Anti-invasive activities of anthocyanins through modulation of tight junctions and suppression of matrix metalloproteinase activities in HCT-116 human colon carcinoma cells

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Abstract. Claudins are a family of proteins that are the most important components of the tight junctions. Recently it has been reported that these proteins are overexpressed in cancers and there is a positive correlation between suppression of the expression of these proteins and anti-invasive activity. Matrix metalloproteinases (MMPs) have been implicated as important mediators in cancer invasion. Here, we investigated the effects of anthocyanins on tight junctions (TJs) and the expression of claudins as well as MMPs. The inhibitory effects of the anthocyanins on cell proliferation, motility and invasiveness were found to be associated with tightening TJs, which was demonstrated by an increase in transepithelial electrical resistance (TER). The expression of claudin proteins was suppressed by anthocyanins. Furthermore, the activities of MMP-2 and -9 were dose-dependently suppressed by anthocyanin treatment. These effects were related to activation of 38-MAPK and suppression of the PI3K/Akt pathway in HCT-116 human colon cancer cells.

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Introduction

Tight junctions (TJs) represent one mode of cell-to-cell adhesion in epithelial or endothelial cells. These serve as a physical barrier to prevent solutes and water from passing freely through the paracellular space. They also contribute to maintenance of epithelial cell polarity by acting as a diffusion barrier (1). In precancerous and cancerous lesions of the epithelial origin, TJ strands become disorganized or lost altogether, and TJs become 'leaky', which can be indicated by decreased resistance to electrical current (transepithelial electrical resistance; TER) and increased paracellular permeability of markers (2).

Claudins are small [20-27 kilodalton (kDa)] transmembrane proteins that are the most important components of the TJs that can form homodimers or heterodimers to produce paired strands between adjacent cells, thereby establishing the paracellular barrier that controls the flow of molecules in the intercellular space between the cells of an epithelium. They have four transmembrane domains, with the N-terminus and the C-terminus in the cytoplasm (3).

Recently it has been reported that claudin-1, -3 and -4 are overexpressed in colorectal cancer as well as in other cancers (4-6) and the inhibition of claudin-3 and -4 reduced cancer invasive potential in ovarian cancer cells (7) although the exact functional importance of the overexpressed claudins in cancers still remains unclear.

Matrix metalloproteinases (MMPs), a family of Zn-dependent endopeptidases, are known to prossess a broad spectrum of cell surface molecules and to function in several important biological processes. MMPs are also collectively capable of cleaving virtually all extracellular matrix (ECM) substrates, and play an important role in cancer invasion. Particularly, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are involved in proteolytic digestion of the basement membrane by degrading type IV collagen. In addition, it has been reported that MMP-2 and -9 are highly expressed in colon

cancer areas compared to normal areas (8), and high expression of MMP-2 is a poor prognostic factor in colon cancer (9).

Recently many researchers have been studying the dietary agents for chemoprevention and treatment because phytochemicals or food substances are known to safely modulate physiological function and enhance anti-cancer activity. With the growth of ecological movements, natural products and herbal remedies have become more popular for the prevention or treatment of cancer. *Vitis coignetiae Pulliat* (Meoru in Korea) is a fruit that has traditionally been used in Korean folk medicine for inflammatory lesions. It has a dark red husk, which contains an abundance of anthocyanins belonging to a class of flavonoids. Recently, *in vitro* and *in vivo* anti-cancer activities of anthocyanins have been reported regarding anti-angiognesis and cancer invasion (10-12). Here, we investigated the effects anthocyanins from the fruit *Vitis coignetiae Pulliat* on tight junctions (TJs) and the expression of claudins as well as MMPs.

