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Original article

Induction of apoptosis by Moutan *Cortex Radicis* in human gastric cancer cells through the activation of caspases and the AMPK signaling pathway

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ABSTRACT

Moutan *Cortex Radicis*, the root bark of *Paeonia* × *suffruticosa* Andrews, Paeoniaceae, has been widely used in traditional medicine therapy. Although it has been shown to possess many pharmacological activities, the molecular mechanisms of its anti-cancer activity have not been clearly elucidated. In the present study, we investigated the pro-apoptotic effects of the ethanol extract of Moutan *Cortex Radicis* in human gastric cancer AGS cells. Moutan *Cortex Radicis* treatment inhibited the cell viability of AGS cells in a concentration-dependent manner, which was associated with apoptotic cell death. Moutan *Cortex Radicis*'s induction of apoptosis was connected with the upregulation of death receptor 4, death receptor 5, tumor necrosis factor-related apoptosis-inducing ligand, Fas ligand, and Bax, and the downregulation of Bcl-2 and Bid. Moutan *Cortex Radicis* treatment also induced the loss of mitochondrial membrane potential ($\Delta\psi m$), the proteolytic activation of caspases (-3, -8, and -9), and the degradation of poly(ADP-ribose) polymerase, an activated caspase-3 substrate protein. However, the pre-treatment of a caspase-3 inhibitor significantly attenuated Moutan *Cortex Radicis*-induced apoptosis and cell viability reduction. In addition, Moutan *Cortex Radicis* treatment effectively activated the adenosine monophosphate-activated protein kinase signaling pathway; however, a specific inhibitor of AMPK significantly reduced Moutan *Cortex Radicis*-induced apoptosis. Overall, the results suggest that the apoptotic activity of Moutan *Cortex Radicis* may be associated with a caspase-dependent cascade through the activation of both extrinsic and intrinsic signaling pathways connected with adenosine monophosphate-activated protein kinase activation, and Moutan *Cortex Radicis* as an activator of adenosine monophosphate-activated protein kinase could be a prospective application to treat human cancers.

Introduction

Apoptosis is a well-known type of programmed cell death mediated by the extrinsic and intrinsic pathways. The extrinsic pathway is activated by an interaction between death receptors (DR) and their ligands at the plasma membrane, which causes the subsequent activation of caspase-8 (Fulda and Debatin, 2006; Kantari and Walczak, 2011). The intrinsic pathway, termed the mitochondrial pathway, is initiated by the loss of mitochondrial membrane potential (MMP, $\Delta\psi m$) and the release of pro-apoptotic proteins, including cytochrome *c*, leading to the activation of caspase-9 and -3 (MacKenzie and Clark, 2008; Hensley et al., 2013). The extrinsic pathway can also cross talk with the intrinsic pathway through the caspase-8-mediated truncation of Bid, a member of the Bcl-2 family of proteins, with an ultimate amplification of the intrinsic apoptotic pathway (Lovell et al., 2008; Shamas-Din et al., 2011).

AMP-activated protein kinase (AMPK) is a metabolic-sensing protein kinase and plays a critical role as an energy-sensor in

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ATP-deprived conditions (Gwinn et al., 2008; Shackelford and Shaw, 2009; Tran et al., 2016). An increased ADP/ATP ratio leads to AMPK phosphorylation at Thr172, contributing to the activation of the ATP-generating catabolic processes, such as fatty acid oxidation and glycolysis, as well as the suppression of the ATP-utilizing anabolic pathways, including glycogen, protein, and lipid synthesis (Woods et al., 2003; Shaw et al., 2004). Recently, a variety of studies have reported that AMPK regulates cell proliferation and apoptosis via multiple signaling pathways, such as phosphorylation and the subsequent stabilization of tumor suppressor p53, the upregulation of cyclin-dependent kinase inhibitors, and the downregulation of the mammalian target of rapamycin complex-1 activity (Gwinn et al., 2008; Monteverde et al., 2015). In particular, it has been demonstrated that AMPK activation is responsible for inducing apoptosis via the hmitochondrial pathway (Nieminen et al., 2013; Fiarola et al., 2015; Chen et al., 2015). On the other hand, AMPK is also known to play a protective role in cancer cells under metabolic stressed conditions via the maintenance of energy homeostasis and modulation of autophagy, making its influence on cancer progression controversial (Laderoute et al., 2006; Rubinsztein et al., 2007; Jeon, 2016).

Moutan *Cortex Radicis*, the root bark of *Paeonia* × *suffruticosa* Andrews, Paeoniaceae, has been widely used to regulate human sickness, such as eliminating heat, promoting blood flow, and removing blood stasis in traditional medicine (Hirai et al., 1983; Poon et al., 2011). It also has been reported that the extracts of Moutan *Cortex Radicis* and its phenolic compounds exhibit antioxidant (Rho et al., 2005), hepato-protective (Park et al., 2011), neuro-protective (Kim et al., 2014), anti-inflammatory (Fu et al., 2012; Yun et al., 2013), and anti-tyrosinase effects (Peng et al., 2013). More recently, the anti-cancer activities of Moutan *Cortex Radicis* have been suggested. Extracts and several components isolated from MCR were proven to induce cell cycle arrest in the G1 phase (Gao et al., 2015) and apoptosis (Choi et al., 2012; Mukudai et al., 2013; Fan et al., 2013; Li et al., 2014; Gao et al., 2015), inhibit invasion (Wang et al., 2012), and overcome drug resistance (Cai et al., 2014) in a variety of cancer cell lines. However, the effects of Moutan *Cortex Radicis* on AMPK signaling-mediated apoptosis in cancer cells and the underlying detailed mechanisms have not been elucidated to date. In the current study, we explored the anti-cancer effects of an ethanol extract of Moutan *Cortex Radicis* (MCR) in AGS human gastric cancer cells and investigated the underlying mechanism. We found that MCR triggered caspase-dependent apoptosis through the activation of both the intrinsic and extrinsic pathways, indicating that AMPK is a critical regulator of MCR-induced apoptosis. To our knowledge, this is the first publication to suggest the involvement of AMPK in the anti-cancer activity of MCR.

