Full Paper

Embelin Induces Apoptosis in Human Glioma Cells Through Inactivating NF- κ BSang-Yoon Park^{1,2}, Sung-Lyul Lim^{1,2}, Hyeung-Jin Jang^{1,3}, Jun-Hee Lee^{3,4}, Jae-Young Um^{1,2,3}, Sung-Hoon Kim^{1,2,3}, Kwang Seok Ahn^{1,2,3,*a}, and Seok-Geun Lee^{1,2,3,*b}¹Department of Science in Korean Medicine, ²Cancer Preventive Material Development Research Center,³Institute of Korean Medicine, ⁴Department of Clinical Korean Medicine, College of Korean Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea

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Abstract. Aggressive tumor growth and diffuse tissue invasion are hallmarks of malignant glioma. Embelin is an active compound identified as a novel XIAP inhibitor from the *Embelia ribes* that exhibits various medicinal effects including anti-inflammatory and anti-cancer activities. In the present study, we investigated whether embelin could have a therapeutic effect in glioma. We found that embelin suppressed proliferation of human glioma cells, but not in normal immortalized human astrocytes. In addition, embelin induced apoptosis in human glioma cells by inhibiting NF- κ B, which is a crucial transcription factor associated with several human diseases including cancer and controls multiple genes involved in tumor progression such as cell proliferation and survival. Intriguingly, embelin had no inhibitory effect on XIAP in glioma cells even though discovered as an XIAP inhibitor, but instead inhibited NF- κ B activity by reducing nuclear translocation of p65 through decreasing phosphorylation and proteasomal degradation of I κ B α in glioma cells. Furthermore, p65 overexpression decreased embelin-induced apoptosis in glioma cells. Taken together these results indicate that embelin could be a potent novel therapeutic modality for glioma via blocking cancer cell proliferation and inducing apoptosis by inhibiting NF- κ B activity.

Keywords: embelin, glioma, proliferation, apoptosis, NF- κ B

Introduction

Tumors of the central nervous system are the most prevalent solid neoplasms of childhood in South Korea and the United States and the second leading cancer-related cause of death in young adults between the ages of 20 – 39 years in the United States (1, 2). Malignant gliomas, including glioblastoma multiforme (GBM), a grade IV astrocytoma, and anaplastic astrocytomas are the most common and most aggressive primary brain tumors in humans (3). GBM is an extremely aggressive, invasive, and destructive malignancy with much faster proliferation rate than grade III tumors (3, 4). Extensive surgical resection is not curative due to the highly invasive capacity of glioma cells into normal brain paren-

chyma (5). Moreover, malignant glioma is largely resistant to current treatments based on cytotoxic approaches targeting replicating DNA, such as chemotherapy or radiotherapy (3, 4). Median survival of the patients, therefore, is still around 14 months from the time of diagnosis despite multimodality treatment with aggressive surgery, radiation, and chemotherapies, and even in the most favorable situations most patients die within two years (3, 6).

NF- κ B has been associated with many disease states such as chronic inflammation, neurodegenerative disorders, diabetes, and stroke (7, 8). In various types of cancer including glioma, NF- κ B is constitutively active by diverse mechanisms including mutation of upstream components of the pathway (9, 10). NF- κ B is composed of homo- and heterodimers of Rel family protein including p65/RelA, RelB, c-Rel, p50/p105, and p52/p100 (11, 12). NF- κ B in the cytoplasm is anchored by a family of inhibitory proteins called the I κ Bs that mask the

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nuclear localization signal (8, 9). Various NF- κ B activating signals induce phosphorylation and degradation of I κ Bs regulated by the multi-protein I κ B kinase complex, resulting in nuclear translocation of NF- κ B to activate the expression of multiple target genes that are involved in survival and anti-apoptosis in cancer cells (8, 9, 13).

Embelin a small-molecule inhibitor of XIAP identified from the fruit of the *Embelia ribes* BURM. (Myrsinaceae) was originally discovered by screening a library of natural products derived from Oriental traditional medicine (14). Embelin has been shown to have anti-inflammatory, anti-tumor, and analgesic properties, and considerable interest in its anti-tumor activity revealed that embelin induces growth inhibition and apoptosis in various types of human cancer cells through the inhibition of NF- κ B, XIAP, STAT3, and PPAR γ (14 – 19). In addition, a recent study showed that embelin enhanced TRAIL-mediated apoptosis in U251 and LN229 glioma cells by reducing the expression of c-FLIP (20). However, the therapeutic effect of embelin by itself in glioma was not investigated yet. In the present study we examined if embelin could be a therapeutic agent for glioma and revealed the mechanism by which embelin induced its therapeutic effects.

