 Renders Apoptotic Resistance to Glioma Cells through Activation of the IKK-Nuclear Factor- κ B Pathway

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One of the features of malignant gliomas is their deviant resistance to cellular apoptosis induced by cytotoxic reagents. Bmi-1, an oncoprotein, has been linked to oncogenesis and cancer progression in various types of human cancers including gliomas. However, the mechanisms underlying Bmi-1 antiapoptotic function remain largely unknown. In this study, we report that Bmi-1 renders apoptotic resistance to glioma cells through nuclear factor- κ B (NF- κ B). In glioma cells, ectopic expression of Bmi-1 significantly inhibits doxorubicin-, BCNU-, or UV irradiation-induced apoptosis through reduction of activated caspase-3 and PARP, and induction of Bcl-X_L. Cellular depletion of Bmi-1 enhances the sensitivity of glioma cells to apoptosis induced by doxorubicin, BCNU, or UV irradiation. Bmi-1 activates NF- κ B through stimulation of I κ B phosphorylation, nuclear translocation, and transcriptional activity of NF- κ B and expression of downstream genes of NF- κ B including caspase-3, PARP, Bcl-X_L, and c-Myc. Inhibition of the IKK-NF- κ B pathway abrogates the antiapoptotic effect of Bmi-1 on glioma cells. In high-grade gliomas, Bmi-1 and NF- κ B are co-expressed in the cell nucleus. Up-regulation of Bmi-1 also correlates with tumor progression and poor survival of patients with gliomas. Together, our data demonstrate that Bmi-1 bestows apoptotic resistance to glioma cells through the IKK-

NF- κ B pathway and suggest Bmi-1 as a useful indicator for glioma prognosis. (Am J Pathol 2010, 176:699–709; DOI: 10.2353/ajpath.2010.090502)

Malignant gliomas are the most commonly seen primary tumors in the central nervous system. Histopathologically, high-grade gliomas are characterized by dense cellularity, pleomorphic cellular and nuclear morphology, including prominent multinucleation, extensive vascular proliferation and angiogenesis, intratumoral necrosis, and diffuse infiltration of the surrounding brain parenchyma.¹ Moreover, gliomas are also highly resistant to cell death-inducing treatments such as radiation and chemotherapy. The abilities of their intrinsic invasiveness and insidious resistance to cell apoptosis render these malignant tumors virtually incurable with a high frequency of tumor recurrence, despite technical advances in neurosurgery, radiation therapy, and efforts of clinical trials aimed at developing targeted and improved conventional therapies. A mean survival time is only 9 to 12 months for

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patients inflicted with this deadly disease.² Additionally, the complex molecular features with multiple genetic mutations during glioma formation and progression make identification of critical targets that integrate essential glioma pathophysiological processes highly challenging.³ In the clinic, it is urgent to further understand the molecular basis on which glioma cells acquire apoptotic resistance and identify new targets for developing approaches to the treatment of malignant gliomas.

The intense apoptotic resistance of malignant glioma cells has been linked to dysregulation of multiple signaling pathways including activation of receptor tyrosine kinase-mediated signaling pathways and the PTEN-PI3K-Akt-mTOR pathway.³ Recent evidence showed that the nuclear factor- κ B (NF- κ B)-mediated signaling pathway is also constitutively activated in a large proportion of high-grade glioblastoma multiforme (GBM) and glioma cell lines.^{4,5} It has been established that NF- κ B is one of the major factors that modulate the ability of cancer cells, including glioma cells, to resist apoptosis-based tumor surveillance and treatments.⁶ NF- κ B is a transcriptional factor that comprises two subunits, commonly p65 and p50 that are normally held in the cytoplasm by its inhibitors, I κ Bs. I κ Bs is controlled by its kinase, IKK that responds to cellular stimuli and phosphorylates I κ Bs, resulting in ubiquitin-mediated protein degradation of I κ Bs. As a result, NF- κ B is released and translocated into nucleus, stimulating an array of its targeting genes promoting cell proliferation, invasion, and prevents cell apoptosis.⁶ In gliomas, NF- κ B-mediated cell proliferation and invasion have been well documented.⁷ However, mechanisms by which activation of the IKK-NF- κ B pathway that prevent glioma cells from death remain largely unknown.

Polycomb group and epigenetic gene silencer Bmi-1 was first described as an oncogene cooperating with c-Myc during the initiation of lymphoma.^{8,9} Bmi-1 was found to express at high levels in various types of human cancers including prostate, breast, lung, ovarian, and bladder cancers and acute myeloid leukemia.¹⁰ Also, Bmi-1 is implicated in the development and progression of human cancers.^{11,12} The oncogenic function of Bmi-1 was attributed to inhibition of Ink4a and Arf tumor suppressors through directly targeting the Ink4a-Arf locus.¹³ Conversely, embryonic deletion of Ink4a/Arf rescues several genetic defects caused by Bmi-1 deficiency.¹⁴ Meanwhile, increased copy number of Bmi-1 gene was found in a subset of high-grade human gliomas.¹⁵ In a mouse model of glioma, Bmi-1 is not only required for the transformation of Ink4a/Arf-null primary astrocytes to gliomas in the brain, but also controls tumor development in an Ink4a/Arf-independent manner.¹⁴ This multifaceted role of Bmi-1 explains that during the development and tumorigenesis, Bmi-1 expression results in an increase in cell proliferation and a decrease in cell apoptosis.¹⁶ Indeed, in Ink4a/Arf-null mice, Bmi-1 collaborates with Myc in enhancing proliferation and transformation of primary embryo fibroblasts in an Ink4a-ARF dependent manner by prohibiting Myc-mediated induction of Arf and cell apoptosis.¹⁷ Other studies have shown that Bmi-1 protects keratinocytes from stress-induced apoptosis¹⁸ and

abrogates MYCN-induced sensitization of SHEP1 cells, increasing cell survival.¹⁹ However, whether Bmi-1 expression promotes gliomagenesis and tumor progression by rendering apoptotic assistance to glioma cells has not been documented.

