SPANDIDOS PUBLICATIONS

Anthocyanins are novel AMPKα1 stimulators that suppress tumor growth by inhibiting mTOR phosphorylation

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Received July 6, 2010; Accepted August 26, 2010

DOI: 10.3892/or_00001007

Abstract. AMP-activated protein kinase (AMPK) has emerged as a therapeutic target of cancer. AMPK functions as an upstream regulator of proliferative signals such as mammalian target of rapamycin (mTOR), tuberous sclerosis complex (TSC), p70S6 and elongation factor-2, indicating that AMPK can be applied for the inhibition of cancer cell proliferation via modulating the proliferative signaling network. The Akt/mTOR signaling pathway is activated in colon cancer. The well known mTOR inhibitor rapamycin has a disadvantage of feedback stimulation of Akt. Anthocyanins are naturally-occurring mTOR inhibitor possessing Akt inhibitory activities. We have investigated the mTOR inhibitory effect of anthocyanins through the activation of AMPK. In this study, anthocyanins were applied to colon cancer cells and tumor-bearing xenograft models to investigate their anti-proliferative and pro-apoptotic effects, and elucidate the mechanisms that link AMP-activated protein kinase (AMPK) α1 activation to the survival signal of mTOR. Our results indicated that anthocyanins significantly decreased phospho-mTOR comparable to rapamycin, a synthetic mTOR inhibitor, and this inhibitory effect of anthocyanins on mTOR was completely abrogated by inactivating AMPKα1. Furthermore, suppression of cell growth with anthocyanins was also alleviated in the absence of noticeable AMPKα1 activities. For the first time we have found anthocyanins as novel AMPKα1 activators, and in conditions of AMPKα1 inactivation, anthocyanins lost their ability to inhibit mTOR in HT-29 colon cancer cells. The activation of AMPKα1, and the deactivation of mTOR and Akt were observed in anthocyanins-treated tumor-bearing xenograft models. The results from this study suggest that there is a complex interaction between AMPKα1 and mTOR signaling, and anthocyanins are powerful AMPKα1 activators that inhibit cancer cell growth by inhibiting mTOR phosphorylation.

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Key words: anthocyanins, AMP-activated protein kinase, mTOR, colon cancer, apoptosis

Introduction

Anthocyanins are a group of phenolic pigments responsible for the bright blue or red colors of berries, cherries and other fruits, including grapes and vegetables (1,2). A broad range of physiological properties, such as anti-oxidative, anti-inflammatory and anti-cancer activities, have been attributed to the consumption of anthocyanins present in natural food (3-6). *Vitis coignetiae Pulliat*, generally referred to as Meoru in Korea, is an anthocyanin-containing fruit that belongs to the grape family. Anthocyanins previously characterized by high performance liquid chromatography (HPLC)-MS/MS techniques from the extracts of Meoru skin include delphinidine-3,5-diglucoside and other components (7). Previous efforts to determine the mechanisms that mediate the anti-cancer effects of AIMs (anthocyanins isolated from Meoru) demonstrated that AIMs suppress colon cancer cell proliferation and induce apoptosis through the regulation of anti-apoptotic proteins, and the strong association of apoptotic potential with activation of p38 mitogen-activated protein kinase (MAPK) and the down-regulation of Akt was suggested (8). In addition, AIMs have indicated anti-invasive properties through the inhibition of nuclear factor (NF)-κB-regulated matrix metalloproteinase (MMP)-2 and MMP-9 expression in HT-29 colon cancer cells (9). Several epidemiological studies have suggested anthocyanins as effective natural chemopreventive agents. Repeated consumption of fruits and vegetables containing high levels of anthocyanins reduces the risk of tumor development in breast and colon cancers. In addition, anthocyanin intake inhibits tumor proliferation in patients with colorectal cancer (6,10). Studies have also indicated that various anthocyanins derived from fruits and vegetables are effective in the prevention or reduction of solid tumor development in animal models (11-13). Altogether, these data indicate that using dietary chemopreventive

agents, such as AIMs, might be a promising strategy for controlling colon cancer.

