Embelin induces apoptosis of human gastric carcinoma through inhibition of p38 MAPK and NF-κB signaling pathways

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Abstract. Embelin is a small-molecule inhibitor of X-linked inhibitor of apoptosis protein (XIAP), and it induces apoptosis in tumor cells via the inhibition of XIAP. The aim of the present study was to investigate the anticancer effect of embelin on human gastric carcinoma cells and the mechanisms underlying this effect. Cell proliferation of SGC7901 human gastric carcinoma cells was measured using MTT assay, following treatment with embelin (5, 10 and 15 μM) on days 1, 3 and 5. Caspase-3 and nuclear factor (NF)-κB p65 activation in SGC7901 cells were assessed using commercial kits. Cellular and nuclear apoptosis were analyzed with an Annexin V-FITC/PI Apoptosis Detection kit and DAPI staining assay, respectively. Phospho (p)-p38 mitogen-activated protein kinase (MAPK), p-inhibitor of NF-κB (p-IκBα) and p-IκB kinase α/β (p-IKKα/β) protein expression levels were analyzed with western blot assay. In the present study, treatment with embelin decreased cell proliferation, induced caspase-3 activation and suppressed NF-κB p65 activation in SGC7901 cells. Furthermore, embelin administration reduced p-IκBα and p-IKKα/β protein expression levels. In conclusion, embelin induces cell apoptosis in human gastric carcinoma through activation of p38 MAPK and inhibition of the NF-κB signaling pathways. It was further suggested that embelin may be used as a potential drug for the treatment of gastric carcinoma.

Introduction

According to the 2012 Chinese cancer registration report and statistics of the World Health Organization, the Chinese population presents a high incidence of gastric carcinoma (1). Gastric carcinoma presented the second largest incidence of malignant tumors in China and the third highest rate of mortality; cases of gastric carcinoma and associated mortality account for half the malignant tumor cases worldwide (2). Gastric cancer in China presents high morbidity and mortality rates, and most cases are diagnosed at the advanced stage. The incidence rate of gastric carcinoma in patients under the age of 30 increased from 1.7% in the 1970s to 3.3% at present in China (3).

Nuclear factor (NF)-κB is a transcription factor observed in various cell types that serves a role in physiological and pathological processes through the NF-κB-inducing kinase (NIK)/IκB kinase (IKK)/NF-κB signaling pathway (4,5). Previous studies have demonstrated that NF-κB is associated with proliferation, differentiation, apoptosis, invasion and metastasis of tumor cells (6-8). In addition, aberrant activation of NF-κB was demonstrated in gastric cancer cells and pathological tissues (9).

X-linked inhibitor of apoptosis protein (XIAP) is an effective caspase inhibitor and the most investigated molecular structure of the inhibitor of apoptosis protein (IAP) family. XIAP selectively binds to caspases-3, -7 or -9 to inhibit their activity and prevent cell apoptosis (10). Embelin is a small-molecule inhibitor of XIAP that binds to the baculoviral IAP repeat 3 structural domain of XIAP and prevents binding to caspases-3, -7 or -9, thus inducing cell apoptosis (11). However, the anticancer effect of embelin in human gastric carcinoma cells and the mechanisms underlying it are poorly understood. The present study hypothesizes that the anticancer effect of embelin induces cell apoptosis in human gastric carcinoma cells through the p38 mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways.

Materials and methods

Chemical reagents. Invitrogen RPMI-1640 and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). MTT was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). DAPI staining assays (cat. no. C1005) and caspase-3 activation commercial kits (cat. no. C1116) were obtained from Beyotime Institute of Biotechnology (Haimen, China). A Pierce BCA Protein Assay kit was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China).

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An Annexin V-FITC/PI Apoptosis Detection kit (cat. no. KGA101) was obtained from KeyGen Biotech Co., Ltd. (Nanjing, China). An NF-κB p65 colorimetric assay kit was obtained from Elabscience Biotechnology Co., Ltd. (Wuhan, China; cat. no. E-EL-H1388c).