In this study, we report that Bmi-1 expression correlates with the progression of malignant human gliomas and poor prognosis of patients with gliomas. *In vitro*, Bmi-1 protects glioma cells from cytotoxic reagent-induced apoptosis, whereas cellular depletion of Bmi-1 potentiates cell death by the apoptotic inducers. We have found that Bmi-1 activates the IKK-NF- κ B pathway and induces expression of various antiapoptotic genes. Furthermore, in an independent set of primary glioma specimens, co-expression of Bmi-1 and p65 protein, a subunit of NF- κ B in tumor cell nucleus is evident. These results reveal a mechanism by which Bmi-1 enables glioma cells to escape cytotoxic killing by conventional treatments through activation of NF- κ B signaling, suggesting that Bmi-1 is an important indicator for prognosis of malignant human gliomas.

Materials and Methods

Cell Culture

Glioma cell lines U87MG and U251MG were from the American Type Culture Collection (Manassas, VA), SNB19 was from Dr. Y.H. Zhou at the University of California, Irvine, and LN229, LN-235, LN-382, LN-464, SNB19, D247MG, LN229, LN443, LN428, LN319, and LN340 were from our own collections.²⁰ These cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 100 units penicillin-streptomycin at 37°C with 5% CO₂ atmosphere in a humidified incubator.

Cell Treatments

Doxorubicin (Sigma, St. Louis, MO), 1, 3-bis(2 chloroethyl)-1-nitrosourea (BCNU; Sigma), IKK inhibitor Wedelolactone, or NF- κ B Activation Inhibitor II JSH-23 (EMD, La Jolla, CA) was dissolved in dimethyl sulfoxide and used to treat glioma cells at indicated concentrations for various time lengths as previously described.^{21,22}

Vectors and Retroviral Infection

pNF- κ B-luc and control plasmid (Clontech, Mountain View, CA) were used to quantitatively examine NF- κ B activity. pBabe-Puro-I κ B α -mut (plasmid 15291) expressing mutant I κ B α was from Addgene (Cambridge, MA). pMSCV/Bmi-1 overexpressing human Bmi-1 was constructed as previously described.²³ To silence endogenous Bmi-1 expression, two RNA interference (RNAi) oligonucleotides (Table 1) were cloned into the retroviral transfer vector pSuper-retro-puro, respectively, and retroviral production and infection were performed as described previously.²⁴ Stable cell lines expressing Bmi-1

Table 1. Primer Sequences used RT-PCR and Real-Time PCR and RNA Interference (RNAi)

Gene	Forward primer	Reverse primer
RT-PCR		
<i>Bmi-1</i>	5'-ATGCATCGAACAACGAGAATCAAGATCACT-3'	5'-TCAACCAGAAGAAGTTGCTGATGACCC-3'
<i>MYC</i>	5'-TTCGGGTAGTGGAAAACCAG-3'	5'-CAGCAGCTCGAATTTCTTCC-3'
<i>CCND1</i>	5'-AACTACCTGGACCGCTTCCT-3'	5'-CCACTTGAGCTTGTTCACCA-3'
<i>Bcl-xL</i>	5'-ATTGGTGAGTCGGATCGCAGC-3'	5'-AGAGAAGGGGTGGGAGGGTA-3'
<i>PTGS2</i>	5'-TGAGCATCTACGGTTTGCTG-3'	5'-TGCTTGTCTGGAACAACCTGC-3'
<i>IL1A</i>	5'-AATGACGCCCTCAATCAAAG-3'	5'-TGGGTATCTCAGGCATCTCC-3'
<i>VEGF</i>	5'-CCCACTGAGGAGTCCAACAT-3'	5'-TTTCTTGCGCTTTCGTTTTT-3'
<i>BIRC3</i>	5'-CTTTGCCTGTGGTGGAAAAT-3'	5'-ACTTGCAAGCTGCTCAGGAT-3'
<i>GAPDH</i>	5'-GACTCATGACCACAGTCCATGC-3'	5'-AGAGGCAGGGATGATGTTCTG-3'
Sequence		
RNAi		
shBmi-1 no. 1	5'-ATGAAGAGAAGAAGGGATT-3'	
shBmi-1 no. 2	5'-AATGGACATACCTAATACT-3'	

or Bmi-1 short hairpin RNAs (shRNAs) were selected by treatment of with 0.5 μ g/ml puromycin for 10 days, beginning from 48 hours after infection.

Patients and Tissue Specimens

A total of 297 paraffin-embedded glioma samples and an independent cohort of 40 paraffin-embedded glioma samples were surgically dissected from glioma patients during the years 2000 to 2005, and had been histopathologically diagnosed and verified by experienced pathologists at the First Affiliated Hospital of Sun Yat-Sen University. Four normal brain tissues were obtained from individuals who died of traffic accidents and who were histopathologically confirmed to be free of pre-existing pathological lesions. Clinical information of the samples is described in detail in Supplemental Table S1 (see <http://ajp.amjpathol.org>). Percentage tumor purity in sections adjacent to regions used for RNA extraction was estimated during routine histopathological analysis. For the use of these human materials, prior consents and approval from the Institutional Research Ethics Committee were obtained.

RT-PCR

Total RNA from cells and primary tumor materials were extracted by using the Trizol reagent (Invitrogen) according to the manufacturer's instruction. The extracted RNA was pretreated with RNase-free DNase, and 2 μ g of RNA from each sample was used for cDNA synthesis primed with random hexamers. For PCR amplification of cDNA, an initial amplification using primers was done with a denaturation step at 95°C for 10 minutes, followed by 28 cycles of denaturation at 95°C for 60 seconds, primer annealing at 58°C for 30 seconds, and primer extension at 72°C for 30 seconds. On completion of the cycling steps, a final extension at 72°C for 5 minutes was done before the reaction was terminated and stored at 4°C. Expression levels of various genes were normalized to housekeeping gene *GAPDH* as controls. PCR primers designed by using the Primer Express version 2.0 soft-

ware (Applied Biosystems, Foster City, CA) are listed in Table 1.

Immunoblotting and Immunofluorescence Assays

Immunoblotting (IB) was performed by using the following antibodies as described previously²³: anti-Bmi-1 and anti-EF-1 α antibodies (Upstate, Temecula, CA); anti-NF- κ B p65, anti-I κ B α , and anti-IKK β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-p-I κ B α , anti-p-IKK β , anti-Cyclin D1, anti-caspase-3, anti-PARP, and anti-Bcl-X_L antibodies (Cell Signaling, Danvers, MA). Immunofluorescent staining on various cells was performed on cells grown on coverslips by using anti-human p65 monoclonal antibody (Santa Cruz Biotechnology; 1:200 dilution) and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Jackson Immuno Research, West Grove, PA). Gray level images were acquired under a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany).