An emerging master controller of cancer, AMPK, has been proposed to play important roles in preventing cancer development (14-16). AMPK regulates cancer cell proliferation and apoptosis, and naturally occurring components, such as polyphenols or flavonoids, target AMPK to inhibit cell proliferation and apoptosis induction (17-19). Currently, targeting mTOR has emerged as an attractive way to control cancer with phytochemicals (20-22). mTOR promotes tumorigenesis, and mTOR complex 1 inhibition has received a great deal of attention (23,24). In this study, we focused on the effects of anthocyanins from Meoru on two central cancer regulators, AMPKα1 and mTOR. We found that AIMs strongly activated AMPKα1, and that this led to the inhibition of tumor growth through the suppression of mTOR signaling *in vitro* in HT-29 colon cancer cells and *in vivo* in a xenograft mouse model. We suggest that the modulation of AMPKα1/mTOR pathway by anthocyanins used in this study can further strengthen the use of phytochemicals for cancer control.

Materials and methods

Cell culture and reagents. The HT-29 human colon adenocarcinoma grade II cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and was cultured in RPMI-1640 with 10% fetal bovine serum (Gibco, MD, USA). Rapamycin and Compound C were purchased from Calbiochem (San Diego, CA, USA) and insulin-like growth factor-1 (IGF-1) was obtained from Sigma (St. Louis, MO, USA). Monoclonal antibodies specific for p-AMPKα_1 (Thr^{172}), AMPKα_1, p-mTOR (Ser^{2448}), mTOR and p-Akt (Ser^{473}) were purchased from Cell Signaling (Beverly, MA, USA), and ß-actin antibody was obtained from Sigma.

Isolation of anthocyanins from Meoru. Fruit of Meoru was collected in the middle of September 2007 at Jiri mountain in Korea, freeze-dried and stored in dark glass containers at -20˚C until required for analysis. Anthocyanin pigments were extracted by maceration of the fruits (100 g) in methanol containing 0.1% HCl at 5˚C for 24 h. The extraction procedure was repeated three times. After concentration under reduced pressure (Rotavapor R-124, Buchi, Switzerland), the extract was diluted with distilled water (100 ml) and partitioned against ethyl acetate (100 ml). The water layer containing the pigments was concentrated to 50 ml. The concentrate was purified according to established procedures by means of ethyl acetate/water partitioning and adsorption chromatography on a bed of Amberlite XAD-7 (Sigma, Youngin, South Korea) (9).

Cell proliferation measurements. Cells seeded on 96-well microplates at 4x10^3 cells/well were incubated with test compounds at the indicated concentrations for the indicated time periods. Following incubation with the test compound, the medium was removed, and the cells were then incubated with 100 μl MTT solution (2 mg/ml MTT in PBS) for 4 h. The samples were then solubilized in DMSO and the purple formazan dye, converted from MTT by viable cells, was quantified by absorbance at 560 nm.

Apoptosis detection. Apoptosis was measured using a FITC-Annexin V apoptosis detection kit (BD PharMingen™, San Diego, CA, USA) or Hoechst 33342 chromatin staining dye. For Annexin V/PI staining after treatment with AIMs, cells were harvested by trypsinization, washed with ice-cold phosphate-buffered saline (PBS), and suspended in a binding buffer at a density of 1x10^6 cells/ml. Cells were stained with Annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry (Becton-Dickinson Biosciences, Drive Franklin Lakes, NJ, USA). To examine chromatin condensation, cells were stained with 10 μM Hoechst 33342 for 30 min and fixed with 3.7% formaldehyde for 15 min. Changes in chromatin condensation were observed by fluorescence microscopy (Olympus Optical Co., Tokyo, Japan).

Western blot analysis. After starvation for 12 h in serum-free medium, cells were seeded into 6-well plates and treated with test compounds. Total proteins were extracted using a RIPA lysis buffer [50 mM Tris-HCl (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF] and subjected to Western blot analysis with specific antibodies. The proteins were then visualized by enhanced chemiluminescence (Intron, Kyunggi, Korea) and detected using an LAS4000 chemiluminescence detection system (Fuji, Tokyo, Japan).