Materials and methods

Reagents and antibodies

RPMI-1640 and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 4,6-diamidino-2-phenylindole (DAPI), ethidium bromide (EtBr), 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), and compound C, an inhibitor of AMPK, were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). An Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit and a mitochondrial fractionation kit were purchased from Becton Dickinson (San Jose, CA, USA) and Active Motif (Carlsbad, CA, USA), respectively. *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk), a caspase-3 inhibitor, and caspases colorimetric assay kits were obtained from CalBiochem (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. The primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc. (Beverly, MA, USA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin and an enhanced chemiluminescence (ECL) detection system were obtained from Amersham Co. (Arlington Heights, IL, USA). All other chemicals not specifically cited here were purchased from Sigma–Aldrich Chemical Co.

Preparation of MCR

To prepare MCR, the dried root bark of *Paeonia* × *suffruticosa* Andrews, Paeoniaceae (100 g), was provided from Dongeui Korean Medical Center (Busan, Republic of Korea) and pulverized into a fine powder. The powder was then extracted in 1 l of 70% ethanol by sonication for 3 h. After filtering and concentrating the extracts, the remained powder was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at a 100 mg/ml concentration and stored at 4 °C. The stock solution was diluted with medium to the desired concentration prior to use.

Cell culture

AGS human gastric cancer and WI-38 human normal lung fibroblast cells were obtained from the American Type Culture Collection (Manassas, MD, USA) and cultured in a RPMI-1640 medium containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

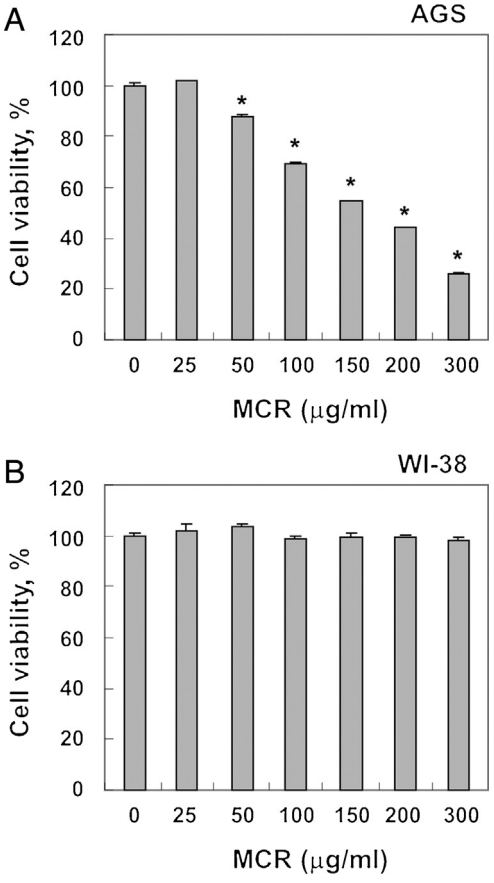

Fig. 1. Effects of Moutan *Cortex Radicis* on the cell viability in AGS and WI-38 cells. AGS (A) and WI-38 (B) cells were treated with various concentrations of MCR for 24 h. The cell viability was measured by an MTT assay. The data are expressed as the mean ± SD of three independent experiments (*$p < 0.05$ vs. untreated control).

Cell viability assay

To investigate the cell viability, AGS and WI-38 cells were seeded in a 96-well plate (2×10^3 cells/well) and stabilized for 24 h. The cells were treated with various concentrations of MCR for 24 h with or without 1 h of pre-treatment of z-DEVD-fmk or compound C. An MTT working solution (0.5 mg/ml) was then added to the media and incubated for further 3 h at 37 °C. After incubation, the culture supernatant was aspirated, and 100 μl of DMSO was added to completely dissolve the formazan crystals. The absorbance of each well was measured at a wavelength of 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular

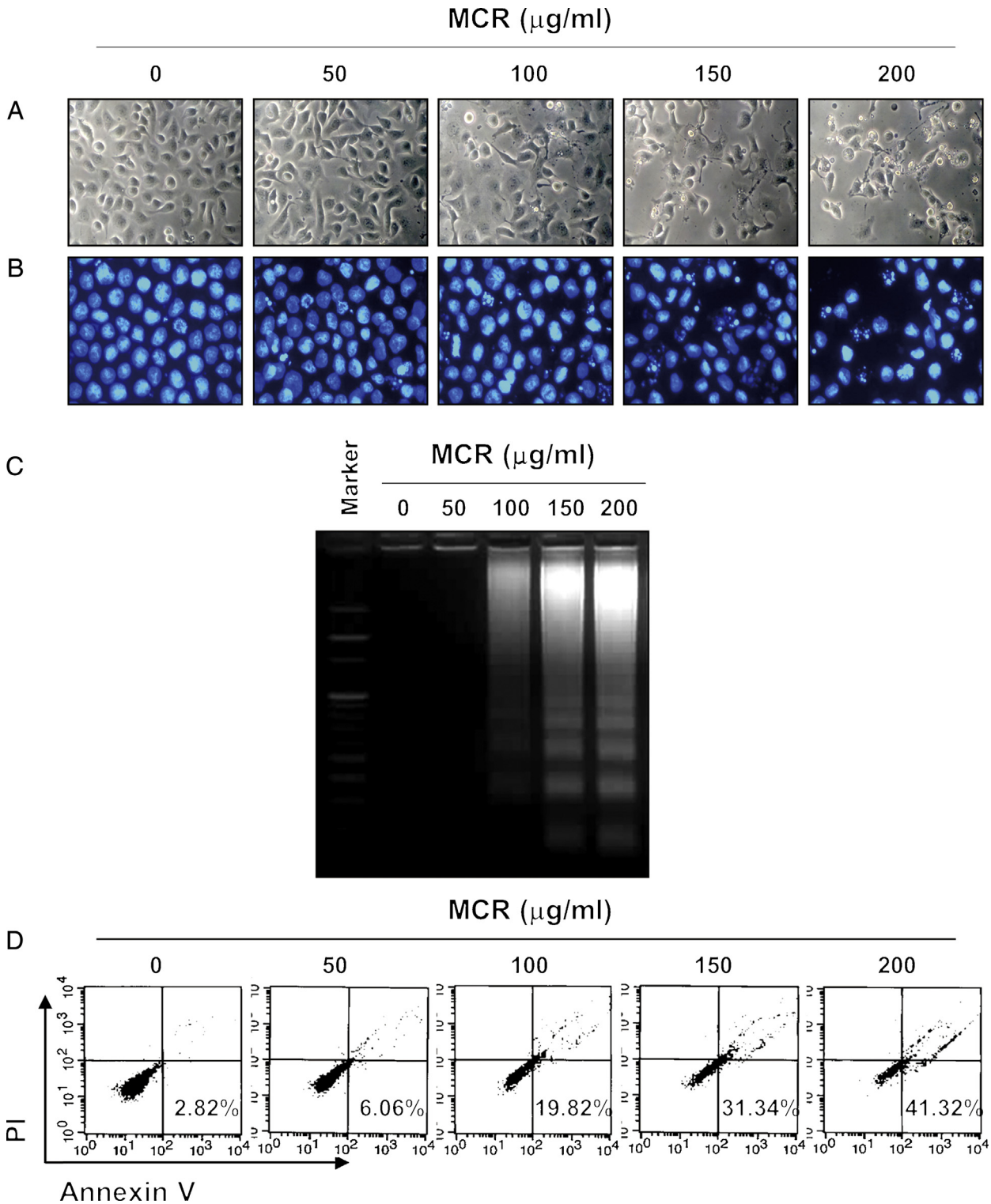

Fig. 2. Induction of apoptosis by Moutan *Cortex Radicis* in AGS cells. The cells were treated with various concentrations of MCR for 24 h. (A) Morphological changes were visualized by an inverted microscope (magnification, ×200). (B) The nuclei were stained with DAPI solution and photographed (magnification, ×400). (C) The fragmented DNA was separated on 1% agarose gel electrophoresis and visualized under UV light after staining with EtBr. (D) The percentages of apoptotic cells were determined by counting annexin V^+/PI^- cells. Data are presented the mean of the two different experiments.