Immunohistochemistry

Immunohistochemical analysis was performed to study altered protein expression in 297 human glioma tissues. The procedure was performed similarly to previously described methods.²⁴ In brief, paraffin-embedded specimens were cut into 4- μ m sections and baked at 65°C for 30 minutes. The sections were deparaffinized with xylenes and rehydrated. Sections were submerged into EDTA antigenic retrieval buffer and microwaved for antigenic retrieval. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 1% bovine serum albumin to block nonspecific binding. Rabbit anti-Bmi-1 antibody (1:500; Upstate) was incubated with the sections overnight at 4°C. For negative controls, the rabbit anti-Bmi-1 antibody was replaced with normal goat serum, or the rabbit anti-Bmi-1 antibody was blocked with a recombinant Bmi-1 polypeptide by co-incubation at

4°C overnight preceding the immunohistochemical staining procedure. After washing, the tissue sections were treated with biotinylated anti-rabbit secondary antibody (Zymed, San Francisco, CA), followed by further incubation with streptavidin-horseradish peroxidase complex (Zymed). The tissue sections were immersed in 3-amino-9-ethyl carbazole, counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted in Crystal Mount.

The degree of immunostaining of formalin-fixed, paraffin-embedded sections was reviewed and scored independently by three observers, based on both the proportion of positively stained tumor cells and the intensity of staining. The proportion of tumor cells was scored as follows: 0 (no positive tumor cells); 1 (<10% positive tumor cells); 2 (10 to 50% positive tumor cells); and 3 (>50% positive tumor cells). The intensity of staining was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow brown); and 3 (strong staining = brown). The staining index (SI) was calculated as staining intensity score $\times$ proportion of positive tumor cells. Using this method of assessment, we evaluated the expression of Bmi-1 in benign breast epithelium and malignant lesions by determining the staining index, which scored as 0, 1, 2, 3, 4, 6, and 9. Cutoff values for Bmi-1 were chosen on the basis of a measure of heterogeneity with the log-rank test statistical analysis with respect to overall survival. An optimal cutoff value was identified: the staining index score of ≥ 4 was used to define tumors as high Bmi-1 expression, and ≤ 3 as low expression of Bmi-1.

Immunohistochemistry (IHC) staining for protein expression in tumor and normal tissues was quantitatively analyzed by using the AxioVision 4.6 computerized image analysis system assisted with an automatic measurement program (Carl Zeiss). The method of mean optical density (MOD) was used to determine the immunostaining intensity of each tested specimen and was performed as previously reported.²⁵ Briefly, the stained sections were evaluated at $\times 200$ magnification, and 10 representative staining fields of each section were analyzed to verify the MOD, which represents the strength of staining signals as measured per positive pixels. The MOD data were statistically analyzed by using the *t* test to compare the average MOD difference between different groups of tissues, and $P < 0.05$ was considered significant.

Cell Survival

Cells (1×10^3 cells/well) were plated in six-well plates and incubated for 24 hours, followed by doxorubicin treatment or irradiation with UV_{245 nm} and a further incubation for 24 hours. Numbers of surviving cells were determined by trypan blue exclusion. The DeadEnd Fluorometric Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) System (Promega, Madison, WI) and ApopNexin™ Fluorescein Isothiocyanate Apoptosis Detection Kit (Millipore, Lake Placid, NY) were used to access cell apoptosis. Various images were captured by fluorescence microscope equipment with a digital camera by using appropriate fluoresce in filters.

Luciferase Reporter Assay for NF- κ B Transcriptional Activity

Fifty thousand cells per well were seeded in triplicates in six-well plates and were allowed to settle for 12 hours. One hundred nanograms of pNF- κ B-luciferase plasmid or control-luciferase plasmid plus 10 ng pRL-TK renilla plasmid (Promega) were transfected into glioma cells by using the Lipofectamine 2000 reagent (Invitrogen). Medium was replaced after 6 hours, and luciferase and renilla signals were measured 48 hours after transfection by using the Dual Luciferase Reporter Assay Kit (Promega) according to a protocol provided by the manufacturer.

Statistical Analysis

All statistical analyses were performed by using the SPSS 10.0 (SPSS Inc, Chicago, IL) statistical software package. The χ^2 test was used to analyze the relationship between Bmi-1 expression and clinicopathological characteristics of gliomas and the relationship between Bmi-1 and NF- κ B p65 expression. Bivariate correlations between each pair of study variables were calculated by using Spearman's rank correlation coefficients. Survival curves were plotted by using the Kaplan-Meier method and were compared by using the log-rank test. Survival data were evaluated by using univariate and multivariate Cox regression analyses. $P < 0.05$ was considered as statistically significant.

Results

Up-Regulation of Oncoprotein Bmi-1 Is Correlated with Glioma Progression and Poor Prognosis of Patients with Gliomas

We first determined whether Bmi-1 expression was associated with glioma of varying World Health Organization grades. A total of 297 archived paraffin-embedded glioma specimens, including 37 cases of polycytic astrocytoma (World Health Organization grade I), 112 cases of diffuse astrocytoma (grade II), 93 cases of anaplastic astrocytoma (grade III), and 55 cases of GBM (grade IV), were analyzed by IHC staining with an antibody against human Bmi-1. As summarized in Supplemental Table S1 (see <http://ajp.amjpathol.org>), we found that Bmi-1 was up-regulated in all four grades of gliomas when compared with that in normal brain tissues. Overall, expression of Bmi-1 protein was detected in 279 of 297 glioma specimens (93.9%). Figure 1A shows examples of IHC stained tumor specimens of each of the four World Health Organization grade gliomas. Specifically, moderate to strong nuclear staining of Bmi-1 protein was evident in tumor cells in these primary glioma tissues (Figure 1A). In contrast, minimal immuno-activity of Bmi-1 was detected in control normal brain tissues (Figure 1A). Quantitative IHC analysis revealed that MOD of Bmi-1 stain in primary gliomas of each grade of I to IV was higher than that in

