BIOMOLECULES
& THERAPEUTICS

A Fermented Ginseng Extract, BST204, Inhibits Proliferation and Motility of Human Colon Cancer Cells

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Abstract

Panax ginseng CA Meyer, a herb from the Araliaceae, has traditionally been used as a medicinal plant in Asian countries. Ginseng extract fermented by ginsenoside-β-glucosidase treatment is enriched in ginsenosides such as Rh2 and Rg3. Here we show that a fermented ginseng extract, BST204, has anti-proliferative and anti-invasive effects on HT-29 human colon cancer cells. Treatment of HT-29 cells with BST204 induced cell cycle arrest at G_1 phase without progression to apoptosis. This cell cycle arrest was accompanied by up-regulation of tumor suppressor proteins, p53 and p21$^{WAF1/Cip1}$, down-regulation of the cyclin-dependent kinase/cyclins, Cdk2, cyclin E, and cyclin D1 involved in G_1 or G_1/S transition, and decrease in the phosphorylated form of retinoblastoma protein. In addition, BST204 suppressed the migration of HT-29 cells induced by 12-*O*-tetradecanoylphorbol-13-acetate, which correlated with the inhibition of metalloproteinase-9 activity and extracellular signal-regulated kinase activity. The effects of BST204 on the proliferation and the invasiveness of HT-29 cells were similar to those of Rh2. Taken together, the results suggest that fermentation of ginseng extract with ginsenoside-β-glucosidase enhanced the anti-proliferative and the anti-invasive activity against human colon cancer cells and these anti-tumor effects of BST204 might be mediated in part by enriched Rh2.

Key Words: BST204, Ginsenoside, Cell cycle, Cell migration, Cell proliferation, Colon cancer

INTRODUCTION

The root of *Panax ginseng* (Araliaceae), commonly known as ginseng, is well known as a health-enhancing adaptogenic herb and a traditional remedy in Asian countries. Ginsenosides, the major effective component in ginseng, have various pharmacological properties such as immunoenhancement, anti-stress, anti-diabetic, ergogenic, anti-oxidizing, anti-carcinogenic, and anti-cardiovascular disease effects (Attele *et al.*, 1999; Kitts *et al.*, 2000; Yun, 2003; Mehendale *et al.*, 2006). Ginsenosides are dammarane-type triterpene saponins and classified into two major structural groups, panaxadiols (Rb1, Rb2, Rc, Rd, Rg3, Rh2, and Rh3) and panaxatriols (Re, Rf, Rg1, Rg2 and Rh1) (Tachikawa *et al.*, 1999). Some components of ginsenosides are well-known and long-sought-after effector ingredients because they arrest the growth or induce apoptosis of cancer cells (Choi *et al.*, 2009; Kumar *et al.*, 2009; Liu *et al.*, 2009).

Among the ginsenosides, Rh2 has attracted considerable attention owing to its potential tumor-inhibitory activity against various cancer cells (Park *et al.*, 1997; Nakata *et al.*, 1998; Fei *et al.*, 2002; Popovich and Kitts, 2002; Choi *et al.*, 2009). In human hepatoma cell line SK-HEP-1, low concentrations of Rh2 (1 μM) arrests cell cycle at the G_1/S transition by inhibiting cyclin-dependent kinase 2 (Cdk2) activity (Lee *et al.*, 1996), whereas, at higher concentrations (12 μM), Rh2 induces apoptotic cell death through activation of caspases-3 and proteolytic cleavage of p21$^{WAF1/Cip1}$ (Jin *et al.*, 2000). Rh2-induced apoptosis in SK-HEP-1 cells is also mediated through caspases-3-dependent protein kinase C delta activity (Oh *et al.*, 2005). In human lung adenocarcinoma A549 cells, treatment of cells with Rh2 (5 μM) resulted in G_1 phase arrest, fol-

lowed by progression to apoptosis through activation of caspases-2, caspases-3 and caspases-8 (Cheng *et al.*, 2005). In addition to the suppressive effects of Rh2 on the growth of various cancer cells, it is also implicated in cancer metastasis. Recently, it has been reported that the gene expression of matrix metalloproteinase (MMP) such as MMP-1, -3, -9, and -14 is repressed by ginsenoside Rh2 in human astroglioma cells (Kim *et al.*, 2007).

