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Down-modulation of Bis reduces the invasive ability of glioma cells induced by TPA, through NF-κB mediated activation of MMP-9

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Bcl-2 interacting cell death suppressor (Bis) has been shown to have anti-apoptotic and anti-stress functions. Recently, increased Bis expression was reported to correlate with glioma aggressiveness. Here, we investigated the effect of Bis knockdown on the acquisition of the invasive phenotype of A172 glioma cells, induced by 12-O-Tetradecanoylphorbol- 3-acetate (TPA), using a Transwell assay. Bis knockdown resulted in a significant decrease in the migration and invasion of A172 cells. Furthermore, Bis knockdown notably decreased TPA-induced matrix metalloproteinase-9 (MMP-9) activity and mRNA expression, as measured by zymography and quantitative real time PCR, respectively. A luciferase reporter assay indicated that Bis suppression significantly down-regulated NF-κB-driven transcription. Finally, we demonstrated that the rapid phosphorylation and subsequent degradation of IκB-α induced by TPA was remarkably delayed by Bis knockdown. These results suggest that Bis regulates the invasive ability of glioma cells elicited by TPA, by modulating NF-κB activation, and subsequent induction of MMP-9 mRNA. [BMB Reports 2014; 47(5): 262-267]

INTRODUCTION

Bcl-2 interacting cell death suppressor (Bis), also known as Bag3 and CAIR-1, has been reported to be involved in the pathways of anti-apoptosis and anti-stress (1-3). Overexpression experiments have demonstrated that Bis can protect cells from various stresses, including heat shock, heavy metals, proteasome inhibitors, HIV infection, and oxidative stresses, both *in vivo* and *in vitro*. The pro-survival activity of Bis was supported by the finding that it is overexpressed in various types of cancers, such as leukemia, thyroid, prostate and pancreatic cancers, as well as gliomas (4, 5). In addition, Bis has been shown to be involved in the differentiation of promyeolocytic lymphocytes, myocytes, and glia cells, suggesting an important role for Bis in the normal development of organisms. Notably, Bis gene mutations were identified in a number of patients with myofibrillar myopathy and dilated cardiomyopathy (6, 7). Bis co-operates with small heat shock proteins (sHSP), to stimulate the autophagic process, promoting the clearance of proteins that are aggregation prone (8, 9). The biochemical basis of the ability of Bis to function in this wide variety of processes seems to be its capacity to interact with various partner proteins, such as Bcl-2, Hsp70, PLC-γ, IKK-γ and HspB8, in response to a variety of cellular stresses (1-3, 8, 10).

Glioblastoma is the most common type of malignant tumor found in the central nervous system. Despite great advances over the past two decades in surgical techniques and therapeutic strategies for this type of tumor, the prognosis of malignant glioma patients remains poor, as median patient survival is only one year (11). It has been recently demonstrated that Bis expression is higher in more aggressive gliomas, particularly glioblastoma (12). Furthermore, down-regulation of Bis in a rat glioblastoma model results in an increased sensitivity to apoptosis *in vitro*, and a decrease in tumor volume (12), implying that increased expression of Bis contributes to the prolonged survival of glioblastoma cells *in vivo*, in unfavorable environments. These data are in keeping with our previous study that demonstrated that the suppression of Bis expression sensitizes glioma cells to oxygen glucose deprivation, a condition that mimics the hypoxic conditions in tumors *in vivo* (13). Together, these results suggest that Bis could be investigated as a therapeutic target for glioblastoma.

The poor clinical outcome after therapeutic intervention for glioblastoma is due to the nature of glioma cells to infiltrate adjacent normal brain tissues; therefore, modulating cell survival does not seem to be an effective strategy for suppressing the development of glioma cells into more aggressive phenotypes. Furthermore, it has been previously shown that Bis regulates adhesion and motility in several types of cancer

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cells, including breast cancer, and that bis-deficient mouse embryonic fibroblasts exhibit a delayed response, in the formation of focal adhesion complexes (7, 14-16). Therefore, to understand the mechanistic link between Bis expression and the aggressive phenotype of glioblastoma, it is crucial to investigate the impact of Bis on the migration and invasion abilities of glioma cells. However, the role of Bis in the migration and invasion of glioma cells remains unknown.