Cell culture and cell proliferation assay. The human gastric carcinoma cell line SGC7901 was acquired from the experimental center of the Second Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) and maintained in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), in a 5% CO_2 atmosphere at 37˚C. SGC7901 cells were seeded in 96-well plates and treated with embelin (5, 10 or 15 μM; purity ≥98%; Sigma-Aldrich) for 1, 3 and 5 days as previously described (12). Cell proliferation was determined using the MTT assay. Briefly, MTT solution (20 μl; 5 mg/l; Sangon Biotech Co., Ltd.) was added to each well for a 4-h incubation in a 5% CO_2 atmosphere, at 37˚C. Following incubation, 150 μl dimethyl sulfoxide was added to each well and shaken for 20 min at room temperature. The optical density was determined using a 96-well multiscanner at 570 nm (ELx808; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Analysis of caspase-3 activity. SGC7901 cells were seeded in 6-well plates and treated with embelin (5, 10 or 15 μM) for 5 days. Following treatment, caspase-3 activity was assessed using caspase-3 activation commercial kits. Briefly, cells were prepared in cell lysis buffer for 30-60 min at 4˚C and centrifuged at 12,000 x g for 10 min at 4˚C. The protein concentrations in the cell lysates were measured with the Pierce BCA Protein Assay kit according to the manufacturer's instructions. Equal amounts of protein were mixed with the Ac-DEVD-pNA reaction buffer and incubated at 37˚C for 2 h in the dark. Following incubation, absorbance was measured at 405 nm with the XL-818 instrument.

Analysis of cell apoptosis. SGC7901 cells were seeded in 6-well plates and treated with embelin (5, 10 or 15 μM) for 5 days. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), collected and resuspended in annexin V binding buffer from the kit according to the manufacturer's instructions. Following resuspension, 5 μl annexin V-FITC and 5 μl propidium iodide were added to the cells, and incubated for 10 min on ice in the dark. Cell apoptosis was immediately detected using a flow cytometer (Epics Altra; Beckman Coulter, Inc., Brea, CA, USA) to identify annexin V- and/or PI-positive cells.

DAPI staining assay. SGC7901 cells were seeded in 6-well plates and cultured with embelin (5, 10 or 15 μM) for 5 days. SGC7901 cells were washed twice with ice-cold PBS and fixed using 0.5 ml paraformaldehyde (4%; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 30 min at 4˚C. Cells were then washed twice with PBS and incubated with sodium citrate (0.1%; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) containing 0.1% Triton X-100 (Sinopharm Chemical Reagent Co., Ltd.) for 5 min, at 4˚C. Cells were incubated with the DAPI staining assay for 10-15 min at 4˚C in the dark. DAPI was excited by ultraviolet light to indicate nuclear apoptosis and images were captured with an Axio Observer A1 fluorescence microscope (Zeiss AG, Oberkochen, Germany) at the excitation wavelength of 340 nm.

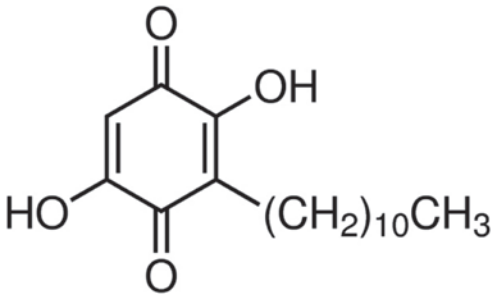

Figure 1. Chemical structure of embelin.

Analysis of NF-κB p65 activation. SGC7901 cells were seeded in 96-well plates and cultured with embelin (5, 10 or 15 μM) for 5 days. NF-κB p65 activation was measured using the NF-κB p65 colorimetric assay kit, according to the manufacturer's instructions.