Tumor formation. Five-week-old male Balb/c nu/nu mice were obtained from SLC (Tokyo, Japan) and housed in sterile filter-topped cages. HT-29 colon cancer cells (1x10^6 cells/0.1 ml) were subcutaneously injected into the left flank of the mice. One week after the injection of HT-29 cells, AIMs was dissolved in PBS and administered i.p. AIMs (50 μg/g/day) for 20 days. The control animals were injected with vehicle (PBS) alone. Tumor size was measured using a caliper at 2-day intervals, and the volume was calculated by the modified formula V= 1/2 (length x width^2). After the 20-day treatment, tumors were removed and frozen in liquid nitrogen for Western blot analysis or fixed with formalin for immunohistochemistry. All animal experiments were approved by the Ethics Committee for Animal Experimentation, Gyeongsang National University.

Immunohistochemistry. Tumor specimens from mice were fixed in 10% formaldehyde, embedded in paraffin, and sectioned into 5-μm thick slices. Sections were deparaffinized with xylene and dehydrated with 98% ethanol. Serial sections were stained using standard immunoperoxidase techniques with primary antibodies against p-Akt (1:100), p-mTOR (1:50), and p-AMPKα_1 (1:50). For epitope retrieval, specimens were microwave treated for 25 min before incubation with primary antibodies. Pre-immune serum was used as a negative control for immunostaining, and positive staining was visualized with diaminobenzidine, followed by a light counterstaining with hematoxylin. All findings were evaluated by a pathologist blinded to the treatment conditions, and samples were evaluated on the basis of stain intensity and