Despite of the significance of the ginsenosides including Rh2 in their anti-tumor activities against broad spectrum of cancer cells, the chemical synthesis of these components is not frequently achieved due to the complicated process and limited yield (Wang *et al.*, 2006). Instead, many efforts have been made to get ginseng with higher concentrations of effector ginsenosides toward therapeutic use. Studies with steamed ginseng showed that it has higher concentration of Rh1, Rg2, Rg3, and Rh2, and, in parallel, augments the anti-proliferative effects on various cancer cell lines (Kim *et al.*, 2000; Wang *et al.*, 2006). Contents of ginsenosides from the fermented ginseng extract prepared by incubation of ginsenoside-β-glucosidase are also quite different from those of crude ginseng extract. Significantly, fermented ginseng contains high concentrations of Rh2 (5.0%) and Rg3 (10.0%), whereas crude ginseng extract contains non-detectable Rh2 and only low concentrations of Rg3 (2.5%) (Seo *et al.*, 2005a).

In the present study, we report that BST204, a fermented ginseng extract with high concentration of effective ginsenosides, arrests the cell cycle at G_0-G_1 through induction of tumor suppressor proteins, p53 and p21[WAF1/Cip1] and down-regulation of the cyclin-dependent kinase/cyclins involved in G_1 or G_1/S transition, and suppresses the motility via the inhibition of MMP-9 activity and extracellular signal-regulated kinase (ERK) activity in human colorectal cancer cell line HT-29.

MATERIALS AND METHODS

Cell culture and reagents

The human colorectal carcinoma cell line HT-29 was obtained from American Type Culture Collection (Rockville, MD) and grown at 37°C humidified atmosphere containing 5% CO_2 in McCoy's 5A (JBI) containing 10% heat-inactivated fetal bovine serum. Ginsenoside Rh2 was purchased from Chengdu Cogon Bio tech Co. Ltd. (China). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and U0126 were from Calbiochem (La Jolla, CA). Antibodies against phospho-/total forms of retinoblastoma protein (pRb), p38 mitogen-activated protein kinase (MAPK), ERK1/2, and c-Jun amino-terminal kinase (JNK) were purchased form Cell Signaling Technology (Beverley, MA). p21[WAF1/Cip1], p53, cyclin D1, cyclin E, Cdk2, poly(ADP)-ribose polymerase (PARP), caspase-3 and α-tubulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of a fermented ginseng extract, BST204

A fermented extract used in this study was described in a previous work under the name BST204 (Seo *et al*., 2005a).

Western blot analysis

Cell lysates were boiled in Laemmli sample buffer for 3 min, and then 30 μg of each protein were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked for 30 min in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder, and incubated overnight with primary antibodies. The membranes were then washed with TBS-0.1% Tween 20, incubated for 1 h with a secondary antibody, and visualized with an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences).

DNA fragmentation assay

DNA fragmentation assay was carried out as reported previsoulsy (Kwon *et al.*, 2002). HT-29 cells were treated with or without 100 μg/ml BST204 or 5 μg/ml Rh2 for 24 h then lysed in extraction buffer (50 mM Tris, pH 7.5, 10 mM EDTA and 0.3% Triton X-100). Cell lysates were treated with RNase A (100 μg/ml) for 30 min at 55°C, and then with proteinase K (400 μg/ml) for 1 h at 55°C. The supernatant was extracted with phenol/chloroform and DNA was electrophoresed on a 2% agarose gel.