Western blotting. SGC7901 cells were seeded in 6-well plates and cultured with embelin (5, 10 or 15 μM) for 5 days. Following treatment, cells were prepared in cell lysis buffer for 30-60 min at 4˚C and centrifuged at 12,000 x g for 10 min at 4˚C. The protein concentrations in cell lysates were measured with the Pierce BCA Protein Assay kit according to the manufacturer's protocol. Equal volumes of proteins were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4˚C with mouse anti-phosphorylated (p)-p38 MAPK monoclonal antibody (1:1,000; cat. no. sc-7973; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-p-IκBα monoclonal antibody (1:1,000; cat. no. ab133462; Abcam, Cambridge, MA, USA), anti-p-IKKα/β polyclonal antibody (1:1,000; cat. no. sc-21661; Santa Cruz Biotechnology, Inc.) and anti-β-actin polyclonal antibody (1:500; cat. no. D110007; Sangon Biotech Co., Ltd., Shanghai, China). Following washing with Tris-buffered saline supplemented with Tween-20, the membranes were incubated with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:1,000; cat. no. sc-2380; Santa Cruz Biotechnology, Inc.). for 2 h. Proteins were visualized using enhanced chemiluminescence (Santa Cruz Biotechnology, Inc.) and detected using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation of at least three independent experiments and analyzed with SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). Differences between groups were analyzed by one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of embelin on tumor growth in gastric carcinoma cells. The chemical structure of embelin is presented in Fig. 1.

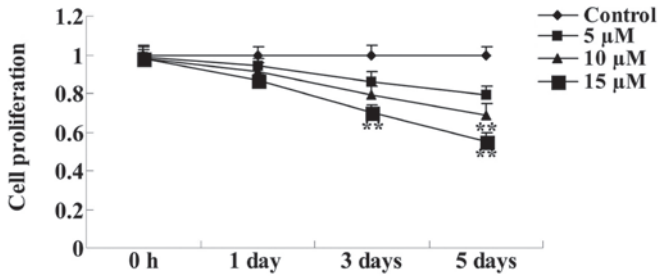

Figure 2. Effect of embelin treatment on the cell proliferation of gastric carcinoma cells. Data represent the mean ± standard deviation. **P<0.01 vs. the control group.

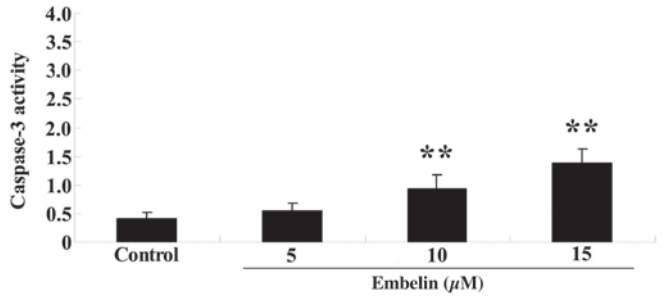

Figure 3. Effect of embelin on caspase-3 activity in gastric carcinoma cells. Data represent the mean ± standard deviation. **P<0.01 vs. the control group.

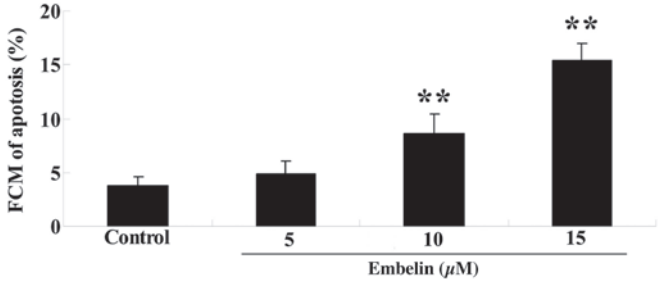

Figure 4. Effect of embelin on cell apoptosis of gastric carcinoma cells. Data represent the mean ± standard deviation. **P<0.01 vs. the control group. FCM, flow cytometry.

To investigate the anticancer effect of embelin on tumor growth, SGC7901 cells were treated with embelin (5, 10 or 15 μM) for 1, 3 or 5 days, and the levels of cell proliferation were determined using the MTT assay. As demonstrated in Fig. 2, 10 and 15 μM embelin significantly suppressed cell proliferation following 5-days of culture, compared with the control group (P<0.01). Therefore, further assays were performed on SGC7901 cells treated with the various concentrations of embelin for 5 days.

Embelin induces caspase-3 activity in gastric carcinoma cells. To investigate the therapeutic effect of embelin on cell apoptosis, SGC7901 cells were treated with embelin (5, 10 and 15 μM) for 4 days, and caspase-3 activity was measured. As demonstrated in Fig. 3, 10 or 15 μM embelin significantly increased caspase-3 activity compared with the control group (P<0.01).