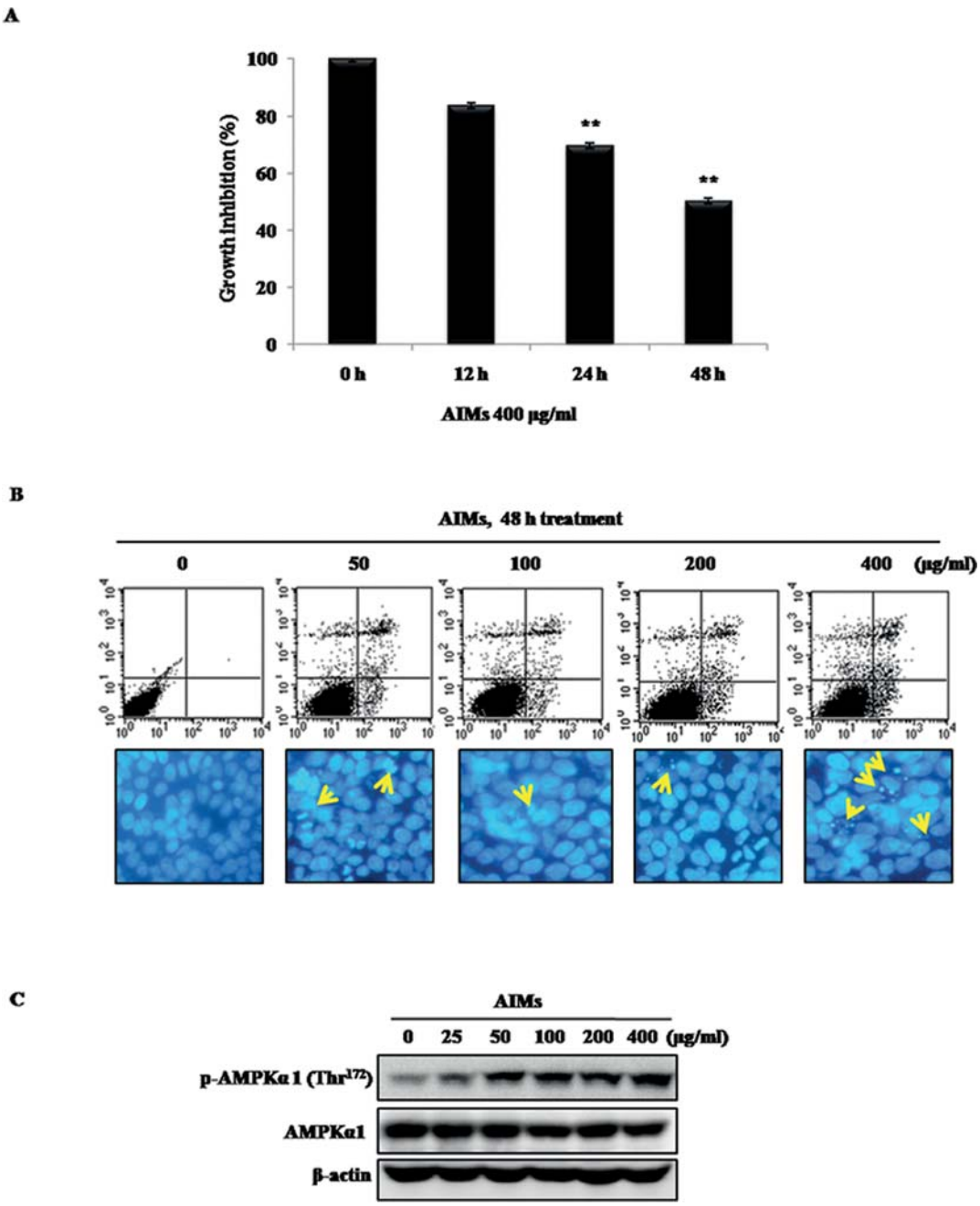

Figure 1. AIMs activate AMPKα1 and indicated apoptotic effects in HT-29 colon cancer cells. (A) Cells were treated with 400 μg/ml of AIMs for indicated times and cell viability was measured by MTT assay. **P<0.05, compared with control (0 h). (B) Cells were treated with AIMs (25-400 μg/ml) for 48 h and stained with Annexin V-FITC and PI or 10 μM Hoechst 33342, and analyzed by flow cytometry or fluorescence microscopy. (C) Cells were treated with AIMs (25-400 μg/ml) for 24 h and total proteins were subjected to Western blot analysis using phospho-AMPKα1, AMPKα1 and ß-actin (loading control) antibodies.

percentage of reactive cells. Images of representative results were recorded.

Statistical analysis. Cell viability and tumor volume data were statistically analyzed using unpaired t-test (SPSS, Chicago, USA). P<0.05 was considered statistically significant.

Results

AIMs activate AMPKα1 in HT-29 colon cancer cells. In a previous study, AIMs were reported to inhibit cell growth and induce apoptosis of HT-29 colon cancer cells. We confirmed the apoptotic effects of AIMs in HT-29 colon cancer cells. Treatment with 400 μg/ml of AIMs inhibited cell growth in a time-dependent manner (Fig. 1A). In addition, AIMs effectively increased apoptotic cell death in a dose-dependent manner (Fig. 1B). To investigate whether AIMs exerted these apoptotic effects through AMPKα1 action, we examined the effects of AIMs on AMPKα1 activation in HT-29 colon cancer cells. Cells were treated with different concentrations of AIMS for 24 h and the phosphorylation status of AMPKα1 was examined. AIMs strongly increased AMPKα1 phosphorylation without affecting total AMPKα1 levels (Fig. 1C). These results suggest that AIMs may induce