In this study, we report that silencing Bis expression noticeably reduces the migration and invasion of A172 glioma cells induced by 12-O-Tetradecanoylphorbol-13-acetate (TPA), a potent tumor inducer. In addition, suppression of Bis expression significantly decreases NF-κB activation, which is responsible for the induction of matrix metalloproteinases (MMP)-9.

RESULTS AND DISCUSSION

Down-regulation of Bis expression inhibits glioma cell migration and invasion *in vitro*

To examine the role of Bis in the aggressive phenotype of glioma cells, A172 glioma cells were transfected with bis-specific siRNA, and the reduction of Bis expression was confirmed by Western blot (Fig. 1A). We subsequently used a Transwell assay, to evaluate the effect of decreasing Bis expression on the migration and invasion abilities of glioma cells treated with TPA, a potent tumor promoter. The knockdown of Bis significantly decreased the migration of A172 cells to 36.6%, compared to the cells transfected with the control siRNA (Fig. 1B). In addition, Bis knockdown suppressed the invasive ability of the A172 cells to 42.8% of the control cells, as measured by the Transwell with Matrigel assay (Fig. 1B). According to previous reports, TPA stimulates the invasion and migration of glioma cells (GBM8401), through NF-κB activation (17). Thus, we compared the regulatory effect of Bis knockdown on the aggressive phenotype of A172 glioma cells with that of p65, a subunit of NF-κB. The effect of p65 down-modulation on cell migration was not noteworthy, while the effect on invasion ability was more severe, than that of Bis down-modulation (Fig. 1B). These results indicate that Bis is involved in regulating the migration and invasiveness of A172 cells via both NF-κB-independent, and dependent mechanisms.

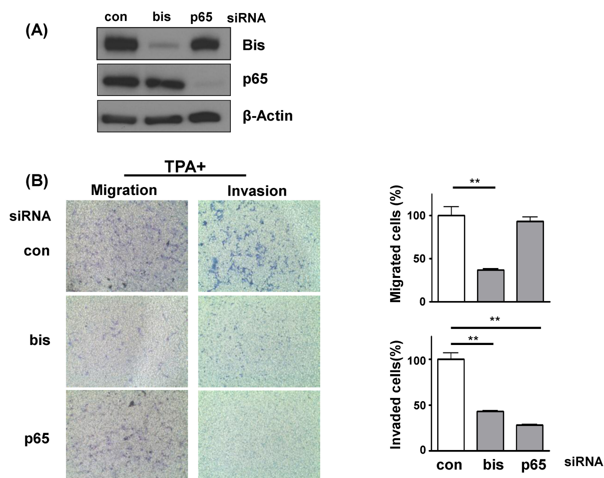

Fig. 1. The effect of Bis knockdown on migration and invasion in A172 glioma cells. (A) A172 cells were treated with specific siRNAs for control (con), Bis or p65 (100 μM) for 48 hr. A significant decrease in Bis or p65 expression is demonstrated by Western blot. (B) The migration or invasion of A172 glioma cells in the presence of TPA was evaluated, using an 8.0-μm-pore-filter Transwell assay for 24 hr, as described in the Materials and Methods. Representative micrographs are shown in the left column. The quantification of migrated cells or invasive cells was evaluated by Image J software, and the relative values compared to control cells are presented as percentages (%, right column). Data are the mean ± SE, from three independent experiments. $^{**}P < 0.005$.