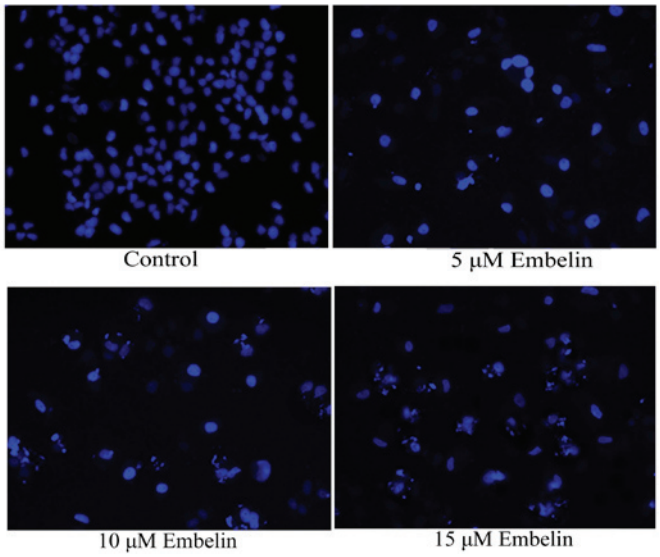

Figure 5. Effect of embelin on nuclear apoptosis in gastric carcinoma cells.

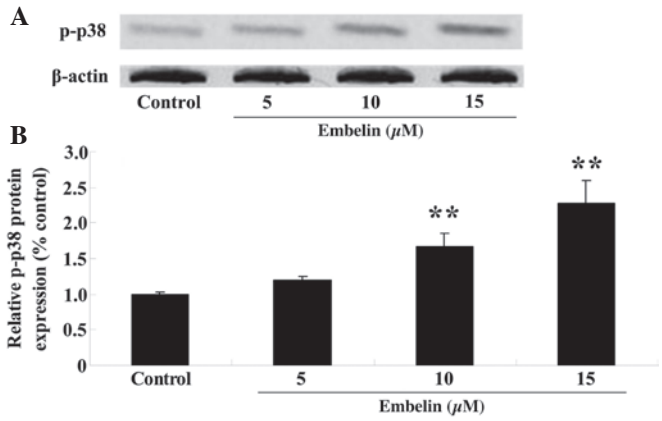

Figure 6. Effect of embelin on p-p38 MAPK protein expression in gastric carcinoma cells. (A) Western blotting assay and (B) quantification of immunoblot. Data represent the mean ± standard deviation. **P<0.01 vs. the control group.

Embelin induces cellular apoptosis in gastric carcinoma cells. In order to examine the effect of embelin on cell apoptosis, SGC7901 cells were treated with embelin (5, 10 or 15 μM) for 4 days, and the cellular apoptosis was determined. As demonstrated in Fig. 4, 10 and 15 μM embelin significantly increased the percentage of cellular apoptosis compared with the control group (P<0.01).

Effect of embelin induces nuclear apoptosis in gastric carcinoma cells. In order to investigate the anticancer effect of embelin on nuclear apoptosis, SGC7901 cells were treated with 5, 10 or 15 μM embelin for 4 days, and nuclear apoptosis was evaluated with the DAPI staining assay. As demonstrated in Fig. 5, nuclear apoptosis was observed following embelin treatment at all concentrations.

Embelin induces p-p38 MAPK protein expression levels in gastric carcinoma cells. SGC7901 cells were treated with embelin (5, 10 or 15 μM) for 4 days, and the p-p38 MAPK protein expression levels were measured using western

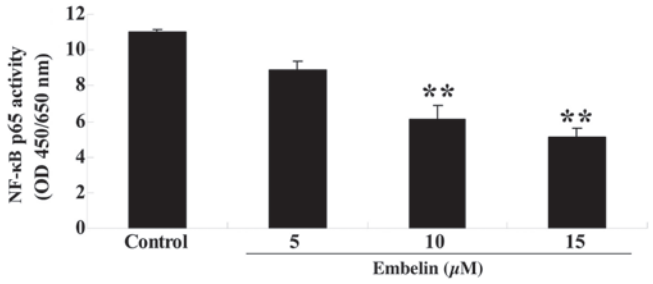

Figure 7. Effect of embelin on the NF-κB p65 activity in gastric carcinoma cells. Data represent the mean ± standard deviation. **P<0.01 vs. the control group. NF-κB, nuclear factor κB.

