RESEARCH ARTICLE

Morin, a Flavonoid from Moraceae, Inhibits Cancer Cell Adhesion to Endothelial Cells and EMT by Down-regulating VCAM-1 and N-cadherin

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Abstract

Morin, a flavonoid found in figs and other Moraceae species, displays a variety of biological actions, exerting anti-oxidant, anti-inflammatory and anti-carcinogenic effects. Here, we investigated the anti-cancer activity of morin focusing on anti-adhesive influence. We performed experiments with MDA-MB-231 human breast cancer cells. Morin inhibited TNF-induced cancer cell adhesion to human umbilical vein endothelial cells (HUVECs) without showing any toxicity. It further inhibited the expression of VCAM-1 on MDA-MB-231 cells as well as HUVECs. Morin also decreased the expression of N-cadherin on MDA-MB-231 cells. In addition, there was apparent anti-metastatic activity *in vivo*. In conclusion, this study suggested that morin inhibits cancer cell adhesion to HUVECs by reducing VCAM-1, and EMT by targeting N-cadherin, and that it features anti-metastatic activity *in vivo*. Further investigation of possible anti-metastatic activity of morin against human breast cancer cells is warranted.

Keywords: Breast cancer cells - morin - metastasis - adhesion - VCAM-1 - N-cadherinn - downregulation

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Introduction

With advances in medical science, the population of elderly cancer patients has been increased. This population has a lack of tolerance to conventional cytotoxic or multi-targeted chemotherapeutic agents. In addition, modern chemotherapy strategy is emphasizing quality of life. Therefore, much interest has been drawn to the possibility of controlling cancer with minimal toxicity using dietary agents from vegetable or fruit because the dietary agents can safely enhance anti-cancer effects (Liu et al., 2007; Kundu and Chun, 2014; Lu et al., 2014; Prasad et al., 2014).

Morin (3,5,7,2',4'-pentahydroxyflavone) is abundant in figs which are used as herbal medicines. It has certain biological activities, including anti-oxidant properties and anti-inflammatory effects (Stavric, 1994). Mulberry trees are widely cultivated in East Asia and its twigs exert significant antioxidative effects; from the extracts of the twigs, five phenolic constituents were identified: maclurin, rutin, isoquercitrin, resveratrol and morin. Among them, maclurin and morin have shown to have superior anti-oxidant activity to the others (Chang et al., 2011). Recently, morin has been reported to exert anti-cancer activity (Jin et al., 2014).

Most of cancer patients eventually die of cancer metastasis. Therefore, inhibition of the metastatic activity is one key to prolong the survival of cancer patients. Metastasis consists of a series of complicated sequential steps. Initially, cancer cells detach from the primary cancer lesion, then invade the basal membrane, and move to other organs though lymphatic or blood vessels. Before making metastatic foci, the cancer cells moving though lymphatic or blood vessels temporarily adhere to endothelial cells, and then extravasate. The ability for cancer cells to adhere to the endothelial cells closely correlates with the capacity for cancer cells to form metastatic lesions (Nicolson, 1988; Orr et al., 2000).

Cell adhesion molecules (CAMs) involve a broad range of normal physiological processes, and a variety of pathologies, especially cancer (Zhang et al., 2012) (Okegawa et al., 2004). Most of CAMs belong to four

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protein families: Ig (immunoglobulin) superfamily (IgSF CAMs), the integrins, the cadherins, and the selectins (Su et al., 2014). Among IgSF CAMs, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) are well known endothelial surface adhesion molecules involved in cancer cells (Orr et al., 2000). Epithelial-mesenchymal transition (EMT) is a hypothesized process that cancer cells acquire invasive properties

The EMT is reported to be involved in cancer cell metastasis and dissemination [2] Therefore, it is important to discover that morin has inhibitory effects on cancer adhesion and EMT. Here, we investigated the anti-cancer activity of morin on ability for cancer cells to adhere to the endothelial cell and EMT with elucidating its mechanism regarding the expression of VCAM-1, ICAM-1, and N-cadherin.