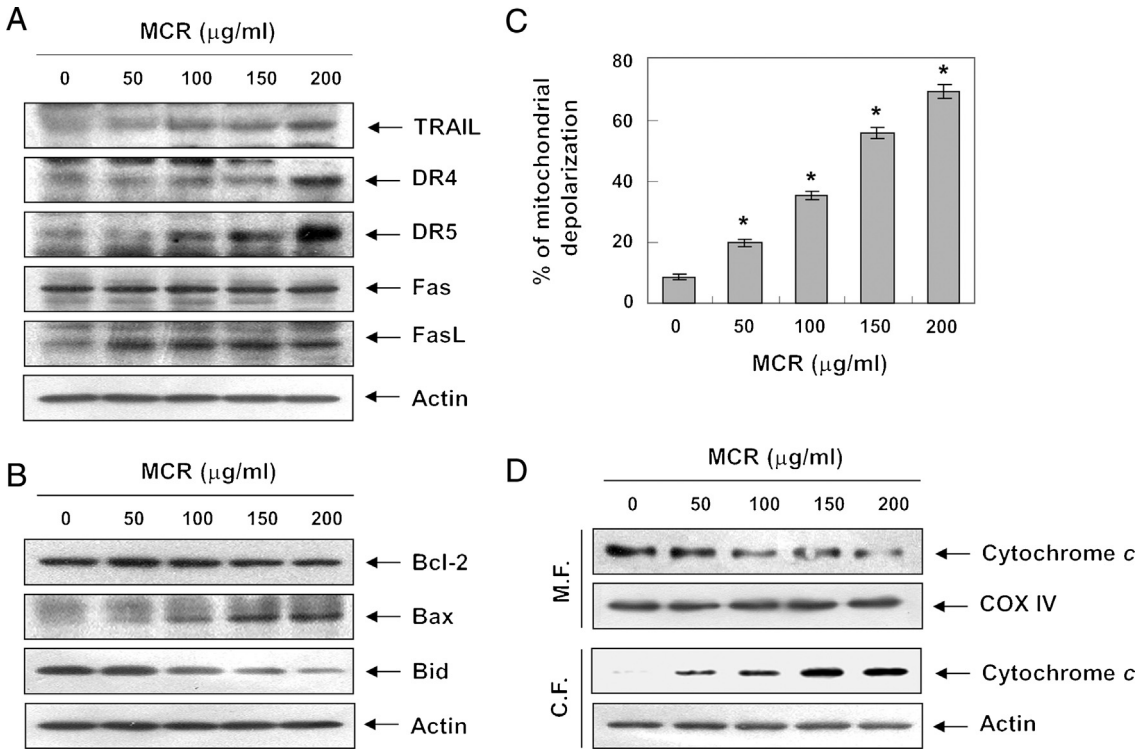

Fig. 3. The effects of Moutan *Cortex Radicis* on the expression levels of apoptosis regulators and MMP values in AGS cells. (A and B) After treatment with MCR, the cell lysates were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an ECL detection system. Actin was used as an internal control. (C) To evaluate the changes in MMP, the cells were stained with JC-1 dye and were then analyzed on a DNA flow cytometer. The data are expressed as the mean ± SD of three independent experiments (*$p < 0.05$ vs. untreated control). (D) The mitochondrial factions (M.F.) and cytosolic factions (C.F.) were separated by SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis using anti-cytochrome *c* antibody. Cytochrome *c* oxidase subunit 4 (COX IV) and actin were used as internal controls for the mitochondrial and cytosolic fractions, respectively.

Devices, Sunnyvale, CA, USA) (You et al., 2016). To assess MCR's effect on cellular morphology, the AGS cells were photographed under an inverted microscope (Carl Zeiss, Oberkochen, Germany).

Nuclear staining with DAPI

AGS cells were treated with various concentrations of MCR for 24 h with or without 1 h of pre-treatment of z-DEVD-fmk or compound C. Then, cells were harvested, washed with phosphate-buffered saline (PBS), and fixed with 3.7% paraformaldehyde for 30 min at room temperature. After they were washed twice with PBS, cells were attached on glass slides using cytospin (Shandon, Pittsburgh, PA, USA) and stained with 2.5 μg/ml of DAPI solution for 10 min at room temperature. The stained cells were washed three times with PBS and analyzed using a fluorescence microscope (Carl Zeiss).

DNA fragmentation assay

Following MCR treatment for 24 h at various concentrations with or without 1 h of pre-treatment of z-DEVD-fmk or compound C, the cells were lysed in a buffer [10 mM of Tris–HCl (pH of 7.4), 150 mM of NaCl, 5 mM of ethylendiaminetetra acetic acid (EDTA), and 0.5% Triton X-100] for 1 h at room temperature. After centrifugation at 18,300 × *g* for 30 min, the supernatant was collected and incubated with proteinase K for 3 h at 50 °C. The fragmented DNA in supernatant was purified using the same amount of neutral phenol:chloroform:isoamyl alcohol solution by rotation for 30 min at room temperature. After centrifugation, the supernatant was added with 0.5 M of NaCl (final concentration) and 1 volume of isopropanol to precipitate the fragmented DNA and incubated overnight at 4 °C. The DNA pellet obtained

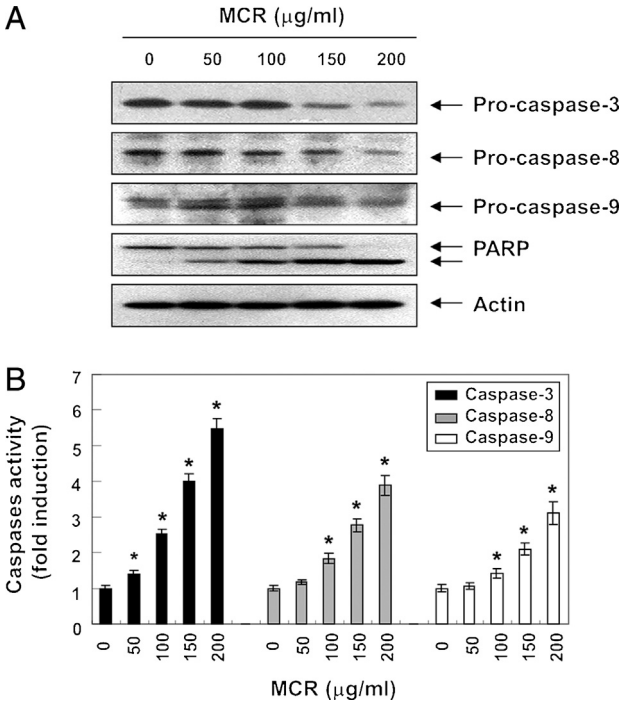

Fig. 4. The activation of caspases by Moutan *Cortex Radicis* treatment in AGS cells. (A) After treatment with MCR for 24 h, the equal amounts of cell lysates were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with the indicated antibodies against caspases and PARP. (B) The activities of caspases were evaluated using caspases colorimetric assay kits. The data are expressed as the mean ± SD of three independent experiments (*$p < 0.05$ vs. untreated control).

by centrifugation was then dissolved in TE buffer (10 mM of Tris–HCl containing 1 mM of EDTA) containing RNase A and separated through 1% agarose gel. The DNA fragmentation pattern was visualized by an ultraviolet light source following EtBr staining.