Figure 1. Up-regulation of Bmi-1 is associated with glioma progression and poor prognosis of glioma patients. **A:** Representative IHC analyses of Bmi-1 expression in normal brain tissue and glioma specimens of varying World Health Organization (WHO) grades. NB, normal brain tissue; PA, polycytic astrocytoma, WHO grade I; DA, diffuse astrocytoma, grade II; AA, anaplastic astrocytoma, grade III; and GBM grade IV are shown. Data are representative of all four WHO grade tumors of a total 297 glioma specimens and four NB tissues. IHC staining was performed in each tumor specimen at least twice with similar stained patterns. **B:** Bmi-1 staining in glioma specimens is markedly increased when compared with that in NB tumors and Bmi-1 expression increases as tumors progress. Statistical quantification of MOD of IHC stains in NB tissue (four cases) and randomly picked 30 tumor cases of each different WHO grade gliomas are shown. The MOD of Bmi-1 staining increases as glioma progresses to higher grades. $P < 0.05$. **C:** Kaplan-Meier analysis of levels of Bmi-1 expression in WHO grades I to IV and survival of patients with malignant gliomas ($P < 0.001$, log-rank test).

normal brain tissue ($P < 0.05$) and increased along with the tumor grades (Figure 1B). To corroborate our observation of Bmi-1 up-regulation in glioma tumor tissues compared with normal brains, we performed IB analyses on four pairs of frozen brain tissues from individuals with high-grade GBM. An increase in Bmi-1 protein expression was found in all tumor tissues compared with the paired adjacent noncancerous brain tissue taken from the same individuals (Supplemental Figure S1, see <http://ajp.amjpathol.org>).

Next, we determined whether Bmi-1 up-regulation correlated with histopathological grades as glioma progresses and prognosis of patients with gliomas. As shown in Supplemental Table S2 (see <http://ajp.amjpathol.org>), the levels of Bmi-1 expression in 297 tumor specimens were closely correlated with their World Health Organization grades ($P = 0.000$) and the survival time of patients with gliomas ($P = 0.005$). These results were further validated by the Spearman correlation analysis (Supplemental Table S3, see <http://ajp.amjpathol.org>). Kaplan-Meier analysis and the log-rank test revealed that levels of Bmi-1 expression in primary glioma specimens were reversely

correlated with patients' survival time with a high degree of statistical significance ($P < 0.001$) and a correlation coefficient of -0.533 ($P < 0.001$, Figure 1C). In addition, a multivariate survival analysis further revealed that higher levels of Bmi-1 expression were correlated with shorter survival time, suggesting that Bmi-1 could be an independent prognostic factor (Supplemental Table S4, see <http://ajp.amjpathol.org>). Taken together, up-regulation Bmi-1 in primary glioma tumors was associated with the progression of glioma and correlated with the poor prognosis of the disease.

Ectopic Expression of Bmi-1 Inhibits Doxorubicin- and UV-Induced Apoptosis of Glioma Cells

Bmi-1 has been shown to promote cell proliferation and protect cells from apoptosis through inhibition of Ink4a/Arf function.^{13,17} We sought to examine whether Bmi-1 up-regulation played a role in the resistance of glioma cells to apoptosis through an alternative mechanism. We

Figure 2. Ectopic expression of Bmi-1 inhibits doxorubicin- and UV-induced apoptosis of glioma cells. **A:** Ectopic expression of Bmi-1 in glioma cell lines. Total cell lysates from U251MG-vector, U251MG-Bmi-1, U87MG-vector, and U87MG-Bmi-1 cells were analyzed by IB by using an anti-Bmi-1 antibody. α -tubulin was used as a loading control. **B:** Bmi-1 expression prevents doxorubicin- or UV irradiation-induced cell death. **Left panel,** representative micrographs of cultured U251MG-vector, U251MG-Bmi-1, U87MG-vector, and U87MG-Bmi-1 cells after treatment with UV irradiation (40 J/m²) followed by a 12-hour culture or doxorubicin (1.0 μ mol/L) for 12 hours. **Middle and right panels,** cell survival of various glioma cells treated by UV irradiation (20 J/m², 40 J/m², and 60 J/m²) followed by a 12-hour culture or doxorubicin (0.5 μ mol/L, 1.0 μ mol/L, and 2.0 μ mol/L) for 12 hours. Survived cells were counted by trypan blue exclusion. Bars represent the average percentages of survived cells compared with the untreated control cells. Data represent three independent experiments with similar results. **C:** Ectopic expression of Bmi-1 inhibits doxorubicin-induced apoptosis as determined by a TUNEL assay. **Left panel,** immunofluorescent images of TUNEL stained cells of U251MG-vector, U251MG-Bmi-1, U87MG-vector, and U87MG-Bmi-1 cells treated with 1.0 μ mol/L doxorubicin for 24 hours. **Right panel,** Quantification of TUNEL stained cells. TUNEL staining-positive cells were counted from 10 random fields after indicated cells were treated by doxorubicin (1.0 μ mol/L) for 24 hours. **D:** Quantification of Annexin V⁺/PI⁺ cells after the indicated cell lines were treated with doxorubicin (1.0 μ mol/L) for 12 hours. Numbers are cells that were counted from 10 random fields. Bars, SD representative images of Annexin V⁺/PI⁺ cells are shown in Supplemental Figure S3A (see <http://ajp.amjpathol.org>). **E:** Bmi-1 expression inhibits doxorubicin-induced proteolysis of pro-caspase-3 and PARP and induced Bcl-X_L expression. Various U87MG and U251MG cells were treated with doxorubicin (1.0 μ mol/L) for 12 hours and the total cell lysates were analyzed for proteolytic cleavage of pro-caspase-3, PARP (**left**), and expression of Bcl-X_L level (**right**) by IB. **Arrowheads** showed the cleaved caspase-3 and cleaved PARP. α -tubulin was used as a loading control. In **A** and **E**, IB analyses were performed at least two independent times with similar results. FITC, fluorescein isothiocyanate. **P* < 0.05.