Materials and methods

Cell culture and MTT assay. HCT-116 human colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and 1% penicillin-streptomycin at 37˚C in a humid environment containing 5% CO_2. Anthocyanins isolated from *V. coignetiae Pulliat* (AIMs) were a generous gift from Dr S.C. Shin (Department of Chemistry, Gyeongsang National University, South Korea) (13) and 100 mg/ml concentration stock solution was made by dissolving AIMs in distilled water. The composition of the anthocyanin mixture was as follows: delphinidin-3,5-diglucoside:cyanidin-3,5-diglucoside:petunidin-3,5-diglucoside:delphinidin-3-glucoside:malvdin-3,5-diglucoside:peonidin-3,5-diglucoside:cyanidin-3-glucoside:petunidin-3-glucoside:peonidin-3-glucoside:malvidin-3-glucoside = 3.5:3.4:7.1:23.9:8.0:9.6:9.1:16.1:5.7:13.4. For the cell viability study, HCT-116 cells were grown to 70% confluence and treated with AIMs. Control cells were supplemented with complete media containing 0.1% DMSO (vehicle control). Following treatment, cell viability was determined by MTT [3-(4,5-dimethyl-2 thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide] assay (Sigma), which is based on conversion of MTT to MTT-formazan by mitochondria.

Wound healing migration assay. HCT-116 cells were grown to confluence on 30-mm cell culture dishes coated with rat tail collagen (20 μg/ml, BD Biosciences, Bedford, MA). Using a pipette tip, a scratch was made in the cell layer. After washing with PBS, serum-free media (to prevent cell proliferation) containing either vehicle (DMSO) or AIMs were added. Photographs of the wounded area were taken immediately after the scratch was made, and again 48 h later to monitor cell movement into the wounded area (14).

Measurement of transepithelial electrical resistance. Transepithelial electrical resistance (TER) was measured with an EVOM Epithelial Tissue Voltohmmeter (World Precision Instruments, FL), equipped with a pair of STX-2 chopstick electrodes. Briefly, HCT-116 cells were seeded into the 8.0 μm pore size insert (upper chamber) of a Transwell® (Corning Costar Corp., NY) and allowed to reach full confluence, after which fresh medium was replaced for further experiments. Inserts without cells, inserts with cells in medium, and inserts with cells with AIMs were treated for 48 h. Electrodes were placed at the upper and lower chambers, and resistance was measured with the voltohmmeter.

In vitro invasion assay. Matrigel invasion assays were used to assess the ability of HCT-116 cells to penetrate the extracellular matrix (ECM) in the presence or absence of AIMs. Briefly, cells were exposed to AIMs for 6 h, and treated cells (50,000) were then plated onto the apical side of the Matrigel-coated filters in serum-free medium containing either DMSO or AIMs. Medium containing 20% FBS was placed in the basolateral chamber to function as a chemoattractant. After 48 h, cells on the apical side were wiped off with a Q-tip. Cells on the bottom of the filter were stained with hematoxylin and eosin (Sigma) and counted (three fields of each triplicate filter) using an inverted microscope (15).

Protein extraction and Western blot analysis. Total cell lysates were prepared in an extraction buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM ethylendiaminetetra acetic acid, 1% nonidet P-40, 0.1 mM sodium orthovanadate, 2 μg/ml leupeptin and 100 μg/ml phenylmethylsulfonyl fluoride]. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Laboratories, Hercules, CA). For Western blot analysis, proteins (30-50 μg) were separated by 8-13% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then electrotransferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Membranes were blocked with 5% skim-milk for 1 h, and were then subjected to immunoblot analysis using the desired antibodies. Proteins were then visualized by the enhanced chemiluminescence (ECL) method, according to the recommended procedure (Amersham Co.). Primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Calbiochem (Cambridge, MA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

Gelatin zymographic analysis of secreted MMPs. Following incubation with AIMs for 48 h, cell culture supernatants were collected and centrifuged at 400 x g for 5 min. Cell-free supernatant was mixed with 2X sample buffer (Invitrogen) and zymography was performed using precast gels (10% polyacrylamide and 0.1% gelatin). Following electrophoresis, gels were washed twice at room temperature for 30 min in 2.5% Triton X-100, subsequently washed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.02% NaN_3 at pH 7.5 and incubated in this buffer at 37˚C for 24 h. Thereafter, gels were stained with 0.5% (w/v) Coomassie Brilliant Blue G-250 (Bio-Rad) for 1 h, then lightly destained in methanol:acetic acid:water (3:1:6). Clear bands appear on the Coomassie stained blue background in areas of gelatinolytic activity. Gels were scanned and images