Wound migration assay

HT-29 cells were seeded in a six-well plate and grown overnight to confluence. The monolayer cells were scratched with a sterile pipette tip to create a wound and replaced with serum-free McCoy's 5A medium after washing twice to remove floating cells. Cells were then treated with 10 ng/ml TPA in the presence or absence of BST204 (100 μg/ml), Rh2 (5 μg/ml) or U0126 (10 μm) for 24 h and photographed after 1 or 2 days.

Gelatin zymography

Gelatin zymography was performed to determine the enzymatic activities of MMP-2 and MMP-9 as reported previously (Maekawa *et al.*, 1999). HT-29 cells that were allowed to grow to confluence were maintained in serum-free medium for 24 h. Cells were then treated with indicated concentrations of TPA with or without BST204 (100 μg/ml) or Rh2 (5 μg/ml) in serum-free McCoy's 5A for 24 h. Conditioned media were collected, mixed with non-reducing sample buffer and subjected to electrophoresis in a 10% polyacrylamide gel containing 0.1% (wt/vol) gelatin from porcine skin. After washing with buffer containing 2.5% Triton X-100 and 50 mM Tris-HCl (pH 7.5), the gel was incubated at 37°C for 24 h with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 40 mM NaN_3. The gel was stained with 0.25% (wt/vol) Coomassie brilliant blue R-250 in 45% (vol/vol) methanol and 1% (vol/vol) acetic acid. The zones of gelatinolytic activity were detected as clear bands against a blue background and photographed on a light box.

Flow cytometry

To analyze the effect of BST204 or Rh2 on the cell cycle progression and apoptosis, the DNA content profile of HT29 cells was determined by flow cytometry as described previously (Kwon *et al.*, 2002). Cells were treated with BST204 (100 μg/ml), or Rh2 (5 μg/ml) for 24 h. After fixing the treated cells with 70% ethanol and treating with 0.25 μg/ml RNase, nuclei were stained with 50 μg/ml propidium iodide, and the relative DNA content was measured using a flow cytometer (Bio-Rad Laboratories, Hercules, CA).

RESULTS

Effects of BST204 on cell cycle distribution and apoptosis

To determine whether a fermented ginseng extract BST204 has an anti-proliferative effect on human colon cancer cells, we first examined the effect of BST204 on cell cycle progression in HT-29 cells. Cells were treated with 100 μg/ml of BST204 for 24 h and the cell cycle distribution was analyzed by flow cytometry. Treatment of cells with BST204 increased the number of cells at G1 from 51% to 69%, whereas the cells at S phase decreased from 25% to 12%, indicating that BST204 inhibits cell cycle at G_0-G_1 (Fig. 1A). The effects of Rh2 (5 μg/ml) on cell cycle distribution were similar to those of BST204. However, we could not find any evidence of apoptosis upon treatment of the cells with either BST204 or Rh2. First, the sub-G_1 population determined by flow cytometry was not affected by treatment of the BST204 or Rh2 (Fig. 1A). Secondly, caspase-3, one of the aspartate-specific cysteinyl proteases that play a pivotal role in the execution of programmed cell death (Grutter, 2000), was not converted from proenzyme form of caspases-3 (32 kDa) to catalytically active effector protease (17 kDa) by BST204 treatment. The cleavage of PARP (poly(ADP)-ribose polymerase), which is accompanied by caspase-3 activation, was not observed (Fig. 1B) as well. Finally, both BST204 and Rh2 did not induce internucleosomal DNA fragmentation (Fig. 1C). Therefore, these results indicate that BST204 has anti-proliferative effect on HT-29 human colon cancer cells without progression to apoptosis.