Flow cytometry analysis

Changes in the MMP were measured by flow cytometry (Becton Dickinson, San Jose, CA, USA) using JC-1, the dual-emission potential-sensitive probe. The cells treated with various concentrations of MCR for 24 h were collected, washed with PBS twice, and incubated with 10 μM of JC-1 for 20 min at 37 °C. in the dark. After centrifugation, the stained cells were washed once with PBS to remove unbound dye and resuspended with PBS. Thereafter, the amount of JC-1 retained by 10,000 cells per sample was measured using a flow cytometer. For Annexin V-propidium iodide (PI) double staining, cells were challenged with MCR for 24 h with or without 1 h of pretreatment of z-DEVD-fmk of compound C. Apoptotic cells were quantitatively identified with the Annexin V-FITC Apoptosis Detection Kit containing FITC-conjugated Annexin V and PI following the protocols provided by the manufacturer (Eom et al., 2015). The data were converted to density plots using CellQuest software for presentation.

Determination of caspase activity

The activities of caspases were determined using colorimetric assay kits containing the synthetic tetrapeptides [Asp-Glu-Val-Asp (DEAD) for caspase-3, Ile-Glu-Thr-Asp (IETD) for caspase-8, and Leu-Glu- His-Asp (LEHD) for caspase-9] labeled with *p*-nitroaniline (pNA) according to the manufacturer's instructions. Briefly, MCR-treated cells were harvested and lysed in the supplied lysis buffer. Supernatants were then collected and incubated at 37 °C with the supplied reaction buffer, dithiothreitol (DTT), and respective substrates. Reaction activities were evaluated by measuring the absorbance at 405 nm using an ELISA reader.

Western blotting analysis

To prepare whole cell lysate, cells were lysed with ice-cold lysis buffer [25 mM of Tris–Cl (pH of 7.5), 250 mM of NaCl, 5 mM of EDTA, 1% Nonidet P-40, 1 mM of phenymethylsulfonyl fluoride (PMSF), 5 mM of DTT] including a complete protease inhibitor cocktail tablet and phosphatase inhibitors (1 mM of Na_3VO_4, 100 mM of NaF, 10 mM of NaPP). In a parallel experiment, the mitochondrial and cytosolic fractions were isolated using a mitochondrial fractionation kit according to the manufacturer's instructions. After centrifugation at 15,800 × *g* at 4 °C for 30 min, the supernatants were collected, and protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Equivalent amounts of protein were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were probed with the specific primary antibodies and corresponding secondary antibodies. The protein-antibody complexes were detected by an ECL detection system according to the manufacturer's protocol.

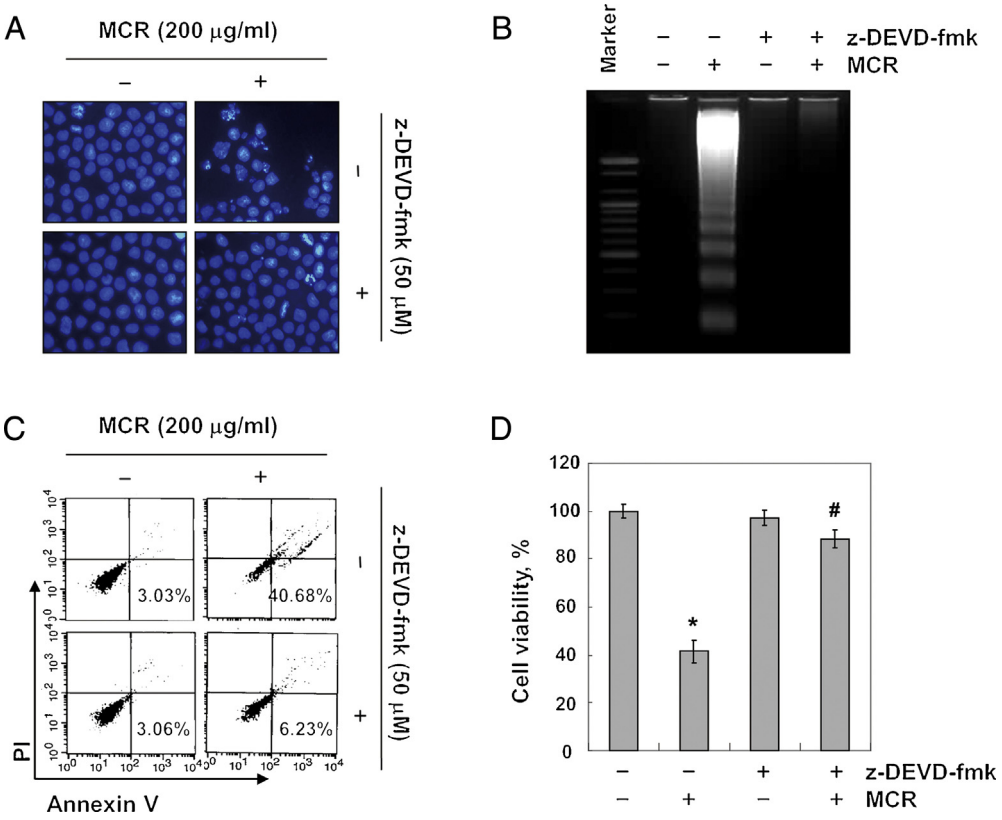

Fig. 5. The suppression of Moutan *Cortex Radicis*-induced apoptosis by the inhibition of caspase-3 in AGS cells. Cells were pre-treated with z-DEVD-fmk for 1 h before treatment with 200 μg/ml of MCR for 24 h. (A) The nuclei were stained with DAPI solution and were photographed. (B) The fragmented DNA was visualized under UV light after staining with EtBr. (C) The percentages of apoptotic cells (annexin V^+/PI^- cells) were measured using DNA flow cytometric analysis. Data are presented the mean of the two different experiments. (D) The cell viability was measured by an MTT assay. The data are expressed as the mean ± SD of three independent experiments (*$p < 0.05$ vs. untreated control; #$p < 0.05$ vs. MCR-treated cells).