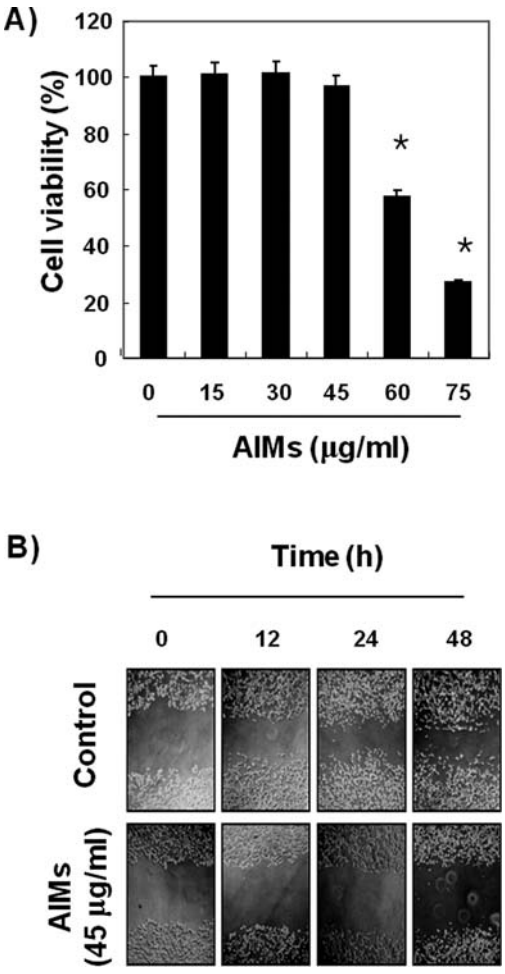

Figure 1. Inhibition of cell growth and motility of HCT-116 cells by AIMs. (A) HCT-116 cells were treated with AIMs at the indicated concentrations for 48 h, and cell viability was estimated by MTT assay. Each point represents the mean ± SD of three independent experiments. Significance was determined by the Student's t-test (*p<0.05 vs. control). (B) Cells were grown to confluency on 30-mm cell culture dishes; a scratch was then made through the cell layer using a pipette tip. After washing with PBS, serum-free media (to prevent cell proliferation) containing either vehicle or AIMs (45 μg/ml) was added for 48 h. Photographs of the wounded area were taken for evaluation of cell movement into the wounded area.