first examined the expression levels of Bmi-1 protein in a panel of human glioma cells. As shown in Supplemental Figure S2 (see <http://ajp.amjpathol.org>), up-regulation of Bmi-1 expression at various levels was evident in all 16 glioma cell lines tested when compared with that in normal human astrocytes. To rule out the possible effect of the Ink4a/Arf and p53 pathways on the antiapoptotic effect of Bmi-1, we chose two Ink4a/Arf null glioma cell lines, namely, U87MG cells with wild-type p53 and U251MG cells with mutant p53 for our studies.²⁶ Ectopic Bmi-1 was stably expressed in these two glioma cell lines (Figure 2A), and the impact of Bmi-1 up-regulation on cytotoxic reagent-induced cell apoptosis was evaluated by exposing these cells to antiglioma agent doxorubicin and UV-irradiation. As shown in Figure 2B, overexpression of Bmi-1 in these two cell lines showed higher degrees of resistance to doxorubicin- and UV-induced cell death in various concentration of the drug or various doses of UV radiation when compared with the vector-control cells. To validate that the increased survival of Bmi-1-expressing cells was due to inhibition of cell apoptosis, TUNEL and Annexin-V binding assays were performed. As shown in Figure 2, C and D, and Supplemental Figure S3A (see <http://ajp.amjpathol.org>), the number of apoptotic vector-control cells was significantly higher than that of the apoptotic Bmi-1-expressing cells after 1.0 μ mol/L doxorubicin treatment.

To further support our observation, we characterized the effect of Bmi-1 on apoptosis protection by assessing its impact on the expression of Bcl-X_L, and cleavage of caspase-3 and PARP proteins critical for cell apoptosis.²⁷ As shown in Figure 2E, ectopic expression of Bmi-1 in U87MG and U251MG cells suppressed cleavages of both pro-caspase 3 and PARP and induced Bcl-X_L expression. Taken together, these results suggest that up-regulation of Bmi-1 enhances the resistance of glioma cells to cytotoxic reagent-induced cell apoptosis by modulating antiapoptotic proteins in these Ink4a/Arf null glioma cells independent of p53 status.

Cellular Depletion of Bmi-1 Enhances Doxorubicin- or UV-induced Apoptosis of Glioma Cells

To further characterize the impact of Bmi-1 on glioma cell apoptosis, we knocked down endogenous Bmi-1 in U87MG and U251MG cells by using specific small-interfering RNAs (siRNAs) and examined the sensitivity of the modified cells to apoptosis. As shown in Figure 3A, two siRNAs targeting Bmi-1 specifically knocked down endogenous Bmi-1 protein in both glioma cell lines. siRNA number 2 with higher efficiency was chosen for the sub-

Figure 3. Depletion of Bmi-1 by siRNA enhances doxorubicin- and UV-induced apoptosis of glioma cells. **A:** knockdown of Bmi-1 in shRNA-stably-transduced glioma cell lines. Total cell lysates from U251MG-vector, U251MG-Bmi-1-RNAi(s), U87MG-vector, and U87MG-Bmi-1-RNAi(s) cells were analyzed by IB using an anti-Bmi-1 antibody. α -tubulin was used as a loading control. **B:** knockdown of Bmi-1 by siRNA enhances doxorubicin- and UV irradiation-induced cell death. **Right panel,** representative micrographs of U251MG-vector, U251MG-Bmi-1-RNAi, U87MG-vector, and U87MG-Bmi-1-RNAi cells that were treated with UV irradiation (40 J/m^2) followed by a 12-hour culture or doxorubicin for 12 hours ($1.0 \mu\text{mol/L}$). **Middle and left panels,** cell survival of various glioma cells treated by UV irradiation (20 J/m^2 , 40 J/m^2 , and 60 J/m^2) followed by a 12-hour culture or doxorubicin ($0.5 \mu\text{mol/L}$, $1.0 \mu\text{mol/L}$, and $2.0 \mu\text{mol/L}$) for 12 hours. Survived cells were counted by trypan blue exclusion. Bars represent the average percentages of survived cells compared with the untreated control cells. Data represent three independent experiments with similar results. **C:** knockdown of Bmi-1 sensitizes glioma cells to doxorubicin-induced apoptosis. **Left panel,** TUNEL staining of various glioma cells treated with $1.0 \mu\text{mol/L}$ doxorubicin for 24 hours. **Right panel,** quantification of TUNEL stained cells. TUNEL staining-positive cells were counted in 10 random fields after indicated cells were treated by doxorubicin ($1.0 \mu\text{mol/L}$) for 24 hours. Numbers are cells that were counted from 10 random fields. Bars, standard deviation. Representative images of Annexin V^+ /PI $^-$ cells are shown in Supplemental Figure S3B (see <http://ajp.amjpathol.org>). **D:** Quantification of Annexin V^+ /PI $^-$ cells after the indicated cell lines were treated with doxorubicin ($1.0 \mu\text{mol/L}$) for 12 hours. Numbers are cells that were counted from 10 random fields. Bars, standard deviation. Representative images of Annexin V^+ /PI $^-$ cells are shown in Supplemental Figure S3B (see <http://ajp.amjpathol.org>). **E:** Depletion of Bmi-1 promotes doxorubicin-induced proteolysis of pro-caspase-3 and PARP and suppresses Bcl-X $_L$ expression. Various U87MG and U251MG cells were treated with doxorubicin ($1.0 \mu\text{mol/L}$) for 12 hours and the total cell lysates were analyzed for proteolytic cleavage of pro-caspase-3, PARP (**left**), and expression of Bcl-X $_L$ level (**right**) by IB. **Arrowheads** showed the cleaved caspase-3 and cleaved PARP. α -Tubulin was used as a loading control. In **A** and **E**, IB analyses were performed at least two independent times with similar results. FITC, fluorescein isothiocyanate. * $P < 0.05$.