Materials and Methods

Cells and reagents

Morin was obtained from Aging Tissue Bank (Pusan, Korea). Human umbilical vein endothelial cells (HUVECs, EA.hy 926 cells) were obtained from ATCC and grown in medium 199 supplemented with 20% FBS, 2 mM L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, 10 μg/ml streptomycin and 50 μg/ml EC growth supplements. Cells were cultured on 100 mm dishes in a humidified 5% CO_2 incubator. HUVECs were used between passage number 3 and 6. Human breast cancer cell line, MDA-MB-231, were obtained from the Korea Cell Line Bank (Seoul, Korea) and grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 25 mM $NaHCO_3$, 100 IU/ml penicillin and 10 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The antibody against β-actin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against ICAM-1, VCAM-1, N-cadherin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human tumor necrosis factor (TNF) was purchased from R&D system (Minneapolis, MN, USA).

Adhesion assay.

HUVECs were seeded into 6-well plate and treated with the indicated reagents as a previous study (Youn et al., 2013). Briefly, HUVECs were washed with serum-free medium, and MDA-MB-231 cells (7.5 X 10^5 cells/ml) was grown in RPMI 1640 medium were applied onto HUVECs at 37°C. After 30 min, cell suspensions were withdrawn, and the HUVECs were gently washed with PBS. MDA-MB-231 cells adhered to HUVECs were counted under the light microscope. Analyses were repeated three times, over the same region, and the results are expressed and the mean values of the three independent experiments.

Western blot analysis

The cells were washed in ice-cold PBS and lysed in PRO-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea). The samples were centrifugated at 13,000 rpm, for 15 min at 4°C. An aliquot of the whole cell lysate was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Membranes, blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 for 2 h at room temperature, were incubated with anti-ICAM-1, VCAM-1, N-cadherin antibodies at 1:1,000 in TBS containing 0.05% Tween-20 and 3% bovine serum albumin (BSA) for overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000) antibody for 1 h at room temperature. After washing, the membranes were developed using the ECL reagent (Bionote, Gyeonggi-do, Korea)

Animal experiments

Athymic nude mice were divided into 3 groups (5 mice/group) and received morin at the dose of 10 mg/kg (daily, i.p.) after 7 days after cell injection. MDA-MB-231 cells ($3x10^6$ cells/100 μl of serum-free RPMI) were injected into the tail vein of the mice. After four weeks of morin treatment, lung tissues were excised. Lung metastasis was quantified by counting metastatic lesions in each section (10 sections per lung). Body weights were measured every 3 days, starting at 7 days after tumor cell injection. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Gyeongsang National University (GLA-120208-M004).

Statistical Analysis

Each experiment was performed in triplicate. The results were expressed as means ± SD. Significant differences were determined using the one-way analysis of variance (ANOVA) with post-test Neuman-Keuls for more than two groups and Student's t test for two group. Statistical significance was defined as $P<0.05$.

Results

Morin inhibited TNF-induced cancer cell adhesion to human umbilical vein endothelial cells (HUVECs)

We previously assessed anti-proliferative effects of morin on MDA-MB-231 cells. MTT test revealed that the growth of MDA-MB-231 cells was not inhibited by 48-hr-morin treatment at the concentration of 200 μM (Jin et al., 2014). Next, we performed adhesion assay to test the inhibitory effects on cancer cell adhesion to endothelial cells at the concentrations (10- 200 μM) where morin did not show anti-proliferative effects. The adhesion assay revealed that morin significantly inhibited TNF-induced cancer cell adhesion to HUVECs from the low dose of 10 μg/ml (Figure 1A and B).

Morin inhibited the expression of VCAM-1 and ICAM-1of MDA-MB-231 cells, and on that of VCAM-1 of HUVECs

We then assessed the effects of morin on the expression of VCAM-1 and ICAM-1 to further investigate this finding at the molecular level. Western blot analysis revealed that morin significantly inhibited VCAM-1 and ICAM-1 expression of TNF-treated MDA-MB-231 cells (Figure 2A). Interestingly, morin also suppressed VCAM-1

expression of TNF-treated HUVECs, but not ICAM-1 expression.