Materials and Methods

Plasmids and reagents

NF- κ B-Luc, Renilla luciferase reporter plasmid (Renilla-Luc) for transfection control, and p65 expression vector were previously described (21). Embelin, DAPI (4',6-diamidino-2-phenylindole), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Propidium Iodide and Alexa Fluor 488 goat anti-rabbit antibody were purchased from Invitrogen (Carlsbad, CA, USA). Annexin V staining kits (ApoScan) were purchased from BioBud (Seoul, Republic of Korea). The antibodies used were purchased from the following commercial sources: phospho-I κ B α , XIAP, c-PARP, phospho-p65, phospho-AKT, ERK, phospho-ERK, caspase-3 (CASP3), cleaved CASP3 (c-CASP3), CASP8, c-CASP8, CASP9, c-CASP9, c-Notch1, cyclin D1, and cyclin E (Cell Signaling Technology, Danvers, MA, USA); I κ B α , p65, AKT, Bcl-XL, and Bcl-2 (Santa Cruz Biotech, Santa Cruz, CA, USA); p50 and phospho-p50 (AB frontier, Seoul, Republic of Korea); and β -actin (Sigma-Aldrich).

Cell lines

Human glioma cell lines T98G, U87MG, and H4 and immortalized primary human fetal astrocytes (IM-PHFA) were previously described (22 – 24). LN18 was a kind gift from Dr. Sin-Soo Jeun (Catholic University of Korea

College of Medicine, Seoul, Republic of Korea). All cells were cultured in DMEM (Lonza, Inc., Allendale, NJ, USA) supplemented with 10% heat-inactivated fetal bovine serum (Lonza) and 100 U/ml of the antibiotic antimycotic (Lonza) at 37°C in a humidified incubator with 5% CO₂.

Cell viability assays, cell cycle, and apoptosis

Cell viability was measured using the MTT assay. Cells ($2 - 5 \times 10^3$ cells/well) were plated in 96-well, flat bottom tissue culture plates and treated with embelin for 1 – 6 days. Cell viability was measured by MTT assays as previously described (25). IC₅₀ values representing the drug concentration causing 50% growth inhibition were determined 3 days after treatment. Cell cycle and apoptosis were monitored respectively by propidium iodide (PI) staining and by Annexin V binding and PI staining followed by flow cytometry analysis using FACS Calibur (BD Biosciences, San Jose, CA, USA) as described (24). Every assay was performed at least three times in triplicate.

Western blot analysis

Whole cell lysates were prepared, and western blotting was performed as described (23). Primary antibodies for phospho-I κ B α (1:1000), I κ B α (1:1000), CASP3 (1:1000), c-CASP3 (1:1000), CASP8 (1:1000), c-CASP8 (1:1000), CASP9 (1:1000), c-CASP9 (1:1000), c-PARP (1:1000), XIAP (1:1000), Bcl-XL (1:1000), Bcl-2 (1:1000), CDK2 (1:1000), CDK4 (1:1000), cyclin D1 (1:1000), cyclin E (1:1000), AKT (1:1000), phospho-AKT (1:1000), ERK (1:1000), phospho-ERK (1:1000), c-Notch1 (1:500), p65 (1:1000), phospho-p65 (1:1000), p50 (1:1000), and phospho-p50 (1:1000) were used for immunoblotting followed by horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (1:3000; Cell Signaling Biotechnology, Danvers, MA, USA) for 1 h and were detected using a chemiluminescence system (Corebio, Seoul, Republic of Korea).

Immunofluorescence staining

For analyzing localization of p65, cells were plated in 8-well chamber slides and treated with 50 μ M of embelin for 24 h. Then the cells were fixed and immunostaining was performed with rabbit polyclonal anti-p65 antibody (1:200) and Alexa Fluor 488 goat anti-rabbit antibody (1:1000) and DAPI as described (23). The images were taken with the FluoView FV1000 confocal microscope (Olympus, Tokyo).

Transient transfection and luciferase assays

T98G and U87MG cells were transfected with NF- κ B-Luc and Renilla-Luc in a 10-cm plate using LipofectAMINE 2000 (Invitrogen, Grand Island, NY,

USA). One day after transfection, cells were collected and replated into a 48-well plate (6×10^4 cells/well). Then the cells were treated with embelin for 24 h. Luciferase assays were performed using a dual-luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were measured using a luminometer (BMG Labtech, Ortenberg, Germany). Transient transfection and luciferase assays were performed at least three times in triplicate.