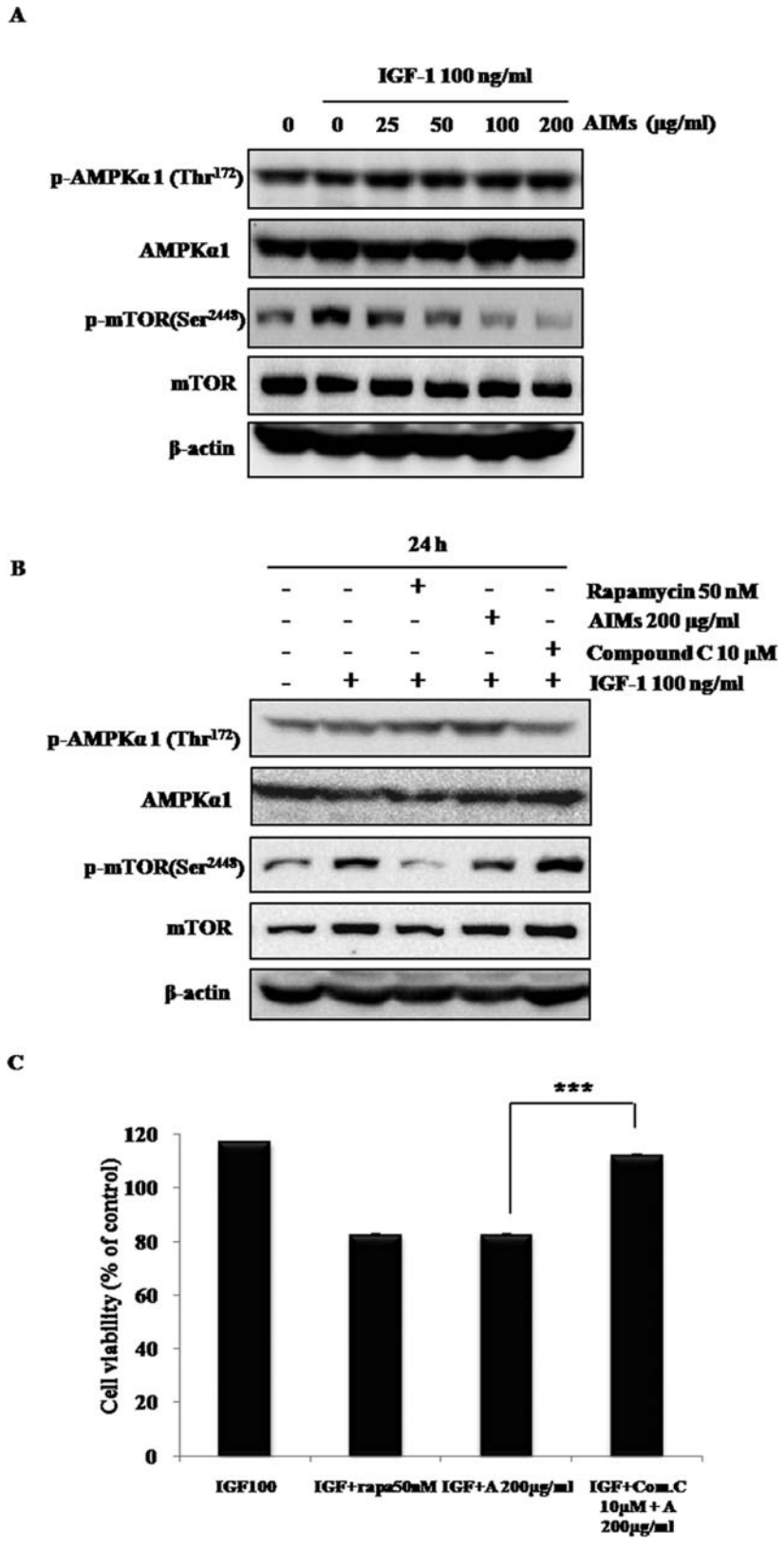

Figure 2. AIMs-activated AMPKα1 inhibits cell growth through mTOR inhibition in HT-29 colon cancer cells. (A) Serum-starved (for 12 h) HT-29 cells were pretreated with IGF-1 (100 ng/ml) for 30 min and then treated with AIMs (25-400 μg/ml) for 24 h. Total proteins were subjected to Western blot analysis using phospho-AMPKα1, AMPKα1, phospho-mTOR, mTOR and ß-actin (loading control) antibodies. (B and C) Serum-starved (12 h) cells were pretreated with IGF-1 for 30 min and treated with 50 nM of rapamycin or 200 μg/ml of AIMs for 24 h. Compound C was treated to the cells for 30 min prior to treatment of AIMs. After treatment, cells were analyzed by Western blot analysis to compare protein levels or by MTT assay to determine cell viability. ***P<0.001, IGF-1 and AIMs treated group vs. IGF-1, AIMs and Compound C treated group.

apoptosis of HT-29 colon cancer cells through the activation of AMPKα1.