Bis knockdown decreases MMP-9 activity and expression

The metastatic potential of tumor cells has been reported to be conferred by the secretion of endopeptidases, such as MMPs, which degrade the extracellular matrix (ECM). Among all MMPs, MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) activity have been highly linked to tumor invasion *in vivo* and *in vitro* (18-20). Therefore, we analyzed MMP-2 and MMP-9 activity, using gelatinolytic activity (gelatin zymography) in control and Bis- knockdown glioma cells. TPA treatment of control A172 cells significantly induced MMP-9 activity, while MMP-2 activity was not induced (Fig. 2A). Reduction of Bis expression in A172 cells suppressed the induction of

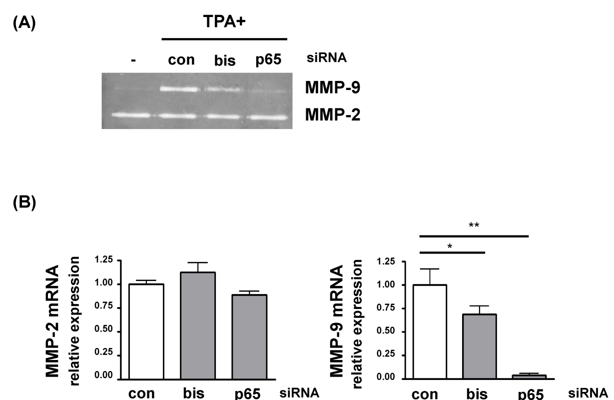

Fig. 2. Bis knockdown decreased MMP-9 activity and mRNA expression. (A) Gelatin zymography was performed with the conditioned medium from A172 glioma cells transfected with control (con), Bis or p65 siRNAs, followed by TPA treatment. (B) Quantitative analysis of MMP-2 and MMP-9 mRNA was evaluated, using Real-Time PCR, as described in Materials and Methods. The value from non-TPA treated cells was arbitrarily designated as 1.0, and the relative values from control, Bis, or p65 siRNA-treated cells are presented. The mean values from three independent experiments are presented. $^{*}P < 0.05$, $^{**}P < 0.005$ vs control cells.

MMP-9 enzyme activity, but to a lesser degree than did suppressing NF-κB expression. The inhibition of MMP-9 enzyme activity by Bis knockdown was attributable to the repression of MMP-9 mRNA transcription (Fig. 2B). Similar to the effect on MMP-9 enzyme activity, transcriptional repression of MMP-9 was stronger in p65- knockdown cells, than in Bis-knockdown cells (68.5% vs. 3.8% of control cells). The transcription of MMP-2 was not significantly affected by bis siRNA, or by p65 siRNA.

TPA -induced NF-κB activation is suppressed by Bis knockdown

It has been previously shown that in several types of glioma cell lines, TPA stimulates migration and invasion, via NF-κB-dependent MMP-9 activation (17, 21-23). These data are consistent with our results obtained in A172 glioma cells, that the effects of reducing Bis expression on cell migration and invasion, and the induction of MMP-9 activity, were all comparable to that of suppressing p65 expression. Furthermore, in osteosarcoma cells, Bis has been involved in the stabilization of IKK-γ, thereby degrading IκB, leading to the subsequent activation of NF-κB (10). We have also previously observed that Bis regulates the survival of glioma cells upon hypoxic stress, by modulating NF-κB activation (13). These results suggest that, in the present study, Bis is involved in the activation of NF-κB upon TPA treatment. Thus, we examined if the activation of NF-κB activity induced by TPA treatment is regulated by Bis, using a NF-κB-directed luciferase reporter assay. TPA treatment radically increased the transcriptional activity of NF-κB 97.3-fold, compared to untreated cells (data not shown). The transfection of Bis siRNA decreased the transcriptional activity of NF-κB elicited by TPA to 38.2% of control siRNA treated cells, while treatment with p65 siRNA decreased this activity to 17.4% of control cells (Fig. 3). Therefore, the significant inhibitory effect of Bis gene silencing on the invasive ability of glioma cells could be attributed to the suppression of NF-κB activation, which ultimately results in a decrease in MMP-9 activity. These results suggest that the high expression of Bis in high grade glioma tissues contributes to the invasion of glioma cells, most likely through a mechanism involving NF-κB activation, and subsequent induction of MMP-9.