Morin inhibited the expression of N-cadehrin of MDA-MB-231 cells

Next, we also assessed the changes in the expression of N-cadehrin of MDA-MB-231 cells by morin treatment to assess the anti-EMT effects of morin on MDA-MB-231 cells. Western blot analysis revealed that morin inhibit N-cadehrin expression in a dose-dependent manner (Figure 3A and 3B). These findings suggest that morin may inhibit TNF-induced EMT of MDA-231 cells by suppressing N-cadherin.

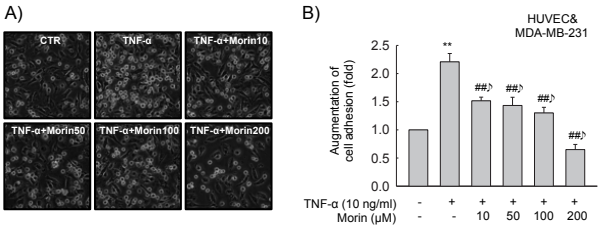

Figure 1. Inhibitory Effects of Morin on Cancer Cell Adhesion to HUVECs. MDA-MB-231 Cells were Seeded at the Density of 5 x 10^4 Cells Per ml. The cells were treated with indicated concentrations of morin for 24 hours. (A) Cells were stimulated with TNF (10 ng/ml) for 6 h. HUVECs were washed with serum-free medium, and MDA-MB-231 cells were added onto HUVECs and incubated for 30 min at 37°C. Cells were gently washed and adhered MDA-MB-231 cells to HUVECs were determined with light microscope (Magnification, X 200) and quantified. (B) Values are the means ± S.E. from three independent determinations. **p<0.01 compared with vehicle-treated group; ##p<0.01 compared with TNF treated group

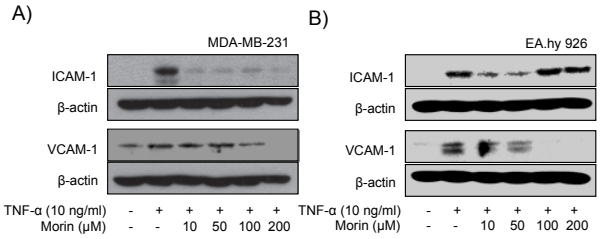

Figure 2. Inhibitory Effects of Morin on the Expression of VCAM-1 and ICAM-1 1of MDA-MB-231 Cells, and on that of VCAM-1 of HUVECs. (A and B) MDA-MB-231 were starved for 16 h, and then pretreated with morin at the indicated concentrations. (A) After 24 h treatment, proteins were extracted from the cells, and ICAM-1 and VCAM-1 protein expression levels were assessed by western blot analysis and quantified by densitometer. (B) EA.hy926 cells were then stimulated with TNF (10 ng/ml) for 6 h. After treatments, proteins were extracted from the cells, and ICAM-1, and VCAM-1 protein expression levels were assessed by western blot analysis.

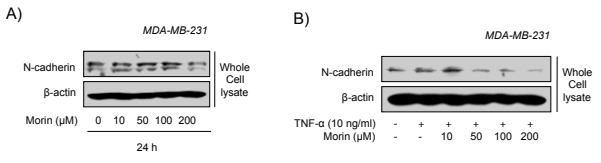

Figure 3. Inhibitory Effects of Morin on the Expression of N-cadherin of MDA-MB-231 Breast Cancer Cells. (A) MDA-MB-231 were starved for 16 h, and then pretreated with morin at the indicated concentrations for 24 h. (B), MDA-MB-231 were starved for 16 h and then pretreated with morin at the indicated concentration for 24 h. Cells were then stimulated with TNF (10 ng/ml) for 6 h. After treatments, proteins were extracted from the cells for N-cadherin. Protein levels were determined by western blot analysis and quantified by densitometer