AMPKα1 inhibits cell growth by suppressing mTOR signal in AIMs-treated HT-29 colon cancer cells. We next examined

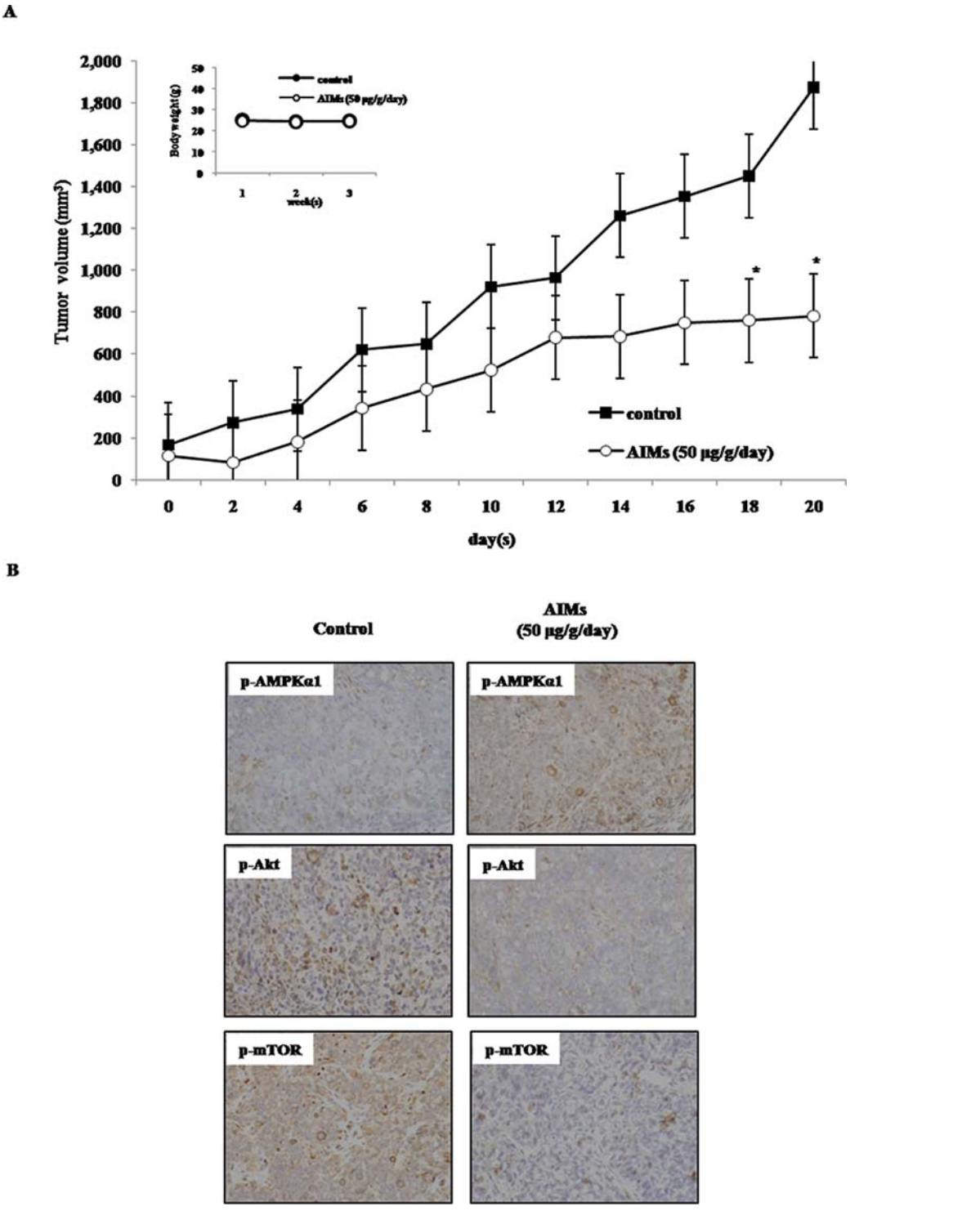

Figure 3. AIMs suppress tumor growth in xenograft models using HT-29 colon cancer cells. (A) HT-29 colon cancer cells ($1x10^6$ cells/0.1 ml) were injected subcutaneously into the left flank of Balb/c nu/nu mice (n=5 per group). After 1 week, mice were treated with AIMs (i.p., 50 μg/g/day) for 20 days. Tumor volume was measured every other day and tumor volume was calculated, as described in Materials and methods. Body weight was measured once a week. *P<0.05, compared with control tumor volume on day 18 or 20. (B) Mice were sacrificed and the levels of p-AMPKα1, p-Akt and p-mTOR were measured by immunohistochemical analysis.

whether AIMs exerted an inhibitory effect on mTOR signal. Serum-starved HT-29 colon cancer cells were treated with insulin-like growth factor (IGF-1) to induce mTOR activation prior to treatment with AIMs, and the levels of phospho-mTOR were determined. AIMs effectively reduced IGF-1-medated mTOR phosphorylation in a dose-dependent manner and activated AMPKα1 (Fig. 2A). To examine whether the inhibitory effect of AIMs on mTOR signal was dependent on AMPKα1 activation, we examined the effect of AIMs on mTOR activity in the presence of Compound C, a synthetic AMPKα1 inhibitor. Our results indicated that treatment of AIMs alone effectively activated AMPKα1 and inhibited mTOR activity, however, treatment of AIMs with Compound C could not activate AMPKα1 or suppress