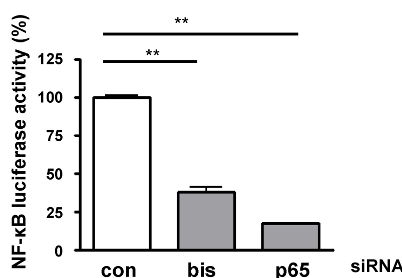

Fig. 3. Bis knockdown reduces the NF-κB-directed luciferase assay. A172 cells were transfected with the Ig-κB-luciferase vector, after silencing of Bis or p65, and transcriptional activity of NF-κB was determined by luciferase activity, using a luminometer. The mean values from triplicate experiments are presented as a percentage of those from the cells transfected with control (con) siRNA. **P < 0.005.

Bis regulates the degradation of IκB-α and phosphorylation of p65

Previous studies have demonstrated that TPA-induced NF-κB activation is mediated by PI3K/AKT or MAP kinase activation, by demonstrating that specific inhibitors of those kinases inhibit phosphorylation and degradation of IκB-α, which leads to subsequent activation of NF-κB directed transcription (21, 24). IκB-α has also been shown to be a target for the regulatory action of Bis, during the growth of osteosarcoma and melanoma cells (10). To clarify whether the effect of Bis knockdown on the invasion of glioma cells is attributable to the regulation of IκB-α expression, we determined the levels of IκB-α expression, as well as the phosphorylation status of IκB-α, following Bis knockdown. TPA treatment of A172 cells resulted in a marked decrease of IκB-α levels, in a time dependent manner that was evident as early as after 15 min of TPA treatment (Fig. 4). The striking contrast in IκB-α levels in control and Bis- knockdown A172 cells seems to be due to a difference in the timing and degree of phosphorylation of IκB-α, in response to TPA treatment. Moreover, even though p65 expression level is not apparently affected by Bis expression, p65 phosphorylation was clearly dependent on Bis expression. Thus, Bis regulates NF-κB signaling, by modulating IKK activity, targeting both IκB-α and p65.

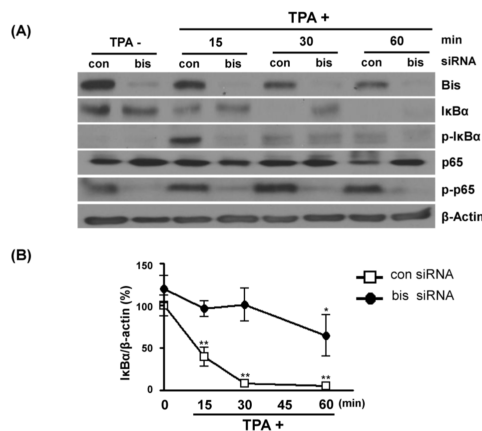

Fig. 4. Knockdown of Bis delayed the degradation of IκB-α upon TPA treatment. (A) Bis expression was suppressed in A172 cells, and cells were then exposed to TPA treatment for indicated times. A Western blot was performed, to determine the expression levels for IκB-α, p-IκB-α, p65 and p-p65. Quantitative analysis showed that Bis knockdown significantly suppressed the degradation of IκB (B). The density ratio of IκB to that of β-Actin in control cells was designated 100%. Data represent the mean value with SE from four independent experiments. *P < 0.05, **P < 0.005 vs TPA non-treated cells.