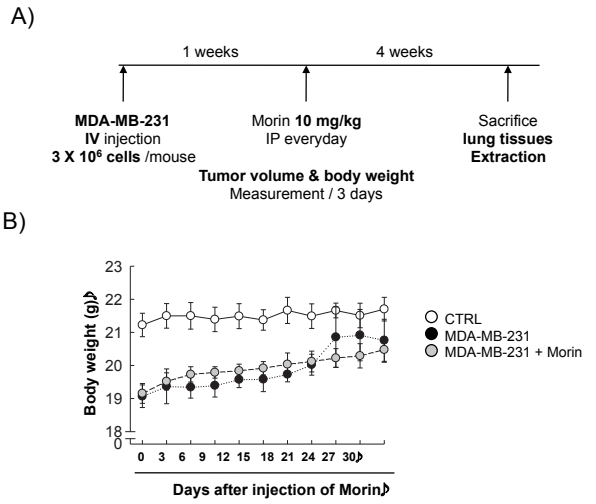

Figure 4. *In vivo* Study Treatment Scheme and Weight Changes after Morin Treatment. Athymic nude mice were divided into 3 groups (5 mice/group) and received morin at the dose of 10 mg/kg (daily, i.p.) after 7 days after cell injection. MDA-MB-231 cells (3x106 cells/100 μl of serum-free RPMI) were injected into the tail vein of the mice. After four weeks of morin treatment, lung tissues were excised. Lung metastasis was quantified by counting metastatic lesions in each section (10 sections per lung). (B) Body weights were measured every 3 days, starting at 7 days after tumor cell injection. There was no difference the two morin-treated and not-treated groups that received cancer cells. Significance compared with control, *P<0.05, **P<0.01

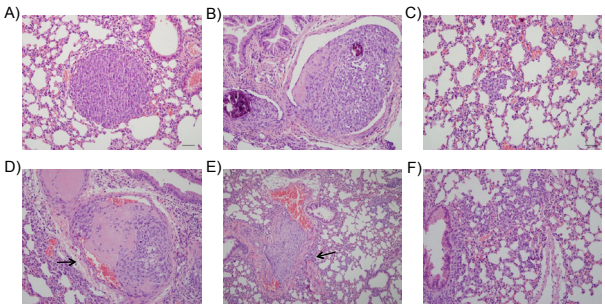

Figure 5. Histologic Evaluation of *in vivo* Effects of Morin on Metastatic Nodules of MDA-MB-231 Cells in the Lung. The metastatic patterns of MB-231 cancer cells varied: (A) pure hematogeneous metastatic nodules, (B) satellite nodules surrounding large hematogeneous metastatic nodules with micro calcification, and (C) interstitial infiltrations of MDA-MB231 cells that is easy to overlooked. (D) necrotic changes in the metastatic nodules (E) irregular shaped with fibrous changes in the cancer nodules. (F) a worse interstitial infiltration pattern of metastasis. (Magnification, X 200, scale bar)

hematogeneous metastatic nodules of human breast cancer, but not interstitial pattern of hematogeneous metastasis in nude mice

We assessed the anti-metastatic effects of morin on MDA-231 human breast cancer in vivo (Figure 4A). There were significant changes in body weights between two groups that received cancer cells or not, but there was no significant difference between the two morin-treated and not-treated groups that received cancer cells (Figure 4B). The pathologic exam of the lung tissue revealed that various pattern of lung metastasis in the group that received cancer cells alone. The metastatic patterns of cancer cells include pure hematogeneous metastatic nodules (Figure 5A), satellite nodules surrounding large hematogeneous metastatic nodules with microcalcification (Figure 5B), tiny interstitial infiltrations of MB231 cells which is easy to overlooked (Figure 5C). Morin-treated group showed that there we also lung metastatic nodules, a certain mouse had a bigger nodules than the not-treated group had, but they have some necrotic changes in the metastatic nodules (Figure 5D) and a certain one was irregular shaped with fibrous changes in the cancer nodules, suggesting shrinkage of a metastatic nodule by treatment (Figure 5E). For the interstitial infiltration pattern of metastasis, morin-treated group appeared to have a worse interstitial infiltration pattern of metastasis (Figure 5F). These finding suggests that there were some different patterns of hematogeneous metastasis and that morin have some anti-cancer effects on the metastatic nodules but not the interstitial pattern of hematogeneous metastasis.