Statistical analyses

Data were presented as the mean $\pm$ standard error of the mean (S.E.M.) and analyzed for statistical significance using the unpaired Student's *t*-test. *P* < 0.05 was considered significant.

Results

Embelin represses proliferation of human glioma cells

Recent studies have remarked on cancer chemoprevention with dietary phytochemicals (26, 27). Embelin, one of the phytochemicals, has already been known to

have various medicinal effects on diabetes (28, 29), ischemia (30), antibacterial activity (31), osteoclastogenesis (32), and several types of cancer such as pancreatic cancer (17), prostate cancer (33), leukemia (34), hepatoma, and colon cancer (18). In the present study, we evaluated the potential of embelin as a therapeutic agent for another fatal disease, malignant glioma. To investigate the possible therapeutic effect of embelin on glioma, we first examined whether embelin affects proliferation of human glioma cells. Human glioma cell lines T98G, U87MG, LN18, and H4 and a normal immortalized astrocyte cell line IM-PHFA were treated with embelin as shown in Fig. 1, and cell viability was measured by MTT assays. Interestingly, embelin repressed cell growth in a dose- and a time-dependent manner in all the glioma cell lines with little effect on normal cells, IM-PHFA (Fig. 1: A – E). In addition, IC₅₀ values at 3 days after embelin treatment indicated that T98G and U87MG cells were more sensitive to the treatment (Fig. 1F). These results suggested that embelin is a potentially good therapeutic phytochemical for malignant glioma.

Fig. 1. Embelin represses proliferation of glioma cells. T98G (A), U87-MG (B), LN18 (C), H4 (D), and IM-PHFA (E) were treated with embelin (0–50 μM). Cell viability was determined by MTT assays at 1, 3, and 6 days after the treatment. Data in the graphs are presented as the mean $\pm$ S.E.M., and values in the graphs indicate absorbance at 570 nm (A₅₇₀). Asterisks indicate statistically significant differences between the untreated control and embelin-treated cells (*P* < 0.05). F) IC₅₀ in each cell line was determined using the data at 3 days after embelin treatment. Data are presented as the mean $\pm$ S.E.M.

Embelin induces apoptosis in glioma cells

The balance between cell proliferation and cell death determines cell growth, and therefore induction of apoptosis or/and inhibition of cell proliferation can decrease the cell number. Results described above demonstrate that embelin could decrease the number of cells in human glioma cell lines. We next investigated whether embelin induces cell cycle arrest or apoptosis. As shown in Fig. 2A, embelin did not obviously alter the cell cycle in IM-PHFA cells. However, embelin treatment in T98G cells significantly increased the cell population of the sub-G1 phase as being indicative of apoptosis (Fig. 2A). To further confirm if embelin's effects are due to apoptosis, we analyzed the induction of apoptosis by Annexin V binding and PI staining followed by fluorescent-activated cell sorting (FACS) analysis. Based on Annexin V staining, embelin induced apoptosis in T98G glioma cells with little effect on IM-PHFA cells, corroborating the data obtained in the cell cycle analysis (Fig. 2B). These results indicated that embelin induces apoptosis in glioma cells, but not in normal cells. We further examined the expression of apoptosis- and cell cycle-related proteins.

As shown in Fig. 2C, embelin in glioma cells had no effect on the expression of XIAP, which is its original target, but instead reduced other anti-apoptotic proteins Bcl-XL and Bcl-2. In addition, embelin decreased cell cycle regulators CDK2, CDK4, Cyclin D1, and Cyclin E (Fig. 2C). We also investigated the effect of embelin on the activity of CASPs, which mediate the induction and execution of apoptosis (35). Embelin increased cleavage of both initiator and executioner CASPs, CASP8, 9 and 3, in T98G cells, indicating their activation. Furthermore, embelin increased PARP cleavage (Fig. 2D). Together these results indicated that embelin induces apoptosis in glioma, but the effect of embelin on glioma was not related with XIAP.