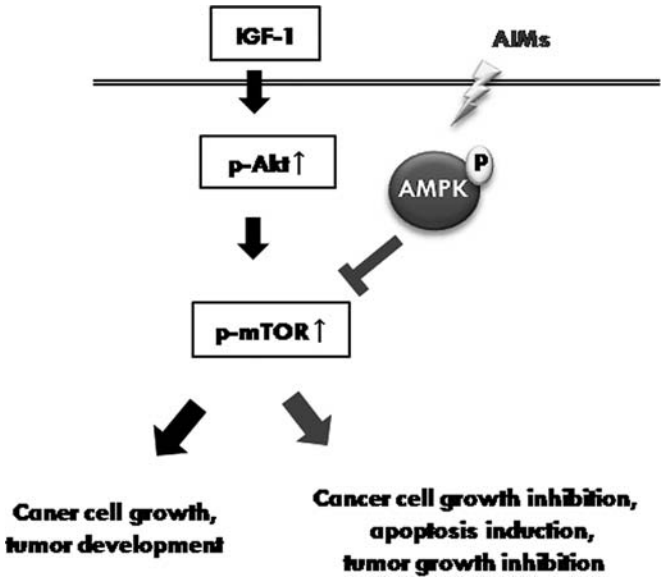

Figure 4. AIMs induced anti-cancer effects through activating AMPKα1. Activated mTOR signal by growth factors including IGF-1 stimulates cell growth and tumor development. Our study demonstrated that AIMs strongly suppressed tumor growth through AMPKα1 activation, which negatively regulates mTOR activity in HT-29 colon cancer cells and *in vivo* xenograft model.

mTOR signal (Fig. 2B). These results imply that AIMs suppress mTOR activity through AMPKα1 activation in HT-29 colon cancer cells and that AMPKα1 is a critical factor in mTOR regulation.

We next examined whether AIM-induced activation of AMPKα1 resulted in the inhibition of HT-29 cell growth, and whether this occurred through mTOR suppression. To test whether blocking mTOR activity inhibited cell growth, IGF-1 treated HT-29 cells were exposed to 50 nM of rapamycin, a synthetic inhibitor of mTOR. Rapamycin effectively reduced cell viability in the presence of IGF-1. In addition, 200 μg/ml of AIMs, which inhibited mTOR activation, effectively decreased cell viability to a level similar to that observed following rapamycin treatment. However, AIM-induced growth inhibition was abolished by treatment with 10 μM of compound C (Fig. 2C). These results suggest that AIM inhibit cell growth by suppressing mTOR activity and that AMPKα1 activity is necessary for this process.