Statistical analysis

Each result is expressed as the mean ± standard deviation (SD) of data obtained from independent triplicate experiments. The statistical analysis was performed using a paired Student's *t*-test. A *p*-value <0.05 was considered statistically significant.

Results

MCR suppressed cell proliferation by inducing apoptosis in AGS cells

To investigate the effects of MCR on the cell viability, AGS cells were treated with various concentrations of MCR for 24 h. As shown in Fig. 1A, MCR treatment reduced the cell viability in a concentration-dependent manner. Morphological changes, including membrane blebbing, diminished cell density, and an increased number of floating cells, were also observed (Fig. 2A). We then determined whether this MCR-mediated inhibition of cell viability was associated with the induction of apoptosis. The results of DAPI staining showed that MCR treatment significantly increased the number of condensed or blebbing nuclei, which generally appear in apoptosis before nuclear fragmentation (Fig. 2B). Consistently, DNA fragmentation was observed by MCR treatment at 100 μg/ml and gradually increased in a concentration-dependent manner (Fig. 2C). MCR also enhanced the population of annexin V^+/PI^-cells, which represents early apoptotic cells (Fig. 2D). In addition, we examined the question of whether or not MCR displayed the antiproliferative effects in human normal lung fibroblast WI-38 cells. Compared with untreated cells, decreased cell proliferation was not observed in normal lung WI-38 cells treated with MCR (Fig. 1B). These data collectively suggest that MCR suppressed cell proliferation by inducing apoptosis in AGS cells.

Both extrinsic and intrinsic apoptosis pathways were activated by MCT in AGS cells

Given that there are two classical pathways in apoptosis, the extrinsic and intrinsic pathways, we examined which pathway is involved in MCR-induced apoptosis. Our results showed that the expressions of TNF-related apoptosis-inducing ligand (TRAIL), DR4, DR5, and Fas ligand (FasL) were concentration-dependently increased by MCR treatment even though the expression of Fas was not changed, suggesting that MCR might regulate the extrinsic pathway (Fig. 3A). Additional immunoblotting data indicated that anti-apoptotic Bcl-2 expression was moderately downregulated by MCR treatment, but MCR markedly increased the expression of pro-apoptotic Bax (Fig. 3B). Accordingly, MCR treatment reduced the MMP in a concentration-dependent manner (Fig. 3C), which was consistent with the release of cytochrome *c* from the mitochondria into the cytosol (Fig. 3D), indicating that mitochondrial damage may also contribute to MCR-induced AGS cell apoptosis.

MCR induced apoptosis by activating caspases in AGS cells

The extrinsic and intrinsic signaling pathways activate caspase cascades, which is a key hallmark of apoptosis. To examine whether MCR activates caspases, we investigated the expression and activity of two initiator caspases of the extrinsic and intrinsic apoptosis pathways, caspase-8 and -9, respectively, and caspase-3, a typical effector caspase. As shown in Fig. 4A, MCR treatment apparently suppressed the expression of pro-caspase-9, -8, and -3. The subsequent increase of cleaved poly(ADP-ribose) polymerase (PARP) was also observed (Fig. 4A). Consistently, the *in vitro* activity of the caspases was significantly enhanced by MCR treatment (Fig. 4B). Although we did not observe the truncated Bid (tBid), which might

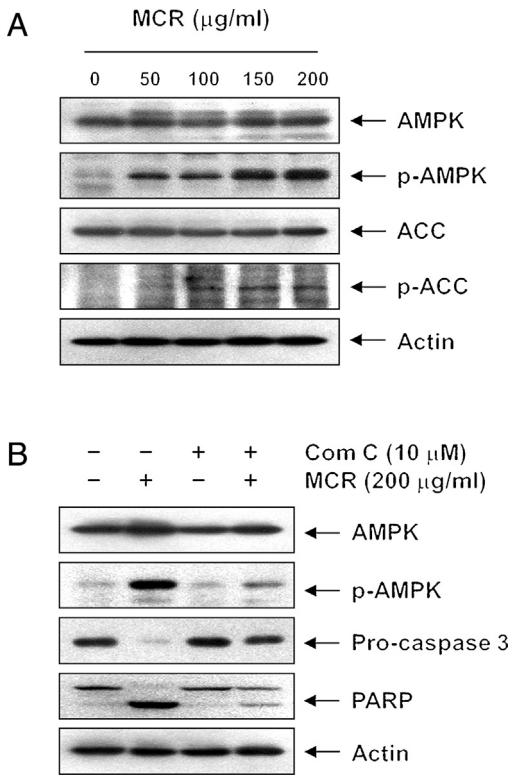

Fig. 6. AMPK activation by Moutan *Cortex Radicis* treatment in AGS cells. The cells were treated with MCR for 24 h (A) or pre-treated with compound C (Com C) for 1 h and then treated with MCR for 24 h (B). Equal amounts of cell lysate were resolved by SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with the indicated antibodies.

be involved in the regulation of the extrinsic apoptosis pathway (Lovell et al., 2008; Shamas-Din et al., 2011), MCR caused the progressive downregulation of the total Bid protein (Fig. 3B), presumably resulting from truncation by activated caspase-8. These results indicate that the cytotoxic effects induced by MCR could be mediated through DR-mediated apoptosis, thereby accentuating crosstalk between the intrinsic and extrinsic apoptosis pathways. These results are in accordance with those from Fig. 3, suggesting that MCR regulated both the extrinsic and intrinsic pathways.