Effect of BST204 on the expression of cell cycle-related genes in HT-29 cells

BST204 was found to inhibit G1-S progression in the cell cycle of HT-29 cells (Fig. 1). Because changes in expression of genes essential for G_1-S progression are mostly observed
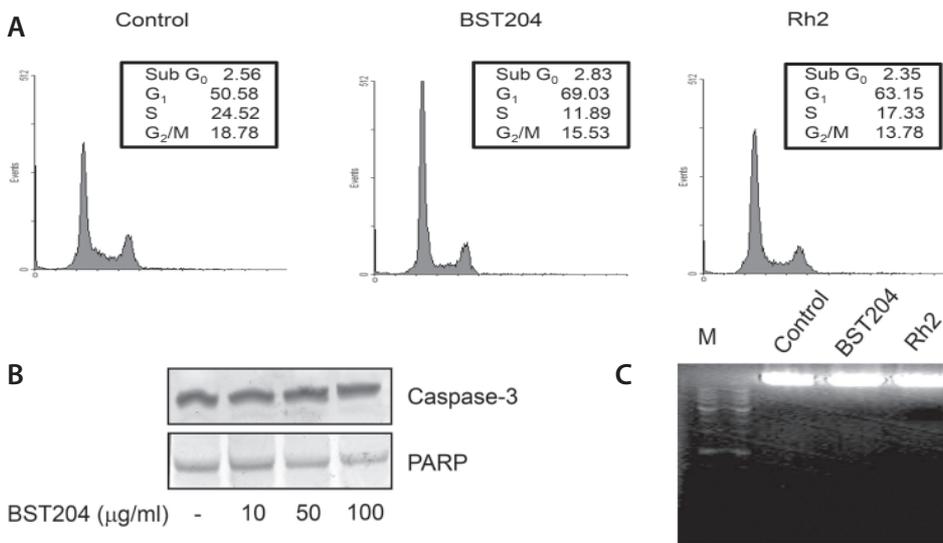

Fig. 1. Effect of BST204 on cell cycle progression of HT-29 cells. (A) HT-29 cells were treated with DMSO (0.1%), BST204 (100 μg/ml), or Rh2 (5 μg/ml) for 24 h. Cell cycle distribution was determined by flow cytometry as described in Materials and methods. The percentage of cells in sub-G_0, G_1, S, and G_2 phase is shown inside each box. (B) HT-29 cells were treated with indicated concentrations of BST204. The presence of active forms of caspase-3 or PARP was determined by Western blot analysis. (C) HT-29 cells were treated with DMSO (0.1%), BST204 (100 μg/ml), or Rh2 (5 μg/ml) for 24 h. Genomic DNA was then extracted and resolved on 2% agarose gel. Lane M indicates DNA size marker.
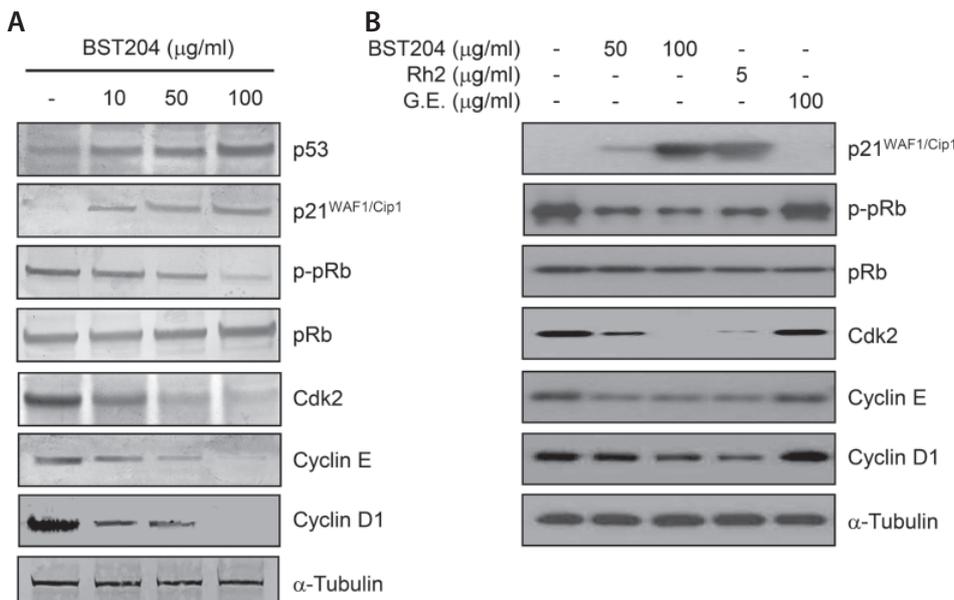