Embelin inhibits NF- κ B activity in glioma cells

In order to investigate the mechanism by which embelin induces apoptosis in glioma cells, we first examined the effect of embelin on the signaling molecules such as AKT, MAPK, Notch, and NF- κ B related with cell proliferation as well as survival in glioma (4, 10, 36–40). As shown in Fig. 3A, embelin did not change the levels

Fig. 2. Embelin induces apoptosis in glioma cells. A) IM-PHFA (upper panel) and T98G (lower panel) cells were treated with 50 μ M embelin for 36 h. Then the cells were stained with PI, and the cell cycle was immediately analyzed by flow cytometry. B) T98G and IM-PHFA cells were treated with 50 μ M embelin for 36 h. Then the cells were stained with Annexin V and PI and immediately analyzed by flow cytometry. The percentage of early and late apoptotic cells was calculated. Asterisk indicates statistically significant differences between the untreated control and embelin-treated cells ($P < 0.05$). C, D) T98G cells were treated with 50 μ M embelin, and whole cell lysates were prepared and then subjected to western blot analysis with the indicated antibodies. Anti- β -actin antibody was used as an internal control.

of c-Notch1 and the phosphorylation of AKT and ERK. However, embelin decreased p65 phosphorylation in a dose-dependent manner with no effect on p50 phosphorylation (Fig. 3B). These results suggest that embelin may induce apoptosis of glioma cells by inhibiting NF- κ B. NF- κ B is constitutively activated in many types of human cancer including malignant glioma with critical roles in survival and proliferation of tumor cells (4, 10, 41). Thus, inhibition of NF- κ B in tumor displays effective suppression of tumor cell survival, proliferation, invasion, angiogenesis, and inflammation (11, 41). A recent study also suggested that embelin is a potent inhibitor of NF- κ B (16). Therefore, we further investigated the effect of embelin on NF- κ B activity. Since nuclear translocation and activation of NF- κ B is regulated by I κ Bs (9, 10), we next examined whether embelin

could regulate phosphorylation of I κ B α . As shown in Fig. 3, C and D, embelin decreased phosphorylation of I κ B α in a dose- and a time-dependent manner. These results indicated that embelin activates I κ B α that is a negative regulator of NF- κ B. In addition, we found that embelin reduced nuclear translocation of NF- κ B (Fig. 3E) and furthermore decreased NF- κ B activity as a transcriptional activator (Fig. 3F). Consistently, we already found that embelin repressed NF- κ B target genes such as Bcl-2, Bcl-XL, and cyclin D1 (Fig. 2C). Together these results clearly indicated that embelin is a potent NF- κ B inhibitor in glioma.

Embelin induces apoptosis in glioma cells by inhibiting NF- κ B

Activation of NF- κ B plays a crucial role in cell sur-

Fig. 3. Embelin inhibits NF- κ B activity in glioma cells. A, B, C, D) Cells were treated with embelin as indicated. After the treatment, cell lysates were prepared and then subjected to western blot analysis with the indicated antibodies. Anti- β -actin antibody was used as an internal control. C, D) Values below each panel indicate fold normalized expression ratio of phospho-I κ B α to I κ B α relative to that of no embelin treatment taken as 1. E) T98G cells were treated with 50 μ M of embelin for 24 h. The p65 immunostaining and DAPI staining were evaluated by confocal microscopy. F) Cells were transfected with NF- κ B Luc vector together with Renilla-Luc plasmid as an internal control. Then the transfected cells were treated with 50 μ M of embelin. Data in the graphs are presented as the mean $\pm$ S.E.M., and values are presented as fold-normalized activity relative to that of untreated cells taken as 100. Asterisks indicate statistically significant differences between the untreated control and embelin-treated cells ($P < 0.05$).

vival, proliferation, and anti-apoptosis of cancer cells (4, 10, 41), and the results described above indicated that embelin induces apoptosis and reduces NF- κ B activity. Together these results suggested that embelin might induce apoptosis by inhibiting NF- κ B in glioma cells. To prove the hypothesis we overexpressed p65 in T98G cells, and then treated the transfected cells with embelin. To confirm the transfection efficiency the Renilla-Luc reporter was cotransfected with each vector, and we

found a similar luciferase activity in every transfection, indicating similar transfection efficiency between the control vector and the p65 expression plasmid (data not shown). We also confirmed the p65 expression by western blotting (Fig. 4A, lower panel). As shown in Fig. 4A (upper panel), embelin induced apoptosis in T98G cells transfected with the control vector. However, p65 overexpression significantly reduced embelin-mediated apoptosis in T98G cells (Fig. 4A, upper panel), indicating that NF- κ B is crucial for the embelin-induced apoptosis. Furthermore, we found that NF- κ B activity in T98G and U87MG is much higher than that of LN18 and H4 (Fig. 4B), corroborating the efficacy of embelin in the glioma cells as shown in Fig. 1. In total, these results indicate that embelin induces apoptosis by inhibiting NF- κ B in glioma.