The association of Bis with the activity of NF-κB was previously demonstrated by several reports, but those results are not consistent with Bis activation of NF-κB. Rosati *et al.* showed that the protective role of Bis in reactive astrocytes infected with HIV is due to the suppressive activity of Bis on NF-κB-induced activation of the long terminal repeat sequence of HIV-1 (25). However, Bis increased NF-κB activity, which probably enhances the survival of tumor cells, such as osteosarcoma and melanoma (10), supporting our results that Bis is involved in the activation of NF-κB, in relation to the invasive ability of glioma cells. At present, the molecular mechanism by which Bis modulates IκB phosphorylation, and subsequent degradation, is not clear. A previous study showed that Bis alters the interaction between IKKγ and HSP70, thereby increasing the availability of IKKγ to degrade IκB, which, in turn, enhances NF-κB activity and cell survival (10). Recently, it has been shown that Bis stabilizes MCl-1, preventing its Hsp70-dependent degradation (26). Thus, it is probable that Bis might be involved in the maintenance of the stability or degradation of IKKα/IKKβ complex or IKKγ, by regulating their interaction with Hsp70, or the proteasome complex. However, the exact molecular mechanism of how Bis modulates NF-κB activity, to promote the invasive ability of a tumor, remains to be clarified in future studies.

The previous study suggests that the anti-apoptotic activity of Bis is the critical factor contributing to the expansion of a glioma, by showing that Bis suppression sensitizes glioma cells to apoptotic stimuli, in a xenograft model. However, the results from our data clearly indicate that the invasive ability of glioma cells is absolutely dependent on the Bis expression status, suggesting that, in addition to its anti-apoptotic activity, increased Bis expression could confer invasive potential to glioma cells, initiating local infiltration, and thereby determining the clinical outcome.

Although our study suggests that NF-κB activation is the critical event in the Bis-mediated promotion of invasion by glioma cells, it should be noted that in contrast to invasion, the migration ability was more significantly suppressed by Bis knockdown, than by p65 knockdown; whereas, MMP-9 activity was more severely reduced by NF-κB suppression, than by Bis suppression. Thus, the molecular mechanism underlying the regulatory effect of Bis on the migration of glioma cells appears to be different, from that on the invasion of glioma cells.

MATERIALS AND METHODS

Cell culture and transfection

The human glioma cell line A172 was obtained from ATCC (Manassas, VA, USA). Cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal-bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific), and maintained at 37°C in a humidified incubator, containing 5% CO_2. Small interfering RNAs (siRNAs), targeted for Bis and p65, were synthesized by Bioneer (Daejeon, Korea). Cells (1.5×10^5/ml) were transfected with specific siRNAs (100 μM) for Bis (5'-AAGGUUCAGACCAUCUUGGAA-3') or p65 (5'-CGG AUUGAGGAGAAACGUAAA-3'), or a control siRNA (5'-AAG GUUCAGACCAUCUUGGAA-3'), using G-Fectin (Genolution Pharmaceuticals, Seoul, Korea), for 48 hr. The transfected cells were incubated for 24 hr in serum-free media, followed by treatment with 50 ng/ml of TPA (Sigma-Aldrich, St. Louis, MO, USA), for the indicated times.

Western blotting

Total cell lysates were prepared, as previously described (13). An equal amount of protein for each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The blots were incubated with anti-Bis serum (1) (1 : 10,000), anti-p65 (1 : 1,000, Santa Cruz Biotechnology, Dallas, TX, USA), anti-IκB-α (1 : 500, Santa Cruz Biotechnology), anti-p-IκB-α (1 : 500, Cell signaling Technology, Danver, MA, USA), or anti-phospho-p65 (1 : 1,000, Cell signaling Technology). The blots were then incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG (1 : 1,000, Promega, Madison, WI, USA). The visualization of immune-reactive bands was performed, using enhanced chemiluminescence (Thermo Fisher Scientific). Quantification of the intensities of each band was carried out, using Image J software, provided by the National Institute of Health (NIH, Bethesda, MD, USA).