Fig. 2. Changes of endogenous gene expression by BST204 and Rh2. (A) HT-29 cells were treated with 0.1% DMSO or indicated concentrations of BST204 for 24 h. The expression of the proteins was analyzed by Western blot analysis using specific antibodies described in Materials and methods. α-tubulin was used as a control. (B) HT-29 cells were treated with 0.1% DMSO or indicated concentrations of BST204 (100 μg/ml), Rh2 (5 μg /ml), or a crude ginseng extract (G.E., 100 μg/ml) for 24 h. Proteins were analyzed as in Fig. 2A.

upon treatment of various cancer cells with anti-cancer agents that arrest cell cycle at G_1 phase (Saya, 2009), we next tried to determine the levels of p53, p21$^{WAF1/Cip1}$, Cdk2, cyclin E, and cyclin D1, as well as Rb phosphorylation by Western blot analysis. Consistent with the effect of BST204 on the cell cycle distribution, BST204 treatment significantly up-regulated the expression of tumor suppressor genes, p53 and Cdk inhibitor, p21$^{WAF1/Cip1}$, while the protein levels of Cdk2, cyclin E, and cyclin D1, the cyclin-dependent kinase/cyclins involved in G_1 or G_1/S transition, were down-regulated in a concentration-dependent manner. In parallel, Rb protein (pRb), which was hyperphosphorylated in the absence of the extract, was hypophosphorylated without loss of protein stability by treatment with BST204. The protein level of constitutively expressed cytoskeletal protein, α-tubulin, remained unchanged by increasing concentrations of BST204 (Fig. 2A). In addition, the effects of Rh2 on the expression of the genes mentioned above were generally correlated with those of BST204 (Fig. 2B). In all cases, treatment of cells with crude ginseng extract (GE) or a vehicle, DMSO, did not alter the expression level of the genes. Thus, these results indicate that the anti-proliferative activity of BST204 might be attributed to changes in expression of genes associated with G_1 arrest.

Inhibition of TPA-induced cell migration by BST204

12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a potent tumor promoter that induces a variety of cellular responses including differentiation, proliferation, and apoptosis (Wu *et al.*, 2002; Chou Chou and Hsu, 2009). In addition, invasive phenotype of cancer cells is often associated with increased expression of metalloproteinase-2 (MMP-2) and/or MMP-9, which degrade type IV collagen, the major structural collagen of the basement membrane (Ura *et al.*, 1989), and TPA up-regulates MMP-9 expression (Arnott *et al.*, 2002). Based on this notion, we next tried to determine whether a fermented ginseng extract, BST204, has the ability to inhibit TPA-induced migration of colon cancer cells using wound migration assay. Confluent HT-29 cells was scratched to create a wound and incubated with either 100 ng/ml TPA or vehicle for 48 h. Indeed, BST204 inhibited TPA-induced cell migration to the levels observed in control cells (Fig. 3A). Treatment of cells with Rh2 showed similar but moderate effect on the cell migration (Fig. 3B). We then examined the effect of BST204 on the activities of MMP-2 and MMP-9. Treatment of cells with TPA increased the activity of MMP-9 as evidenced by gelatin zymography assay. The activity of MMP-2 was only slightly increased (Fig. 3C). Importantly, BST204 as well as Rh2 were able to abrogate the MMP-9 activity induced by TPA treatment down to the background levels. This result is well correlated with the ability of BST204 or Rh2 to inhibit cell migration, indicating that this anti-invasive effect of a fermented ginseng extract, BST204, could be mediated by the inhibition of MMP-9 activity.