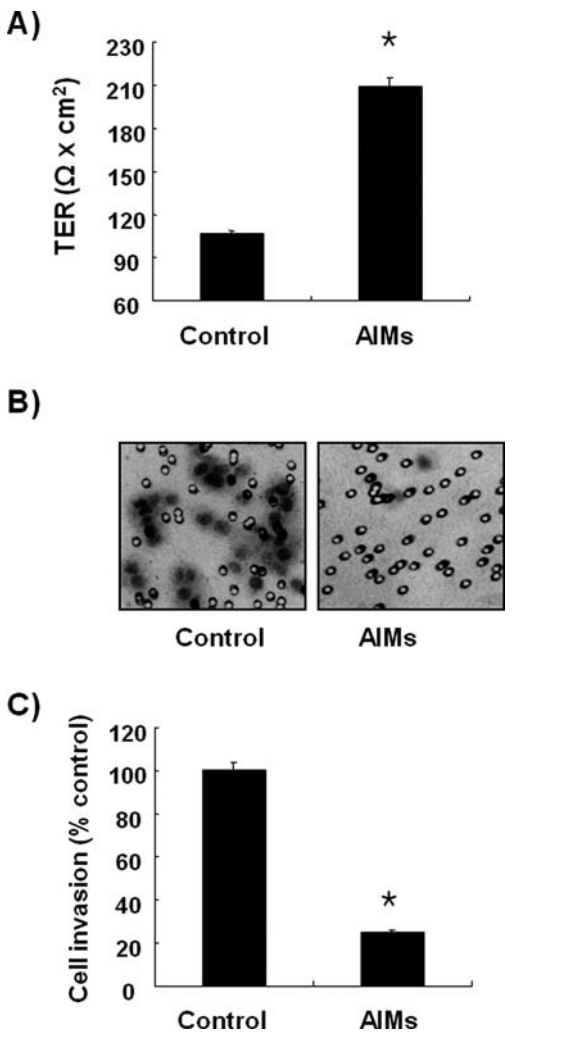

Figure 2. Increases in TER values and decreases of invasion by AIMs in HCT-116 cells. (A) Cells were treated with AIMs at a concentration of 45 μg/ml for 48 h, and TER values were measured. Results are shown as the mean ± SD of three independent experiments. Significance was determined using a Student's t-test (*p<0.05 vs. control). (B and C) Cells pretreated with 45 μg/ml of AIMs for 6 h were plated onto the apical side of matrigel coated filters in serum-free medium containing either vehicle or AIMs. Medium containing 20% FBS was placed in the basolateral chamber to act as a chemo-attractant. After 48 h, cells on the apical side were wiped off using a Q-tip. Next, cells on the bottom of the filter were stained using hematoxylin and eosin, and then counted. Data are shown as the mean of triplicate samples and represent invasive cell numbers compared with those of control cells. Significance was determined using a Student's t-test (*p<0.05 vs. control).

were processed by extraction of the blue channel signal, converting it to black and white, and inverting it for quantification of gelatinolytic activities from the integrated optical density (16).

Statistical analysis. All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's t-test. A value of $p<0.05$ was accepted as an indication of statistical significance. All of the figures shown in this report were obtained from at least three independent experiments.

Results

AIMs inhibit cell growth and migration of HCT-116 cells. To investigate the effects of AIMs on cell viability, HCT-116 cells were treated with various concentrations of AIMs for 48 h and subjected to MTT assay. Data showed that treatment with AIMs decreased the viability of HCT-116 cells in a concentration-dependent manner. When compared with control, treatment with 60 and 75 μg/ml of AIMs caused ~43 and 72% inhibition of cell growth, respectively (Fig. 1A). Wound healing experiments were performed to determine whether or not AIMs inhibit the motility of HCT-116 cells. Wound healing assay revealed that cancer cell motility was time-dependently suppressed by 45 μg/ml of AIMs, which was not cytotoxic, as shown by MTT assay (Fig. 1B).

AIMs increase TER values and decreased cell invasion in HCT-116 cells. TER values can be used as a measure for the degree of tightness of TJ (17). Here we measured the effects of AIMs on TER and cancer cell invasion of HCT-116 cells. As shown in Fig. 2A, incubation of cells with AIMs substantially increased their TER levels in a concentration-

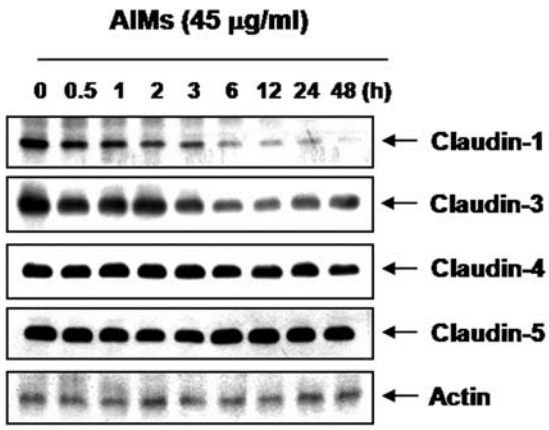

Figure 3. Effects of AIMs on the expression of claudins in HCT-116 cells. Cells were treated with 45 μg/ml of AIMs for the indicated times. Equal amounts of cell lysate were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Western blotting was then performed using the indicated antibodies and an ECL detection system. Actin was used as an internal control.