sequent studies. When these modified glioma cells were treated with $1.0 \mu\text{mol/L}$ doxorubicin or 40 J/m^2 UV irradiation, enhanced sensitivities to these apoptotic inducers were found in cells depleted in Bmi-1 compared with vector control cells (Figure 3B). The apoptotic nature of induced cell death was confirmed by TUNEL and Annexin-V binding assays on Bmi-1 knocked-down cells and control cells treated with $1.0 \mu\text{mol/L}$ doxorubicin (Figure 3, C and D, and Supplemental Figure S3B, see <http://ajp.amjpathol.org>). Furthermore, inhibition of Bmi-1 markedly induced cleavage of PARP and caspase-3 and attenuated expression of Bcl-X $_L$ (Figure 3E). Moreover, we evaluated the impact of Bmi-1 up-regulation on the cell apoptosis induced by alkylating agent BCNU, which represents a widely used type of chemotherapeutic drugs in the clinic. TUNEL and Annexin-V binding assays revealed that Bmi-1 overexpressing cell lines showed stronger resistance to BCNU-induced apoptosis, whereas the number of apoptotic cells, when treated with BCNU, in Bmi-1 knocked-down cells was significantly higher than that in vector-controlled cells (Supplemental Figure S4 see <http://ajp.amjpathol.org>). Taken together, our data indicated that cellular depletion of Bmi-1 impaired the ability of glioma cells to resist cytotoxic reagent-induced cell death and the expression of anti-apoptotic proteins.

Bmi-1 Activates NF- κ B and Induces Expression of NF- κ B-Targeted Genes

Our data showed that Bmi-1 expression modulated Bcl-X $_L$ expression in glioma cells. Since Bcl-X $_L$ is a target gene of NF- κ B, and the NF- κ B-mediated signaling pathway and Bcl-X $_L$ are critical for cancer cell survival,²⁸ we were prompted to determine whether Bmi-1 expression affected the NF- κ B signaling. We first assessed the impact of Bmi-1 modulation on the transcriptional activity of NF- κ B in glioma cells by using a NF- κ B reporter luciferase activity assay. As shown in Figure 4A, when compared with vector control cells, ectopic expression of Bmi-1 increased the transcription activity of NF- κ B by 15% to 40%, whereas siRNA depletion of Bmi-1 attenuated the activity of NF- κ B by 22% to 35% in U87MG and U251MG cells, respectively. Since the NF- κ B transcriptional activity closely correlates with nuclear accumulation of NF- κ B, we further examined whether Bmi-1 expression altered the nuclear translocation of NF- κ B. We fractionated cell lysates into cytoplasmic and nucleic fractions and examined subcellular localization of p65 protein, the catalytic unit of NF- κ B, by IB analysis. As shown in Figure 4B, expression of Bmi-1 increased the amount of NF- κ B p65 protein in the nuclear fraction, whereas knockdown of Bmi-1 by siRNA reduced nucleus

Figure 4. Bmi-1 activates the NF- κ B pathway in glioma cells. **A:** Bmi-1 expression modulates transcriptional activity of NF- κ B in glioma cells. Various glioma cells were co-transfected with plasmids of pNF- κ B-luciferase plus pRL-TK renilla and analyzed by luciferase reporter activity assays. Error bars represent mean $\pm$ SD from three independent experiments with similar results. **B:** subcellular fractions of NF- κ B in various glioma cells. Cytoplasmic and nuclear fractions of indicated cells were analyzed by IB analyses. Nuclear protein p84 was used as a nuclear protein marker and EF-1 was used as a loading control. **C:** immuno-fluorescent staining of subcellular localization of NF- κ B (p65 subunit) in glioma cells. U87MG-vector, U87MG-Bmi-1, U87MG-Bmi-1-RNAi, U251MG-vector, U251MG-Bmi-1, and U251MG-Bmi-1-RNAi cells were immunostained with an anti-p65 antibody and DAPI. Various stained cells were analyzed by epi-fluorescent microscopy. Data are representative from two independent experiments with similar results. **D:** modulation of Bmi-1 expression alters expression of NF- κ B-targeted genes. Total RNA from U87MG-vector, U87MG-Bmi-1, U87MG-Bmi-1-RNAi, U251MG-vector, U251MG-Bmi-1, and U251MG-Bmi-1-RNAi cells were analyzed by RT-PCR analyses for expression of seven indicated NF- κ B-targeted genes. Expression of *GAPDH* was used as a control of gene expression. Data were obtained from two independent experiments with similar results.

accumulation of the NF- κ B p65 protein. Impact of Bmi-1 expression on p65 nucleus translocation was further examined by immunofluorescent staining in these cells in which Bmi-1 were overexpressed or knocked down. We found that Bmi-1 expressing-cells exhibited a marked increase in nuclear p65, while Bmi-1 knocked-down cells displayed a significant decrease in nuclear p65 (Figure 4C, both panels), suggesting that Bmi-1 enhanced the transcriptional activity of NF- κ B via promoting its nuclear translocation.

Since activation of NF- κ B modulates the transcription of its targeted genes critical for cell apoptosis, in addition to Bcl-X_L, we performed semiquantitative PCR analyses and examined the expression levels of six other NF- κ B target genes including IL1A, MYC, CCND1, PTGS2, BIRC3, and vascular endothelial growth factor in Bmi-1-expressing-, Bmi-1 knocked-down, and vector-control glioma cells. As shown in Figure 4D, when compared with vector control-cells, up-regulation of Bmi-1 induced the transcription of all seven genes, whereas depletion of

Bmi-1 in these cells markedly inhibited their mRNA levels. Taken together, our results demonstrated that Bmi-1 expression modulated NF- κ B activity and the expression of NF- κ B-targeted genes in glioma cells.

Inhibition of I κ B, I κ B Kinase, or NF- κ B Abrogates Bmi-1 Protection of Glioma Cells from Apoptosis

NF- κ B activity is under the control of its inhibitors (I κ Bs) and the kinase of I κ Bs, IKK, in response to various cellular stimuli, resulting in ubiquitin-mediated protein degradation of I κ Bs and subsequent NF- κ B translocation into the nucleus.⁶ To investigate how Bmi-1 regulates NF- κ B activity, we assessed the impact of Bmi-1 expression on the phosphorylation of I κ B and IKK β . As shown in Figure 5A, on tumor necrosis factor- α treatment, the phosphorylation levels of IKK β and I κ B in Bmi-1-expressing cells were significantly induced, whereas marked reduction in