Inhibition of TPA-induced ERK activation by BST204

In multicellular organisms, there are three well-characterized subfamilies of mitogen-activated protein kinases (MAPKs): the extracellular signal-regulated kinases (ERKs) that function in the control of cell division; the c-Jun amino-terminal kinase (JNKs) that are critical regulators of transcription; and p38 MAPKs that are activated by inflammatory cytokinses (Johnson and Lapadat, 2002). Since several reports show that MAPK pathways are involved in the promotion of the invasive
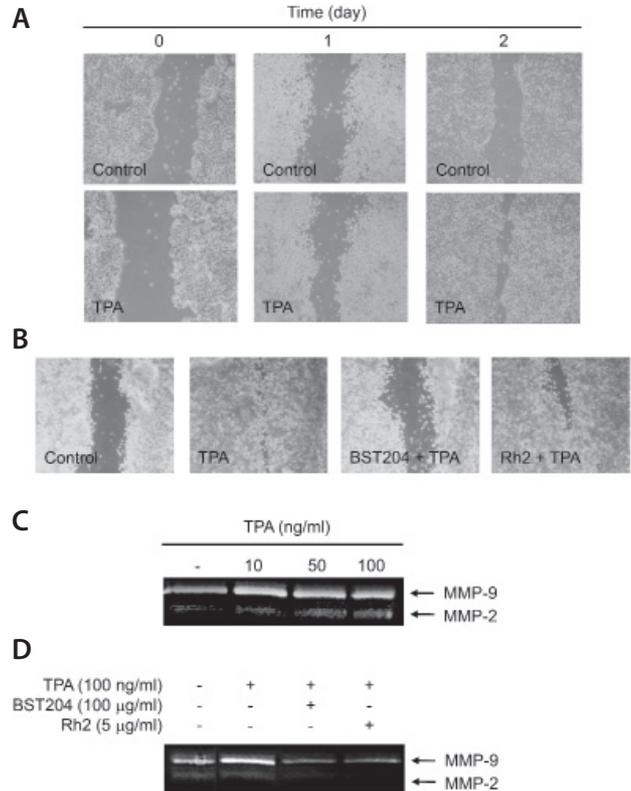

Fig. 3. BST204 inhibits TPA-induced cell migration. (A) HT-29 cells were scratched with a pipette tip and treated with or without 100 ng/ml TPA for 24 h. Migrating cells were photographed after 1 or 2 days. (B) HT-29 cells were treated with 0.1% DMSO or 100 ng/ml TPA in the presence or absence of BST204 (100 μg/ml), or Rh2 (5 μg/ml) for 24 h and photographed after 2 days. (C) HT-29 cells were treated with 0.1% DMSO or indicated concentrations of TPA for 48 h. The supernatants were analyzed zymographically for the MMP activities. (D) HT-29 cells were treated with 0.1% DMSO or 100 ng/ml TPA in the presence or absence of BST204 (100 μg/ml), or Rh2 (5 μg/ml) for 48 h. Gelatin zymography was performed as in Fig. 3C.