Cell migration and invasion assays

For the cell migration assays, A172 (5×10^4) cells were seeded in 200 μl DMEM without FBS, on a fibronectin-coated polycarbonate membrane insert, in a Transwell plate (Costar, Tewksbury, MA, USA). In the lower chamber, 800 μl DMEM with 5% FBS was added, as a chemo-attractant. After the cells were incubated with TPA for 24 hr, the insert was washed with phosphate buffered saline (PBS), and cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface of the filter were fixed, and stained using a Diff-Quick kit (Fisher Scientific, Pittsburgh, PA, USA), and analyzed using Image J software (NIH), to assess the area stained with a single color (27). All assays were repeated independently, at least three times.

The procedure for the cell invasion assay was similar to the cell migration assay, except that the Transwell membranes were pre-coated with 1.4 mg/ml Matrigel (BD bioscience, San Jose, CA, USA) in serum-free medium, and the cells were incubated for 48 hr with TPA (50 ng/ml). Cells adhering to the lower surface were counted the same way, as in the cell migration assay.

Gelatin zymography

The enzymatic activities of MMP-2 and MMP-9 were determined by gelatin zymography (28). Briefly, the conditioned media were

collected 24 hr after the stimulation with TPA, and concentrated with Vivaspin 500 (Sartorius Stedim Biotechnology, Goettingen, Germany). The concentrated medium was mixed with 5X Laemmli sample buffer, without reducing agent. After electrophoresis through an 8% SDS-PAGE gel containing 0.1% (w/v) gelatin at 4°C, the gel was washed with a washing buffer containing 2.5% Triton X-100 in dH_2O, and subsequently incubated in reaction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 40 mM NaN_3), at 37°C overnight. The gel was stained with 0.25% (w/v) Coomassie brilliant blue (Biosesang, Gyeonggi-do, Korea), in 45% (v/v) methanol and 1% (v/v) acetic acid, and washed with de-staining solution (5% MeOH, 7% acetic acid/dH_2O). The clearance zone was examined, using Gel-Doc (Bio-Rad, Hercules, CA, USA).

RNA isolation and Real-time PCR analysis

Total RNA was isolated, using an AcuZol RNA extraction kit (Bioneer); and 2 μg of total RNA was converted to cDNA, using a M-MLV reverse transcriptase kit (ReverTra Ace qPCR RT kit, Toyobo, Osaka, Japan), according to the manufacturer's protocol. The PCR product of each sample was analyzed by quantitative Real-Time PCR, using a kit (SYBR premix Ex Taq, Takara Biotechnology, Shiga, Japan), and an Applied Biosystems 7300 Real-Time PCR machine (Carlsbad, CA, USA). The PCR primers were as follows: Bis, 5'-AGCCC TCAGCACTGCCCCTGCAGAA-3' and 5'-GCAGCTCTTTGGT CAAATACTCTTC-3'; p65, 5'-CCCATCTTTGACAATCGTGC-3' and 5'-ATCAGCTTGCAGAAAGGCGC-3'; MMP-2, 5'-GCAA GGAGTACAACAGCTGC-3' and 5'-GAAGCGGAATGGAAAC TTGCA-3'; MMP-9, 5'-CACTGTCCACCCCTCAGAGC-3' and 5'-GCCACTTGTCGGCGATAAGG-3'; and β-actin, 5'-TGAAG GTCGGTGTGAACGGATTTGGC-3' and 5'-CATGTAGGCCAT GAGGTCCACCAC-3v.

Ig-κB reporter gene assay

Twenty-four hours after transfection with siRNA, the cells were transfected with a luciferase reporter construct, which includes the κB response element in the promoter region (IgκB-Luc, 0.5 μg) (29), using Fugene Extreme (Roche Applied Science, Indianapolis, IN, USA). After treatment with TPA for an additional 24 hr, the luciferase activity was measured with a Dual Luciferase kit (Promega). A pRLTk plasmid was used to normalize the luciferase activity.

Statistical analyses

All experiments were repeated at least three times, and data appear as the mean ± the standard error (SE). Statistical significance was determined using Student's *t*-test. A P value of < 0.05 was considered to be significant.

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