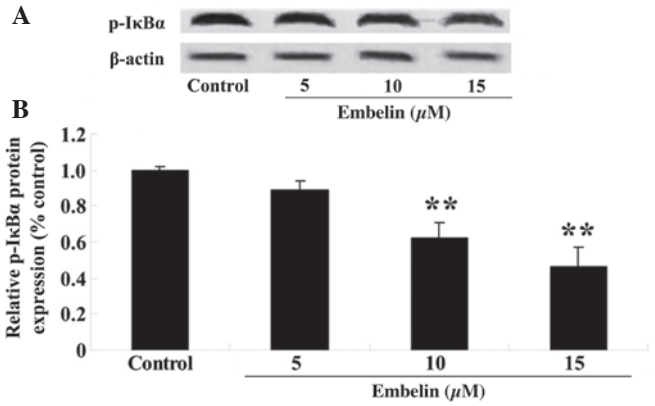

Figure 8. Effect of embelin on the phosphorylated IκBα protein expression levels in gastric carcinoma cells. (A) Western blot assay and (B) quantification of immunoblot. Data represent the mean ± standard deviation. **P<0.01 vs. the control group. IκBα, inhibitor of NF-κB.

blotting. The results demonstrated that administration of embelin treatment (10 or 15 μM) significantly upregulated the relative p-p38 MAPK protein expression levels compared with those of the control group (P<0.01; Fig. 6).

Embelin inhibits NF-κB p65 activity in gastric carcinoma cells. SGC7901 cells were treated with embelin (5, 10 or 15 μM) for 4 days, and the NF-κB p65 activity was measured. As demonstrated in Fig. 7, embelin treatment (10 or 15 μM) significantly reduced NF-κB p65 activity compared with the control group (P<0.01).

Embelin inhibits the phosphorylation of IκBα in gastric carcinoma cells. SGC7901 cells were treated with embelin (5, 10 or 15 μM) for 4 days, and the p-IκBα expression levels relative to β-actin were determined with western blotting. As demonstrated in Fig. 8, embelin treatment (10 or 15 μM) significantly suppressed p-IκBα protein expression compared with the control group (P<0.01).

Embelin inhibits the phosphorylation of the IKKα/β in gastric carcinoma cells. To investigate the anticancer therapeutic effect of embelin treatment on IKKα/β protein expression, SGC7901 cells were treated with embelin (5, 10 or 15 μM) for 4 days, and the p-IKKα/β protein expression levels relative to β-actin were determined with western blotting. As demonstrated in Fig. 9, embelin treatment (10 or 15 μM)

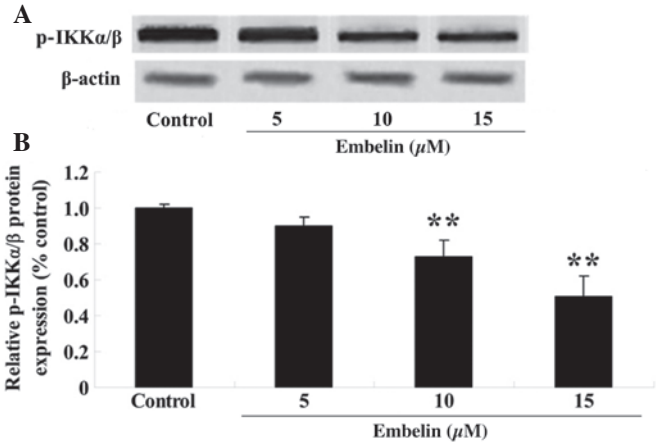

Figure 9. Effect of embelin on the phosphorylated IKKα/β protein expression levels in gastric carcinoma cells. (A) Western blotting assay and (B) quantification of immunoblot. Data represent the mean ± standard deviation. **P<0.01 vs. the control group. IKKα/β, IκB kinase α/β.

significantly reduced the relative p-IKKα/β protein expression levels compared with the control group (P<0.01).