phenotype in many cell systems (Tanno *et al.*, 2001; Nishimoto and Nishida, 2006; Cho *et al.*, 2007; Bocca *et al.*, 2009), we set to test whether the MAPK pathways are associated with TPA-induced cell invasion and migration and BST204 may inhibit the activity of the MAPKs in HT-29 cells. Using antibodies specific for the phosphorylated forms of ERK, JNK, and p38 MAPK, we examined whether MAPKs were activated by TPA. When HT-29 cells were treated with increasing concentrations of TPA up to 100 ng/ml, significant increase in ERK phosphorylation was observed at low concentration of TPA (10 ng/ml) with no further increase at higher concentrations. The phosphorylation of JNK was only slightly increased, whereas that of p38 MAPK remained unchanged (Fig. 4A). We next tried to determine whether inhibition of ERK may lead to suppression of cell migration. Consistent with the effect of BST204 on the cell migration as shown in Fig. 3B, pretreatment of the cells with U0126, an inhibitor of the ERK pathway (Nishimoto and Nishida, 2006), led to the inhibition of TPA-induced cell migration (Fig. 4B). In parallel, BST204 significantly inhibited the phosphorylation of ERK in TPA-treated HT-29 cells (Fig. 4C), indicating that the anti-invasive effect of BST204 might be at-
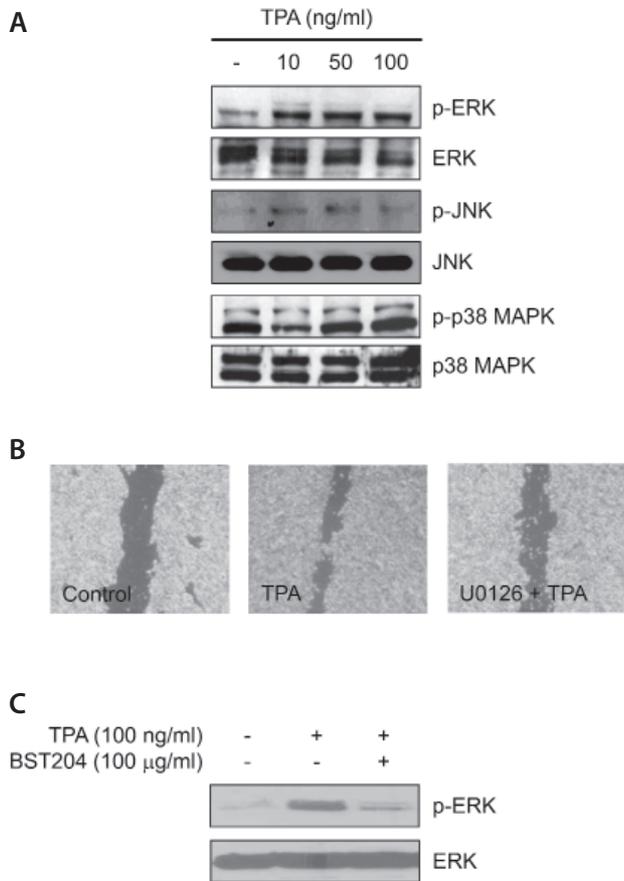
A

B

C

Fig. 4. BST204 inhibits TPA-induced ERK activation. (A) HT-29 cells were treated with 0.1% DMSO or indicated concentrations of TPA for 30 min. Phosphorylation of each MAPK was analyzed by Western blot analysis using specific antibodies described in Materials and methods. (B) HT-29 cells were treated with 0.1% DMSO or 100 ng/ml TPA in the presence or absence of U0126 (10 μm) for 48 h. Migrating cells were photographed after 2 days. (C) HT-29 cells were treated with 0.1% DMSO or 100 ng/ml TPA in the presence or absence of BST204 (100 μg/ml) for 48 h. Phosphorylation of ERK was analyzed as in Fig. 4A.

tributed to the inhibition of the ERK activity.

DISCUSSION

Cell cycle arrest at G1 phase by BST204 in HT-29 cells was accompanied by changes in the endogenous gene expression: up-regulation of tumor suppressor proteins, p53, and p21$^{WAF1/Cip1}$, and down-regulation of Cdk2, cyclin E, and cyclin D1 which mediate G_1/S transition (Fig. 2). Among these proteins, p21$^{WAF1/Cip1}$ was first identified as a Cdk binding protein, named CIP1 (Cdk-interacting protein) (Harper et al., 1993), and as the product of a gene activated by wild-type p53, known as WAF1 (wild-type p53-activated factor) (el-Deiry et al., 1993). Although p21$^{WAF1/Cip1}$ is induced through both p53-dependent and -independent pathways (Gartel and Tyner, 1999), it is likely that increased level of p53 in response to BST204 (Fig. 2A) might transactivate and induce Cdk inhibitor, p21$^{WAF1/Cip1}$, which then inhibits Cdk2 activity. Hypophosphorylated form of