Figure 5. Bmi-1 modulate NF- κ B pathway through activation of IKK-I κ B. **A:** Bmi-1 regulates I κ B and IKK phosphorylation. **Left panel**, ectopic expression of Bmi-1 activates the I κ B/IKK pathway. U251MG-vector, U251MG-Bmi-1, U87MG-vector, and U87MG-Bmi-1 cells were treated with 1 nmol/L tumor necrosis factor- α for 24 hours and were analyzed for phosphorylated IKK β (p-IKK β), total IKK β , phosphorylated I κ B α (S32/36) (p-I κ B α), and total I κ B α levels by IB. **Right panel**, knockdown of Bmi-1 inhibits phosphorylation of I κ B and IKK. U251MG-vector, U251MG-RNAi, U87MG-vector, and U87MG-RNAi cells were treated with 1 nmol/L tumor necrosis factor- α for 24 hours and were analyzed for phosphorylated IKK β (p-IKK β), total IKK β , phosphorylated I κ B α (S32/36, p-I κ B α), and total I κ B α levels by IB. In both panels, α -Tubulin was used as a loading control. **B:** Inhibition of I κ B α , NF- κ B, and IKK suppresses Bmi-1-stimulated transcriptional activity of NF- κ B in glioma cells. U87MG-Bmi-1 and U251MG-Bmi-1 cells were transfected with the NF- κ B luciferase reporter plasmid alone (control), co-transfected with an I κ B mutant (I κ Bmu), or pretreated with a NF κ B inhibitor JSH-23 (30 μ mol/L, NF- κ B in), an IKK inhibitor Wedelolactone (100 μ mol/L, IKK in). After 48 hours, cells were subjected to a luciferase activity assay. * P < 0.05. **C:** Inhibition of I κ B α , NF- κ B, and IKK attenuates Bmi-1 impacts on proteolytic cleavages of pro-caspase-3, PARP, and Bcl-X $_L$ expression. U251MG-Bmi-1 cells in **B** were analyzed by IB for cleavages of caspase-3 and PARP and Bcl-X $_L$ expression. **Arrowheads** showed the cleaved caspase-3 and cleaved PARP. α -Tubulin was used as a loading control. Data in **A–C** were from two independent experiments with similar results.

IKK β and I κ B phosphorylation was observed in Bmi-1 knocked-down cells, while the levels of total IKK β and I κ B proteins remained unchanged.

To further confirm Bmi-1 modulation of the IKK/I κ B/NF- κ B pathway, we examined the impacts of inhibition of IKK, I κ B, and NF- κ B by specific inhibitors on NF- κ B activity in Bmi-1-expressing cells. As shown in Figure 5B, the transcriptional activity of NF- κ B substantially decreased in U251MG Bmi-1-expressing cells that expressed a dominant-negative mutant (I κ Bmu) of I κ B or were treated with 30 μ mol/L of JSH-23, an NF- κ B inhibitor that selectively blocks the nuclear translocation of NF- κ B and its transcription activity without influencing I κ B degradation,²¹ or with 100 μ mol/L of Wedelolactone, an specific IKK inhibitor that selectively and irreversibly inhibits IKK kinase activity without affecting p38 MAPK or AKT.²⁰ In parallel, U251MG Bmi-1-expressing cells were treated with 1.0 μ mol/L doxorubicin and separately treated with I κ Bmu (by expression), 30 μ mol/L of JSH-23, or 100 μ mol/L of Wedelolactone. As shown in Figure 5C, proteolytic cleavage of caspase-3 or PARP, and Bcl-X $_L$ expression, were increased on the treatment of doxorubicin. As expected, inhibition of I κ B, IKK, or NF- κ B in the Bmi-1-expressing cells reversed the effects, namely, increasing caspase-3 and PARP degradation and decreasing

Bcl-X $_L$ expression in the treated cells as compared with U251MG Bmi-1-expressing cells untreated (Figure 5C). Together, these results demonstrate that the IKK/I κ B/NF- κ B pathway is important in mediating the antiapoptotic function of Bmi-1 in glioma cells.

Bmi-1 Is Co-Expressed with NF- κ B in Cell Nucleus in Primary Human Glioma Specimens

To determine whether up-regulation of Bmi-1 and increased NF- κ B activity are relevant to human glioma biology, we examined the expression of Bmi-1 and cellular localization of NF- κ B in a separate cohort of 40 human glioma specimens by IHC staining. Figure 6A shows examples of two glioma specimens analyzed in our IHC study. We found that Bmi-1 staining was positive in 37 out of 40 cases (92.5%), in consistence with our observation made in the first cohort of glioma specimens shown in Figure 3. Among a total of 40 samples analyzed, Bmi-1 was identified to be of high-level expression in 23 cases (57.5%), while the remaining 17 cases (42.5%) exhibited low- or no Bmi-1 expression. Notably, 18 out of 23 (78.3%) glioma specimens that displayed high expression of Bmi-1 exhibited increased nuclear

Figure 6. Bmi-1 and NF- κ B are co-expressed in tumor cell nucleus in primary human glioma specimens. **A:** IHC staining of Bmi-1 and nuclear NF- κ B p65 subunit in primary glioma biopsies. In a diffuse astrocytoma (DA; World Health Organization [WHO] grade II), Bmi-1 expression was low and NF- κ B p65 expression was restricted to the cytoplasm in glioma cells. In a high-grade GBM (grade IV), strong nuclear staining of both Bmi-1 and NF- κ B p65 were observed. Data are representative from positive stained samples of a total of 40 glioma specimens. **B:** correlation of Bmi-1 expression and nuclear NF- κ B p65 staining (P = 0.04). Low-expression scoring: 0 = no staining; 1 = low expression. High-expression scoring: 2 = moderate expression; 3 = high expression. Each point represents one glioma specimen.

NF- κ B p65 proteins (Figure 6A). In contrast, cytoplasmic NF- κ B p65 was found in 13 of 17 glioma specimens with low-level or lack of Bmi-1 expression (76.5%) (Figure 6A). The correlation between Bmi-1 expression and nuclear localization of NF- κ B was statistically significant (Figure 6B, $P = 0.04$), which was further validated by the Spearman correlation analysis ($P < 0.001$). Taken together, IHC analyses of this second cohort of primary glioma specimens further support the notion that Bmi-1 promotes the NF- κ B nuclear translocation and activates the NF- κ B pathway rendering glioma cells resistant to apoptosis.