AIMs inhibit tumor growth in a xenograft mouse model. To investigate whether AIMs also have anti-cancer activities *in vivo*, we examined the effects of AIMs on tumor growth in a xenograft mouse model using HT-29 cells. AIMs (50 μg/g/day) were administered intraperitoneally once a day for 20 days beginning 1 week after the initial injection of cells. AIMs significantly inhibited tumor growth compared to the control group, while body weights remained unchanged in all groups (Fig. 3A). In addition, we examined the levels of AMPKα1, mTOR and Akt phosphorylation in the tumors of both groups by immunohistochemical analysis. Compared to the control group, AIMs treatment strongly increased the levels of phospho-AMPKα1 and decreased the levels of phospho-mTOR and Akt, an upstream signaling component of mTOR (Fig. 3B). These results suggest that AIMs have potent anti-tumor effects both *in vitro* and *in vivo* possibly through regulating the AMPKα1-mTOR pathway (Fig. 4).

Discussion

In this study, we determined whether AIMs inhibit cancer cell growth by suppressing mTOR signaling and whether AMPKα1, an upstream regulator of mTOR, is involved in the anti-proliferative and pro-apoptotic affects of AIMs. Our results show that mTOR is a characteristic stimulator of cancer cell growth, and that increased cell growth mediated through mTOR activity is reduced by AMPKα1 activation in cancer cells or xenograft tumors treated with AIMs.

mTOR is a well-established stimulator of cell growth and tumor progression, and it is supported by the evidence that most of colon cancer patients have highly activated mTOR expression in their tumors (23,25,26). We previously reported that selenium-mediated activation of AMPKα1 inhibits the growth of colon cancer cells and xenograft tumors by suppressing mTOR signaling through Akt-dependent or independent pathways (27). In addition, several other studies have revealed that AMPKα1 is necessary for the inhibition of mTOR activity in cancer cells (28-30). In a recent report, AMPKα1 was shown to suppress mTOR signaling by direct phosphorylating raptor, a component of mTOR (31). AMPKα1 can also suppress mTOR by activating a downstream molecule in the mTOR pathway, tuberous sclerosis 2 (TSC2), which inhibits Rheb-induced mTOR activation by using its intrinsic GTPase-activating activity to keep Rheb in a GDP-bound form (32,33). Although we could not determine the exact regulatory mechanism between AMPKα1 and mTOR for the involvement of mTOR downstream signals, our observations support that AMPKα1 activated by AIMs negatively regulates mTOR signals for inhibiting growth or inducing apoptosis of colon cancer cells.

In addition, our results suggest the possibility that AMPKα1 may have molecular targets other than mTOR, since mTOR inhibition induced by AIMs was relatively weaker than that induced by rapamycin, a synthetic mTOR inhibitor. This implies that AMPKα1 may target other cell survival signals or apoptosis-related proteins.

According to previous analysis AIMs are composed of various different anthocyanins than other anthocyanins isolated from fruits or vegetables (9). AIMs are largely composed of aglycoside anthocyanins, such as delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside. There are claims that the potent anthocyanins responsible for anticancer activities include delphinidin, cyanidin and malvidin (34,35). Although the major anthocyanins exerting most of anti-cancer activities of AIMs could be delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside, the diverse and unique composition of AIMs may represent the overall anti-cancer activities observed in this study. In conclusion, we demonstrated that AIMs can stimulate AMPKα1, and that AMPKα1 activation is responsible for AIM-mediated inhibition of tumor proliferation. Tumor suppression via AMPKα1 activation thus appears to be one of the prime regulatory mechanisms by which AIMs control

cells *in vitro* as well as *in vivo* in a xenograft mouse model. Modulation of the AMPKα1/mTOR pathway by phyto-chemicals such as anthocyanins further supports the potential use of phytochemicals in controlling cancer.

Acknowledgements

This study was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. R01-2008-000-20131-0).

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