Discussion

Embelin was identified about 50 years ago as an active component of the *Embelia ribes* BURM. (Myrsinaceae), which has been used for thousands of years to treat diverse diseases such as fever, inflammatory diseases, and various gastrointestinal ailments in traditional medicine and thus should have minimum toxicity (14, 15, 32, 42). Embelin has been recently noticed as an inhibitor of NF- κ B, STAT3, XIAP, and PPAR γ to induce growth suppression and apoptosis in human cancer cells of various organs including pancreas, colon, and prostate and leukemia (17, 18, 43, 44). Additionally, recent studies have shown that embelin enhanced TRAIL-mediated apoptosis (20, 45). These results suggested that embelin may be a good anti-cancer agent with less toxicity in normal cells, and therefore we investigated whether embelin could be a good new modality of glioma treatment.

As shown in Figs. 1 and 2, we found that embelin suppresses cell proliferation and induces apoptosis in human glioma cells, but interestingly has no toxic effect in normal astrocytes. In addition, embelin treatment led to repression of anti-apoptotic proteins Bcl-2 and Bcl-XL and cell cycle regulators CDK2, CDK4, Cyclin D1, and Cyclin E as well as activation of both CASP8 and CASP9, resulting in the subsequent activation of CASP3 and PARP cleavage (Fig. 2). Since CASP8 and CASP9 are, respectively, involved in the extrinsic and the intrinsic pathway of apoptosis (46), these results suggest that embelin induces apoptosis in glioma cells via both apoptotic pathways.

NF- κ B that is constitutively active in glioma mainly constitutes a heterodimer of p65/RelA and p50 and is normally sequestered in the cytoplasm by interacting with I κ Bs (9, 10). Oncogenic signals induces phosphory-

Fig. 4. Embelin induces apoptosis in glioma cells by inhibiting NF- κ B. **A)** T98G cells were transfected with pcDNA3.1 or p65 expression vector. One day after transfection, the cells were treated with embelin for 36 h. Then, the cells were stained with fluorescein isothiocyanate-labeled Annexin V and PI and immediately analyzed by flow cytometry. The percentage of early and late apoptotic cells was calculated. Cell lysates were prepared under the same conditions as above and then subjected to western blot analysis with the indicated antibodies. Anti- β -actin antibody was used as an internal control. **B)** Cells were transfected with NF- κ B-Luc vector together with Renilla-Luc plasmid as an internal control. One day after transfection, cell lysates were prepared and then subjected to luciferase assays. Data in the graphs are presented as the mean $\pm$ S.E.M., and values are presented as fold-normalized luciferase activity relative to that of IM-PHFA cells taken as 1. Asterisk indicates statistically significant differences between the untreated control and embelin-treated cells ($P < 0.05$).

lation of I κ Bs, subsequently resulting in its ubiquitin-mediated proteasomal degradation, which are required for the translocation of NF- κ B to the nucleus and its transcriptional activation (8, 9, 11, 12). We found that embelin reduces phosphorylation of I κ B α and induces phosphorylation of p65 as shown in Fig. 3. In addition, embelin decreases nuclear translocation and transcriptional activation of NF- κ B (Fig. 3). Furthermore, p65 overexpression blocked embelin-induced apoptosis in T98G glioma cells, confirming that inhibition of NF- κ B is crucial for the embelin-induced apoptosis in glioma. Recent studies noticed that curcumin and sulfasalazine suppressed cell proliferation and induced apoptosis in gliomas via inhibiting NF- κ B activity (47, 48). Together these results indicate that NF- κ B is clearly a good therapeutic target for glioma, and its inhibition can induce apoptosis in glioma cells.

Another issue for developing a drug for brain diseases is the blood–brain barrier (BBB), a separation of circulating blood from the brain extracellular fluid in the central nervous system (49, 50). It has been always a critical problem to be solved to develop a drug for brain tumors. Recent studies showed that embelin passes the BBB, and it even has a neuro-protective effect against ischemia-induced brain damage in a rat model (30, 51). All together these results suggest that embelin is a potentially good therapeutic modality for the fatal disease glioma through inducing apoptosis by blocking the NF- κ B pathway, and thus this agent should be further explored for its anti-cancer effects by itself or in combination with other chemotherapeutic agents currently used in the clinic such as temozolomide.

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