Discussion

The current study provides evidence that Bmi-1, an oncogenic polycomb group protein, renders apoptotic resistance to glioma cells through activation of the NF- κ B-mediated pathway. Our data suggest that in addition to constitutive activation of PTEN and PI3K-Akt-mTOR pathways,³ malignant glioma cells acquire intense resistance to cytotoxic treatment-induced cell death by up-regulation of an oncoprotein Bmi-1 during glioma progression. Our data from clinical samples not only corroborate previous reports that Bmi-1 is up-regulated in primary human gliomas,^{14,15,29} but also show that Bmi-1 expression correlates with glioma progression and poor prognosis of patients with gliomas. Bmi-1 was shown to cooperate with H-Ras or c-Myc as an oncogene promoting cell proliferation and inhibiting cell apoptosis.^{17,30} Our results further establish that Bmi-1 protects glioma cells from cytotoxic reagent-induced cell death. We show that ectopic expression Bmi-1 renders glioma cells resistant to doxorubicin- and UV irradiation induced cell death, whereas knockdown of endogenous Bmi-1 accelerates cell death when glioma cells were exposed to these reagents. Additionally, Bmi-1 protection of cell death was linked to its activation of the NF- κ B-mediated antiapoptotic pathway. Bmi-1 expression activates NF- κ B and its targeting gene products critical for apoptosis through induction of phosphorylation of IKK β and I κ B α . Inhibition of NF- κ B, IKK β , or I κ B α attenuates Bmi-1-mediated protection against cell death induced by cytotoxic reagents. Finally, by using a separate collection of primary glioma specimens, we reveal that Bmi-1 is co-localized with the catalytic subunit of NF- κ B in the cell nucleus in these tumors. Together, our results reveal a mechanism through which glioma cells acquire devious resistance to cell death by up-regulation of Bmi-1 through activation of IKK-NF- κ B-mediated antiapoptotic pathway.

The oncogenic properties of Bmi-1 have linked Bmi-1 expression to tumor progression and clinical prognosis of several types of human cancers. Elevated Bmi-1 expression has been found in gastric, nasopharyngeal, prostate, breast, and liver cancers and in myeloid leukemia in association with poor prognosis of patients with cancer,^{23,31–33} suggesting Bmi-1 as a useful molecular marker for predicting prognosis. In glioma cells, high-level expression of Bmi-1 in primary glioma specimens has been recently reported.^{15,29} Our data of Bmi-1 ex-

pression in clinical samples extend these findings, showing that up-regulated Bmi-1 closely correlates with malignant glioma grade progression and predicts poor survival of glioma patients. Importantly, an overall increase in co-expression of Bmi-1 with NF- κ B (the p65 subunit) in cell nucleus in an independent subset of high-grade GBM specimens not only validates our observation but also supports our mechanistic data, demonstrating that up-regulated Bmi-1 activates the NF- κ B pathway rendering glioma cells resistant to apoptosis.

Bmi-1 protection of cells from death has been primarily attributed to its inhibition of c-Myc-mediated antiapoptotic pathway through directly targeting Ink4a/Arf during tumorigenesis.¹⁷ Our results provide an alternative molecular mechanism via which Bmi-1 expression contributes to protection of glioma cells from apoptosis. We found that ectopic expression of Bmi-1 increases the ability of glioma cells to resist doxorubicin- or UV irradiation-induced apoptosis, whereas cellular depletion of Bmi-1 substantially enhances the cytotoxic response of glioma cells to these apoptosis inducers. Our data also show that Bmi-1 protects cells from death through modulating the expression of an antiapoptotic protein, Bcl-X_L, and proteolytic cleavage of caspase-3 and PARP. These results establish that Bmi-1 plays an important role in promoting the survival of glioma cells against the insults of apoptosis inducers.

Bmi-1 promotion of cell survival was shown to be primarily through inhibition Ink4a/Arf-mediated apoptotic pathway.^{13,17} Interestingly, in a mouse model for glioma, Bmi-1 was shown to control multiple processes involving genes other than Ink4a/Arf.³⁴ Our results not only provide evidence in support this hypothesis but also link Bmi-1's function to the activation of the IKK-NF- κ B-mediated pathway. We show that ectopic expression of Bmi-1 promotes nuclear translocation of cytoplasmic NF- κ B and increases its transcriptional activity through phosphorylation of I κ B α and activation of the IKK β complex. Conversely, selective inhibition of NF- κ B activity, by an I κ B mutant, a specific NF- κ B inhibitor or a specific IKK inhibitor abrogates the antiapoptotic effects of Bmi-1 in glioma cells. Additionally, modulation of Bmi-1 expression by ectopic expression or siRNA knockdown in glioma cells also affects expression of several NF- κ B targeted genes such as c-Myc, CCND1, Bcl-X_L, and vascular endothelial growth factor, demonstrating the capability of Bmi-1 to activate NF- κ B pathway. Finally, we have witnessed a close correlation of nucleus-located up-regulation of Bmi-1 and NF- κ B in an independent set of high-grade GBM specimens. Considering that one of the major functions of the NF- κ B signaling is to protect cells from death⁶ and this pathway is constitutively activated in gliomas,^{4,5} these results not only provide a mechanism by which Bmi-1 stimulates NF- κ B-pathway protecting glioma cells from apoptosis, but also suggest that Bmi-1 might represent an important target for development of improved tumor response to cytotoxic-based treatments against gliomas.

In summary, for the first time, this study reveals evidence demonstrating a mechanism by which up-regulated Bmi-1 protects glioma cells from apoptosis induced by anticancer drugs and UV radiation. A mechanistic link between Bmi-1 expression and activation of the NF- κ B-

pathway in glioma cells and co-expression of Bmi-1 and NF- κ B in tumor cell nucleus in primary human glioma specimens illustrates a close association of Bmi-1 expression and apoptotic resistance of malignant glioma cells. These results provide new insights into the mechanisms underlying the devious resistance of cell death by gliomas in response to cytotoxic treatment modalities. Understanding the roles of Bmi-1 in glioma progression will not only advance our knowledge of insidious resistance of gliomas to cytotoxic treatment, but also could establish Bmi-1 as a potential therapeutic target for treatment of these deadly brain cancers.

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