Discussion

Malignant tumors are the second leading cause of mortality in China, and stomach cancer presents the highest rate of mortality associated with these tumors (13). China has the highest rate of gastric carcinoma worldwide (14) and the rate has risen over the past 20 years (15). In the present study, embelin treatment significantly suppressed cell proliferation, induced caspase-3 activation, and increased cell and nuclear apoptosis in the SGC7901 cells. Marsh *et al* (16) suggested that embelin suppresses the growth of pancreatic cancer in A549 non-small cell lung cancer (17) and brain glioma cells (18). Embelin is considered to be a potential drug for the treatment of carcinoma.

MAPKs are Ser/Thr protein kinases that mediate the cellular response and apoptosis, react to various cell growth and mitosis promotion factors, and transduct outer signals into cells (19). The signal transmission of the MAPK pathway is completed by the continuous phosphorylation of MAPKKK, MAPKK and MAPK (20). There are five activators of the MAPK pathways; ERK, p38, JNK, ERK3/ERK4 and ERK5 (21). Extracellular stimuli result in different biological responses depending on the MAPK pathway activated (22). In the present study, administration of embelin significantly upregulated the relative p38 MAPK protein expression in human gastric carcinoma cells. Wang *et al* (23) indicated that embelin reduced cell viability in gastric cancer cells via p38 MAPK pathway activation. Avisetti *et al* (24) indicated that embelin induced apoptosis in lung cancer cells through the activation of the p38/JNK pathway. Activation of the p38/JNK pathway may be a potential target for embelin treatment in human gastric carcinoma cells.

The occurrence and development of a tumor is a complex and multistep process that involves a series of genetic changes, including metastasis of cells deriving from the primary tumor into the blood and lymphatic vessels, through adhesion to endothelial cells, resulting in the metastasis of the tumor (25). A previous study demonstrated that the upregulation of NF-κB leads to the occurrence of tumors, and NF-κB mediates

one of the main mechanisms by which tumor cells resist apoptosis during tumor development (26). Upon activation, it produces anti-apoptotic signals to aid the development of the tumor. Royuela *et al* (27) demonstrated that NF-κB induces anti-apoptosis genes, including IAG, cl-2 like factor, TRAF1, TRAF2 and A20D. Similarly, NF-κB promotes tumor formation via a non-apoptotic pathway, activating the proto-oncogenes *c*-myc and cyclin D1 (28). Yang *et al* (28) demonstrated that cyclin D1 was the target gene of NF-κB, and that NF-κB initiated the transcription of cyclin D1, promoting the transit from phase G_1/G_0 to phase S, leading to the cell proliferation, malignant transformation and cancerization (29). In the present study, embelin significantly reduced the NF-κB activity in human gastric carcinoma cells. Ahn *et al* (30) indicated that embelin is a potential suppressor of tumorigenesis, as it suppresses NF-κB-regulated anti-apoptosis. Reuter *et al* (12) demonstrated that embelin suppresses osteoclastogenesis through the inhibition of the NF-κB cell signaling pathway. Therefore, the suppression of NF-κB may be a marker for embelin treatment in human gastric carcinoma cells.

Pathological classification of the tumor may indicate its invasiveness, thus, NF-κB p65 activity may have an effect on tumor metastasis (31). NF-κB serves a role in the activation of the immune system, cell apoptosis and inflammatory cell chemotaxis associated with gene transcription. As a gene encoding inflammatory molecules, NF-κB regulates and controls the expression of numerous inflammation-mediating genes, and NF-κB p65 activation was previously associated with cell infiltration (32,33). In the present study, a significant reduction in the relative p-IκBα and p-IKKα/β protein expression levels was observed following embelin treatment (10 or 15 μM). Park *et al* (34) indicated that the administration of embelin induced apoptosis in human glioma cells through the degradation of p-IκBα and p-IKKα/β. This may have been due to the decrease in p-IκBα and p-IKKα/β protein expression induced by embelin treatment.

In conclusion, the results of the current study suggest that embelin suppresses cell proliferation and induces levels of apoptosis in human gastric carcinoma cells through the inhibition of the NF-κB signaling pathway. These results indicate that the suppression of the NF-κB signaling pathway, due to embelin administration, is a potential therapeutic target for the treatment of gastric carcinoma.

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