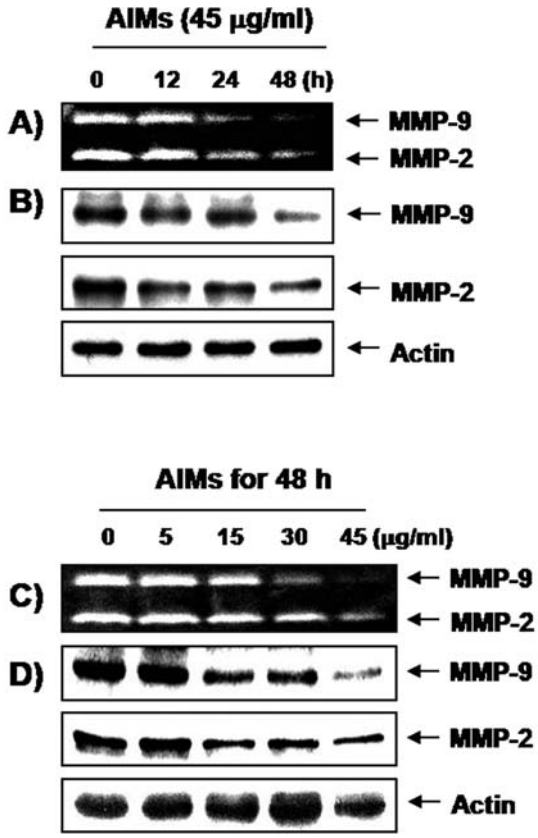

Figure 4. Effects of AIMs on MMP-2 and -9 protein expression and activities in HCT-116 cells. (A and C) After incubation with AIMs under the same conditions as those of (A) and (C), medium was collected, and the activities of MMP-2 and -9 were measured by zymography. (B and D) Cells were treated with various concentrations of AIMs for the indicated times, lysed, and proteins were separated by electrophoresis on SDS-polyacrylamide gels. Western blotting was then performed using anti-MMP-2 and anti-MMP-9 antibodies, and an ECL detection system. Actin was used as an internal control.

dependent manner, suggesting that AIMs increased TJ function in HCT-116 cells. Using a Boyden chamber invasion assay, we next examined the question of whether or not AIMs decrease the activity of cancer cell invasion. As shown in Fig. 2B and C, AIMs treatment reduced cell invasion through the Matrigel chamber in a concentration-dependent manner, suggesting that TER increase by AIMs was associated with the inhibition of HCT-116 cell invasion.

AIMs decrease the expression levels of claudins in HCT-116 cells. Recently, it has been reported that TJ proteins claudin-1, claudin-3, claudin-4 are overexpressed in colorectal cancer (8) and claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion (7). To elucidate the mechanism by which AIMs enhances TJ activity and reduces invasive activity, we determined the levels of claudins, the most important components of the TJ (18), using Western blot analyses. As shown in Fig. 3, AIMs treatment time-dependently decreased levels of claudin proteins (claudin-1, -3 and -4), suggesting that this modulation contributed to TJ tightening and up-regulation of TER by AIMs was associated with inhibition of cell invasion in HCT-116 cells.

AIMs down-regulate expression and activities of MMPs in HCT-116 cells. Cancer invasion is the initial step in metastasis for which the destruction of the basement membrane is primarily mediated by gelatinase MMP-2 and -9. Here, we tested the effects of AIMs on gelatinase activities of MMP-2 and -9 by gelatin zymography. To exclude the possibility that AIMs might inhibit the gelatinolytic activity through chemical interaction, we also measured protein expression of MMP-2 and -9 by Western blotting. As shown in Fig. 4, AIMs decreased MMP-2 and -9 activities in time- and concentration-dependent manner, which was connected with a concurrent down-regulation of their protein levels. These results suggest that the anti-invasive effect of AIMs is associated with inhibition of MMP-2 and -9 protein expression, and activity in HCT-116 cells.

Involvement of p38-MAPK and PI3K/Akt pathways. We found that AIMs showed anti-invasive effects that were related to suppression of MMP-2 and -9 as well as claudins. It has been reported that claudin-3 and claudin-4 expression may enhance invasion and is associated with increased matrix metalloproteinase-2 activity.