the pRb holds gene regulatory proteins such as E2F in an inactive state. Upon phosphorylation of pRb catalyzed by Cdk/cyclin complex, pRb undergoes conformational changes to release and activate the target genes required for entry into S phase (Jackson and Pereira-Smith, 2006). It seems likely that induction of p21$^{WAF1/Cip1}$ by BST204 induces dephosphorylation of pRb through the inhibition of Cdk activity, leading to cell cycle arrest at G_1 phase. However, in our unpublished data, treatment of HT-29 cells with BST204 or Rh2 had no effect on the expression of another Cdk inhibitor, p15^{Ink4b}, which belongs to INK4-family (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, p19^{Ink4d}), although it is known to inhibit Cdk4/6-cyclin D-mediated phosphorylation of pRb (Mittnacht, 1998). Although high concentration of Rh2 induced apoptosis and lower concentration of Rh2 induced G1 phase arrest without progression to apoptosis in SK-HEP-1 cells, neither BST204 nor Rh2 was able to induce apoptosis in HT-29 cells. The discrepancy may be due to the activation of NF-κB by BST204 in HT-29 cells, which is essential for cell survival, because BST204 was able to activate the transcription of NF-κB reporter gene (unpublished data). Consistent with our observation, NF-κB activation has been demonstrated to contribute to resistance of cancer cells to apoptotic potential of HDAC inhibitor apicidin which has been shown to be a potent anti-proliferative agent (Han et al., 2000; Kim et al., 2006a; 2006b; Eun et al., 2007).

MMPs have a function in extracellular matrix breakdown, whose activities have been implicated in various malignant tumors including colon cancer, lung cancer, and liver cancer (Maekawa et al., 1999; Rosenblum et al., 2003). Both MMP-2 and/or MMP-9 are associated with tumor metastatic processes, and the more malignant tumors generally have the more potent MMP-2 and/or MMP-9 activity in stimulating metastasis of tumors (Bourguignon et al., 1998; Shamamian et al., 2001). In addition, different kinds of kinases of cell signal pathways such as PI3K and MAPKs are known to be involved in MMP activation and cancer cell invasion (Tanno et al., 2001; Cho et al., 2007; Bocca et al., 2009). Since BST204 showed potent ability to suppress TPA-induced cell motility as well as MMP-9 and ERK activities in HT-29 cells (Fig. 3 and Fig. 4), it is assumed that the anti-invasive effect of BST204 against human colon cancer cells is probably due to the inhibition of ERK-mediated MMP-9 activity.

Previously we reported that BST204 suppresses the level of cyclooxygenase-2 (COX-2) protein and PGE$_2$ production as well as that of inducible nitric oxide synthase (iNOS) and nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells (Seo et al., 2005a; 2005b). Particularly, ginsenosides such as Rh2 or Rg3 which are augmented by fermentation of ginseng, are thought to exert the most effective pharmacological properties of BST204, because crude ginseng extract had little effect on LPS-induced COX-2 and iNOS expression. In this study, we support the idea that the fermented ginseng extract has pharmacological effects, which is not observed in crude ginseng extract: potential anti-proliferative and anti-invasive activities against human colon cancer cells. BST204 suppressed the growth of HT-29 cells by arresting cell cycle progression at G_1 phase. In accordance with this anti-proliferative activity, the extract had effects on changes in endogenous genes expression associated with this process. In addition, TPA-promoted cell migration was significantly suppressed by BST204, which appears to be mediated by the inhibition of MMP-9 activity and ERK activity.

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In this study, we report that BST204, a fermented ginseng extract with high concentration of effective ginsenosides, enhanced the anti-proliferative and the anti-invasive activity against human colon cancer cells. The anti-proliferative effect was mediated by arrest of cell cycle at G0-G1 through induction of tumor suppressor proteins, p53 and p21$^{WAF1/Cip1}$ and down-regulation of the cyclin-dependent kinase/cyclins, and suppression of motility by BST204 was accompanied by the inhibition of MMP-9 activity and extracellular signal-regulated kinase (ERK) activity. Thus, our findings suggest that BST204 enriched in ginsenosides such as Rh2 and Rg3 may be an effective remedy for human colon cancer.

ACKNOWLEDGMENTS

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