To verify whether MCR-induced apoptosis is caspase-dependent, we next inhibited the activity of caspase-3 using z-DEVD-fmk. Our results clearly showed that MCR-induced apoptotic body formation and DNA fragmentation were absolutely abrogated by z-DEVD-fmk pre-treatment (Fig. 5A and B). The increased population of annexin V^+/PI^-cells and the reduced cell viability were also quite reversed by the inhibition of caspase-3 activity (Fig. 5C and D). Taken together, our results demonstrate that the activation of caspase cascades is essential for MCR-induced apoptosis in AGS cells.

MCR induced apoptosis by activating AMPK in AGS cells

Because AMPK is a representative enzyme activated under low ATP states and recognized as an emerging target of anti-cancer therapy (Hardie, 2004; Shackelford and Shaw, 2009), we assessed the activation of AMPK signaling pathway. Interestingly, AMPK and its downstream target ACC were obviously phosphorylated by MCR treatment in a concentration-dependent manner, indicating that they were converted to the activated state (Fig. 6A). Therefore, we next investigated whether AMPK is involved in MCR-induced apoptosis. As demonstrated in Fig. 6B, treatment with compound C, an inhibitor of AMPK, blocked AMPK phosphorylation and prevented

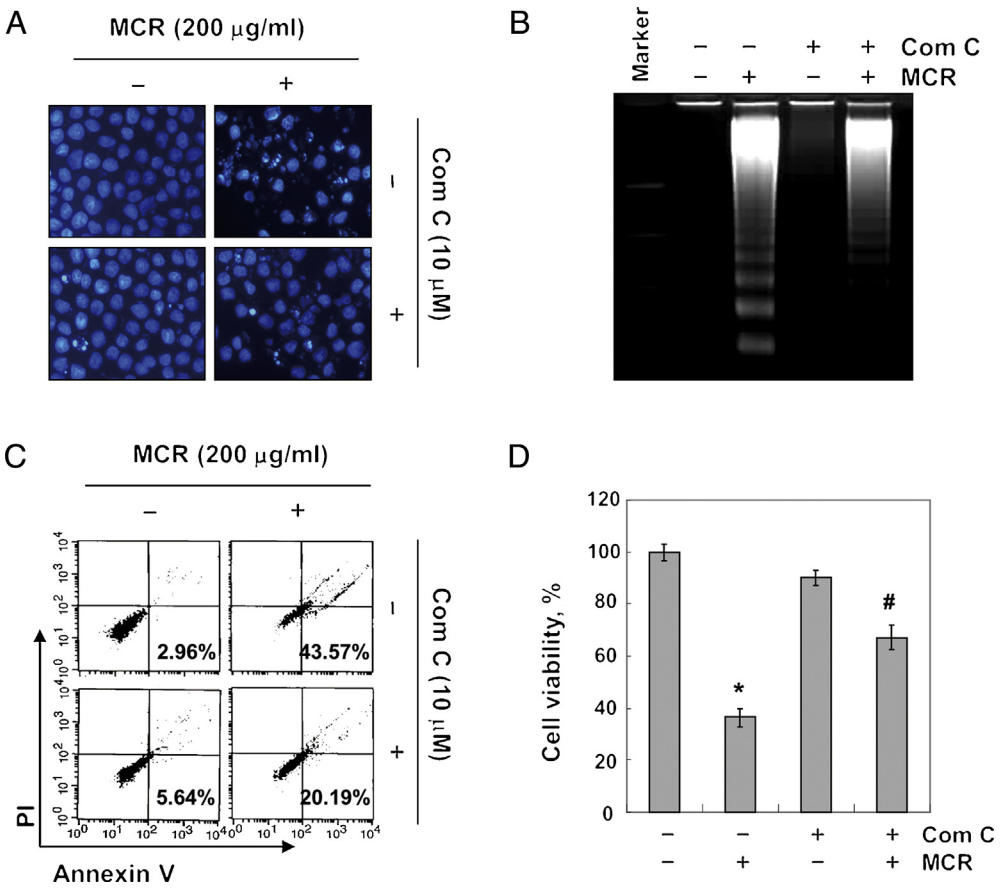

Fig. 7. The suppression of Moutan *Cortex Radicis*-induced apoptosis by the inhibition of AMPK in AGS cells. Cells were pre-treated with compound C for 1 h before treatment with MCR for 24 h. (A) The nuclei were stained with DAPI solution and were photographed. (B) The fragmented DNA was visualized under UV light after staining with EtBr. (C) The percentages of apoptotic cells (annexin V^+/PI^- cells) were measured using DNA flow cytometric analysis. Data are presented the mean of the two different experiments. (D) The cell viability was measured by an MTT assay. The data are expressed as the mean ± SD of three independent experiments ($^*p < 0.05$ vs. untreated control; $^\#p < 0.05$ vs. MCR-treated cells).

the cleavage of caspase-3 and degradation of PARP in MCR-treated cells, implying a linkage between caspase and AMPK activation. In line with these observations, the markers of apoptosis increased by MCR treatment, including the condensed and fragmented nuclei, DNA ladder, and the increased population of annexin V^+/PI^- cells, were significantly reversed by pretreatment with compound C (Fig. 7A–C). Accordingly, the MCR-induced reduction in cell viability was recovered by the addition of compound C (Fig. 7D). These observations collectively suggest that AMPK activation played a crucial role in MCR-induced apoptosis in AGS cells and that AMPK was probably upstream of caspase activation in the signaling pathway involved in this process.