We previously reported that AIM-induced apoptosis is mediated at least in part through the activation of p38-MAPK and suppression of PI3K/Akt pathways in human colon cancer HCT-116 cells (19). Thus, we evaluated the possible roles of p38-MAPK and PI3k/Akt pathways in the AIMs-induced anti-invasive activity on claudin-3 as well as MMP-2 and -9, which are important molecules in the cancer invasion. As shown in Fig. 5, p38-MAPK inhibitor (SB203580) in part attenuated AIM-induced suppression of MMP-2 and -9 gelatinolytic activity, which was also connected with their protein levels. The down-regulation of claudin-3 by AIMs was attenuated by the p38-MAPK inhibitor (Fig. 5A).

PI3k/Akt inhibitor (LY294002) in part augmented the inhibitory effects of AIMs on MMP-2 and -9 gelatinolytic activity, which was also reflected by their protein levels (Fig. 6). The down-regulation of claudin-3 by AIMs was augmented by the PI3k/Akt inhibitor in HCT-116 cells

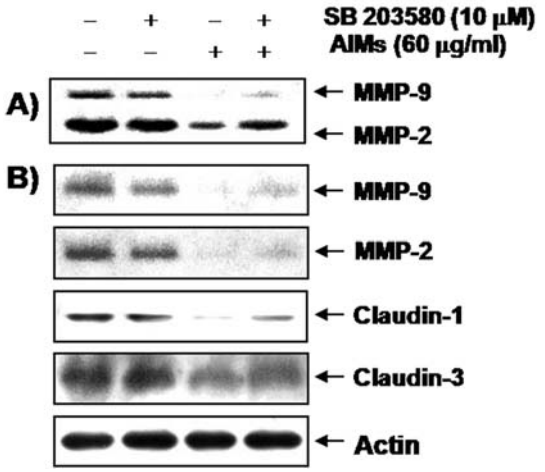

Figure 5. Effects of p38-MAPK inhibitor on the levels of MMPs activity and expression, and claudin-3 expression in HCT-116 cells. HCT-116 cells were treated with indicated concentrations of AIMs for 48 h. SB203580 (10 μM) was given 1 h before the treatment. (A) After incubation with AIMs and/or SB203580 under the same conditions as (A), medium was collected, and the activities of MMP-2 and -9 were measured by zymography. (B) Equal amounts of cell lysate were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with the indicated antibodies. Actin was used as an internal control.

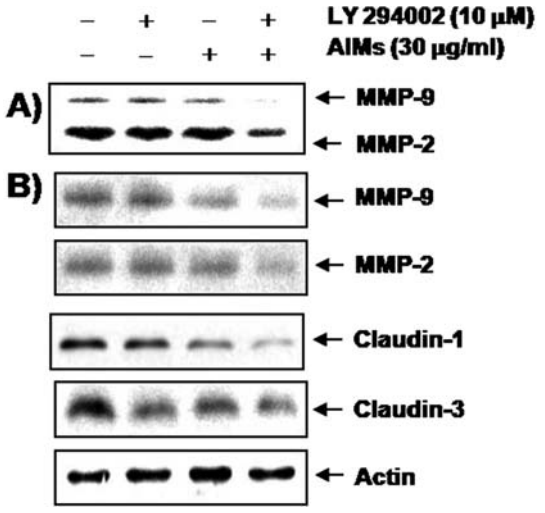

Figure 6. Effects of PI3K/Akt inhibitor on the levels of MMPs activity and expression, and claudin-3 expression in HCT-116 cells. HCT-116 cells were treated with indicated concentrations of AIMs for 48 h. LY294002 (10 μM) was given 1 h before the treatment. (A) After incubation with AIMs and/or LY294002 under the same conditions as (A), medium was collected, and the activities of MMP-2 and -9 were measured by zymography. (B) Equal amounts of cell lysate were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with the indicated antibodies. Actin was used as an internal control.

(Fig. 6A). These findings suggest that AIM-induced down-regulation of MMP-2 and -9 as well as claudin-3 is mediated at least in part by the activation of p38-MAPK and the suppression of the PI3K/Akt pathway.

Discussion

This study was designed to determine the effects of anthocyanins from the fruit *Vitis coignetiae Pulliat* on Tight junctions (TJs) and the expression of claudins as well as MMPs. We found that AIMs have anti-invasive activities through modulation of tightening TJs and inhibition of MMP-2 and MMP-9 expression in HCT-116 human colon cancer cells.