Discussion

This study was designed to investigate the effects of morin on the cancer metastasis focusing on cancer cell adherence to endothelial cells and EMT because adhesive interaction of cancer cells and EMT process are crucial steps for cancer metastasis. Hence, we investigated the effect of morin on adherence of cancer cells to the endothelial cells, and on the expression of VCAM-1, ICAM-1, and N-cadherin in MDA-MB 231 human breast cancer cells. MDA-MB 231 cell is a proper model to study cancer adhesion, EMT and metastasis because the cells are more aggressive, possess high potential to metastasize to distant organ (Anandappa et al., 2000). In this study, we found that morin inhibited TNF-induced cancer cell adhesion to HUVECs through inhibiting ICAM-1 and VCAM-1 expression, and that the inhibitory effects of morin on VCAM-1 expression of HUVECs also appear to be connected to inhibition of TNF-induced cancer cell adhesion to HUVECs. Previous reports demonstrated that TNF induced the two ICAM-1 and VCAM-1 proteins both on vascular endothelial cells and cancer cells (Ahmad et al., 2002). Since previous reports suggested that morin has inhibitory effects on NF-κB (Manna et al., 2007; Sivaramakrishnan and Niranjali Devaraj, 2009), we expected that morin might inhibit both ICAM-1 and VCAM-1 expression induced by TNF. Accordingly, morin significantly suppressed the TNF-induced the expression of VCAM-1 of HUVECs, and ICAM-1 of MDA-MB 231 cells, but morin did not suppress ICAM-1 of HUVECs at the high concentrations (100-200 μM) of morin. It is hard to explain the differential inhibitory effect of morin on the expressions of VCAM-1 and ICAM-1. Previous study suggested that the regulation pathway of VCAM-1 is somewhat different from that of ICAM-1and VCAM-1 is more important in cancer cell adhesion than other adhesion molecules especially for highly metastatic cancer cells (Klemke et al., 2007). Consequently, suppressive effects of morin on VCAM-1 expression of endothelial cells as well as cancer cell may be a very valuable in interfering cancer metastasis. Here, we also assessed the changes in EMT biomarkers to confirm that morin has inhibitory effects on EMT (Figure 3). We only demonstrated that morin suppressed mesenchymal markers N-cadherin because the expression of E-cadherin epithelial marker was not detected in MDA-MB-231 cells (data not shown). In this study, TNF was used to clearly demonstrate the effects of morin on cancer cell adhesion to HUVECs. Therefore, the pathophysiological relevance that TNF is usually increased in patients with advanced cancers (Correia et al., 2007) is supporting that TNF-augmented cancer cell adhesion to HUVECs and EMT process is not an artificial *in vitro* experiment. In vivo experiment, we could not demonstrate the clear anti-metastatic effects of morin on the interstitial infiltration and the comparing of the nodule. In addition, the nodule size and number was not statistically significant even there are some evidence of shrinkage of cancer mass with scar changes in the morin-treated group (Figure 5 E). Therefore, we need a validation with another animal model to measure the anti-cancer activity of cancer adhesion and EMT. In conclusion, this study demonstrated that morin suppressed the cancer cell adhesion to HUVECs through suppression of VCAM-1, and ICAM-1, and and EMT by inhibiting N-cadherin, and that morin has some anti-cancer activities *in vivo*. This study provides evidence that morin might have anti-cancer activity by inhibiting adhesion to HUVECs and EMT process of human breast cancer cells.

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