Discussion

In the current study, we investigated the anti-cancer activity of MCR and explored the underlying mechanism in AGS human gastric cancer cells. Although a variety of researchers have already reported on the anti-proliferative effects of *Evodia rutaecarpa* and its components in various cancer cells, this is the first study, to our knowledge, to propose AMPK as a critical molecule mediating MCR-induced apoptosis.

Our results clearly showed that MCR induced apoptotic cell death in AGS cells. MCR treatment markedly increased the ratio of Bax/Bcl-2 expression, a critical component of the mitochondrial pathway, and subsequently triggered the loss of MMP, which was associated with the increased expression of several DR-related proteins. The mediators of mitochondria-mediated apoptosis have been determined by various preceding reports. The first candidate released from the mitochondria upon apoptotic stimuli is cytochrome *c*, an essential component of the respiratory chain (MacKenzie and Clark, 2008; Hensley et al., 2013). It forms apoptosome with apoptotic peptidase activating factor-1 (Apaf-1) and pro-caspase-9 to activate caspase-9 and the classical caspase cascade (Cory and Adams, 2002; Bröker et al., 2005). Consistently, our results showed that MCR activated caspase-9 as well as caspase-3 and enhanced the cytosolic release of cytochrome *c*, together with the pronounced activation of caspase-8 and reduction in Bid, suggesting that MCR-induced apoptosis occurred by accentuating the crosstalk between the intrinsic and extrinsic apoptosis pathways. As the other candidates released from the mitochondria to elicit apoptosis are apoptosis-inducing factor (AIF) and endonuclease G (endoG), which are particularly involved in the caspase-independent pathway (Li et al., 2001; Cregan et al., 2004). However, based on our present data indicating that z-DEVD-fmk completely blocked the apoptotic cell death induced by MCR, we suggest that AIF and endoG might only play a small role in MCR-induced apoptosis.

Various researchers have suggested that mitochondria and AMPK communicate. When the cellular ATP level drops, AMPK is activated to inhibit mTOR signaling, leading to the attenuation of protein synthesis, thereby modulating cell proliferation (Gwinn et al., 2008; Shackelford and Shaw, 2009). Because the mitochondrial electron transfer chain is the major source of ATP production, mitochondrial dysfunction can drive the reduction of ATP synthase,

which results in AMPK activation (Pan et al., 2004; Fiarola et al., 2015). In the current study, MCR treatment of AGS cells promoted the concentration-dependent phosphorylation of AMPK and of ACC, its downstream target. However, the MCR-induced phosphorylation of both AMPK and ACC was diminished markedly by an inhibitor of AMPK, compound C, suggesting that MCR could be an AMPK activator. Furthermore, the blockage of AMPK activation prevented the MCR-mediated induction of apoptosis and the reduction of cell viability, strongly suggesting that MCR activated AMPK, which led to caspase-dependent cell apoptosis in AGS cells.

Although AMPK might give cancer cells a survival advantage by conserving energy in ATP-depleted conditions (Laderoute et al., 2006; Rubinsztein et al., 2007), it has been generally considered a tumor suppressor based on the following points: first, AMPK deactivates mTOR, which is commonly activated in many cancers; second, most of the tumor suppressor genes have been identified as upstream activators of AMPK (Hardie, 2004; Feng et al., 2007); third, AMPK activators have been reported to inhibit tumorigenesis as well as tumor growth (Buzzai et al., 2007; Sauer et al., 2012; Sui et al., 2014). Notably, metformin and antroquinonol have been reported to activate AMPK to induce apoptosis by inducing mitochondrial stress (Zakikhani et al., 2006; Chiang et al., 2010). Avicin D also disrupts mitochondrial metabolism, leading to decreased ATP levels and AMPK activation, which is followed by autophagic cell death (Xu et al., 2007). These results are in accordance with our present data proposing AMPK as a key molecule to induce apoptosis in AGS cells and support the possibility that the MCR-induced loss of MMP leads to ATP depletion and the subsequent activation of AMPK. However, further studies are warranted to determine the precise upstream activator of AMPK.

In conclusion, our present results verified the anti-cancer effects of MCR in AGS human gastric cancer cells. We demonstrated that MCR induces apoptosis in AGS cells via the caspase-dependent extrinsic and intrinsic pathways by increasing DR-related regulators, MMP loss, and the release of cytochrome *c*, combined with an increase in the Bax/Bcl-2 ratio. We also suggest that AMPK also played a pivotal role in mitochondria-mediated apoptosis in response to MCR treatment. Although the active compound of MCR and the exact mechanism through which AMPK is activated should be further elucidated, our results propose MCR as a prospective clinical option to treat other cancers.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

CP, MHH, SHP and SHH contributed in running the laboratory work, analysis of the data and drafted the paper. GYK and SKM contributed to critical reading of the manuscript. WJK and YHC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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