Changes in permeability properties and loss of cell polarity are hall marks of epithelial cell tumorigenesis. TJs are the structures which are crucial for maintaining these functions in cancers. Higher loss of the TJs was found in colon cancer compared to that in normal tissue. TER values can be used as a measure for the degree of tightness of TJ (17). It was reported that TER of colon cancer tissue was significantly lower that of normal tissue. Therefore, the increased TER induced by AIMs indicated tightening of TJs. This function may contribute to anti-invasive activity of AIMs. This is supported by emerging evidence that the disruption of TJs with dysregulation of TJ proteins is an early event of cancer invasion and metastasis. In addition, it was reported that claudin-1, -3 and -4 are overexpressed in colorectal cancer as well as in other cancers (4-6) and the inhibition of claudin-3 and -4 reduced cancer invasive potential in cancer cells (7). Moreover, overexpression of claudin-3 and -4 is associated with increased MMP-2 activity which is important in cancer invasion. These observations supports that the suppression of claudins by AIMs is one of the mechanisms of anti-invasive activity of AIMs.

Proteolytic digestion of the extracellular matrix (ECM) and cell migration are essential processes for cancer invasion. Previous studies demonstrated that MMP-2 and MMP-9 are key molecules in proteolytic digestion of the extracellular matrix (ECM) (20,21). Therefore, they have been targeted for preventing cancer invasion (22,23) because cancer invasion is prerequisite for cancer metastasis, the ultimate cause of death for most cancer patients. Therefore, MMP-2 and -9 are good target proteins to evaluate anti-invasive activity. Here, we found that AIMs have inhibitory effects on MMP-2 and -9 expressions. This is consistent with the previous reports using anthocyanins from another source (24,25). Similar to the observations in our study, recent studies demonstrated that an edible berry juice, which is classified within a grape family like Meoru, or a dietary anthocyanin inhibited the MMP-2 and -9 in cancer cells.

To investigate upstream signaling pathways, we examined p38-MAPK and PI3K/Akt pathways because we previously reported that AIMs activate p38-MAPK and suppressed PI3K/Akt pathways in human colon cancer HCT-116 cells (19). We found that a P38-MAPK inhibitor (SB203580) attenuated the suppression of claudin-1 and -3 by AIMs (Fig. 5). However, the P38-MAPK inhibitor did not significantly reverse the suppression of MMP-2 and -9 induced by AIMs. These findings suggest that the suppression of claudin-1 and -3 by AIMs is related with activation of P38-MAPK, but the suppression of MMP-2 and -9 may be associated with other mechanisms. This is consistent with the previous report that indicating TJs are regulated through p38 MAPK. We also found that the suppression of claudin-3 and MMP-2 and -9 by AIMs is associated with PI3K/Akt pathways. It is consistent with the finding that MMP-2 and -9 expressions are regulated through PI3K/Akt pathways. The effects of PI3K/Akt pathways were less active on claudin-1 and -3.

Although not all these results are fully understood at present, it is evident that the suppression of PI3K/Akt path-way is also another important upstream signaling pathway in the anti-invasive activity of AIMs.

Here, we used low concentration of AIMs at which AIMs did show anti-proliferative activity. Therefore, the concentration used in the current study is lower than those used in many other studies of the anti-tumor effect of anthocyanins (24-26).

AIMs are composed of a greater variety of anthocyanins than anthocyanins from other sources (25,27). The reason why Meoru has high variety of anthocyanins compared to other vegetables or fruit is not fully understood.

In conclusion, we demonstrated that AIMs tightened TJs and have anti-invasive properties in HCT-116 human colon cancer cells. These activities are mediated at least in part by suppression of cladin-3 through activation of p38-MAPK as well as by suppression of MMP-2 and -9 expressions which are probably mediated through the inhibition of PI3K/AKT pathway. This study provides evidence that AIMs might be useful phytochemicals for inhibiting invasion and metastasis of colon cancer.

Acknowledgements

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