The flavonoid *p*-hydroxycinnamic acid mediates anticancer effects on MDA-MB-231 human breast cancer cells *in vitro*: Implications for suppression of bone metastases

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Abstract. Tumor invasion into bone tissues is associated with osteoclast and osteoblast recruitment, resulting in the liberation of growth factors from the bone matrix, which can feed back to enhance tumor growth resulting in the vicious cycle of bone metastasis. Activated nuclear factor-κB (NF-κB) in breast cancer cells has been shown to play a crucial role in the osteolytic bone metastasis of breast cancer in stimulating osteoclastogenesis. The flavonoid *p*-hydroxycinnamic acid (HCA) mediates bone anabolic and anti-catabolic effects by stimulating osteoblastic bone formation and suppressing osteoclastic bone resorption. However, the capacity of HCA to ameliorate the negative effects of breast cancer on bone cells has not been investigated. The present study was undertaken to determine the anticancer effects of HCA on MDA-MB-231 human breast cancer bone metastatic cells *in vitro* models. Proliferation of MDA-MB-231 cells was suppressed by culture with HCA (10-1000 nM) due to G1 and G2/M phase cell cycle arrest. The suppressive effects of HCA were mediated through signaling pathways that are related to NF-κB, extracellular signal-regulated kinase (ERK), protein kinase C, calcium signaling, phosphatidylinositol 3-kinase (PI3K) and nuclear transcription activity. HCA was also found to induce death of confluent cancer cells. Furthermore, co-culture with MDA-MB-231 cells suppressed mineralization and stimulated osteoclastogenesis in bone marrow cells. These alterations were prevented by HCA (10-250 nM). The present study demonstrates that HCA possesses anticancer properties in MDA-MB-231 human breast cancer cells and alleviates the negative effects on osteoblastogenesis and osteoclastogenesis *in vitro*. HCA may have important applications in the treatment of breast cancer bone metastasis.

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Introduction

Bone homeostasis is regulated through osteoclasts, osteoblasts and osteocytes in bone tissues (1). Bone loss is induced through decreased osteoblastic bone formation and/or increased osteoclastic bone resorption (2,3). Osteoporotic bone loss, which is caused by inflammation, obesity, diabetes and cancer cell bone metastasis, is widely recognized as a major public health threat. Various cancer cells produce bone metastasis that leads to bone loss and fracture. Breast cancer bone metastasis occurs in 70-80% of patients with advanced breast cancer (4-7), leading to severe pathological bone fractures, pain, hypercalcemia, and spinal cord and nerve-compression syndromes (6,8), which are a common cause of morbidity and mortality.

Tumor invasion into bone tissues is associated with osteoclast and osteoblast recruitment, resulting in the liberation of growth factors from the bone matrix, which can feed back to enhance tumor growth resulting in the vicious cycle of bone metastasis (7,8). Breast cancer cells promote the formation of osteoclasts through secreting osteoporotic cytokines, such as parathyroid hormone-related peptide (PTH-rP), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), interleukins (IL-1, IL-6, IL-8, IL-11, IL-15 and IL-17) and leukemia inhibitory factor (LIF) (7,9). Constitutively activated nuclear factor-κB (NF-κB) in breast cancer cells has been shown to play a crucial role in the osteolytic bone metastasis of breast cancer that drive osteoclastogenesis (10). Enhanced NF-κB stimulates production of granulocyte macrophage-colony stimulating factor (GM-CSF) in breast cancer cells that has been shown to enhance osteoclast development from monocytes (10). Progesterone receptor-positive mammary epithelial cancer cells express receptor activator of NF-κB ligand (RANKL), a key osteoclastogenic cytokine, that also mediates epithelial proliferation and carcinogenesis (11). Matrix metalloproteinases (MMPs), which contribute to bone degradation, are increased in breast cancer

cells (9). Differentiation and activation of osteoclasts is stimulated by production of RANKL, which is mediated by several osteoclastogenic cytokines including PTH-rP, TNF-α and interleukins, in osteoblasts (12). In addition, osteoblasts are negatively affected by breast cancer cells as evidenced by an increase in apoptosis and a decrease in proteins required for new bone formation (9). Thus, breast cancer cell bone metastasis-induced bone loss is due to both activation of osteoclastic bone resorption and suppression of osteoblastic bone formation. However, its mechanism is complex.

Drugs, which target osteoclastogenesis, such as bisphosphonates or anti-RANKL antibody (denosumab), are the current standard care for patients with bone metastasis (13). Bisphosphonates inhibit bone resorption but do not promote new bone formation and actually suppress it. Denosumab suppresses the maturation of osteoclasts by inhibiting the binding of RANKL to RANK, which is the receptor of RANKL in preosteoclasts and mature osteoclasts. Development of osteogenic compounds, that stimulate osteoblastic bone formation to repair bone destruction are needed.

The flavonoid HCA, which is an intermediate-metabolic substance in plants and fruits, is synthesized from tyrosine. HCA has been found to possess anabolic effects on bone metabolism *in vitro* and preventive effects on bone loss in osteoporosis animal models *in vitro* and *in vivo* (14-19). Among botanical factor cinnamic acid-related compounds (cinnamic acid, HCA, ferulic acid, caffeic acid and 3,4-dimethoxycinnamic acid), HCA has been shown to possess a specific anabolic effect on bone metabolism *in vitro* (14). HCA has also been found to possess suppressive effects on osteoclastogenesis by antagonizing RANKL-induced NF-κB activation (17) and potent stimulatory effects on osteoblastogenesis and mineralization through inhibiting TNF-α-enhanced NF-κB signaling *in vitro* (15-17). HCA was also found to stimulate osteoblastogenesis and suppress adipogenesis in bone marrow cells through regulating MEK/ERK signaling *in vitro* (17,20). Moreover, oral administration of HCA has been shown to mediate anabolic effects on bone calcification in the femoral tissues of normal rats *in vivo* (22), and was demonstrated to prevent bone loss in ovariectomized rats (18), an animal model for postmenopausal osteoporosis, and in streptozotocin-induced diabetic rats (19), an animal model for type 1 diabetic osteoporosis *in vivo*.

The present study was undertaken to determine whether HCA has the potential to prevent the bone loss induced by cancer cell bone metastasis. We utilized a common *in vitro* osteoclastogenesis and osteoblastogenesis system involving murine bone marrow cells which were co-cultured with MDA-MB-231 human breast cancer cells (21). We demonstrated that HCA mediates anticancer effects on MDA-MB-231 cells, and that prevents the suppression of osteoblastogenesis and stimulation of osteoclastogenesis induced by co-culture of bone marrow cells with MDA-MB-231 cells. HCA may have important applications in the treatment of breast cancer bone metastasis.

Materials and methods

Materials. Dulbecco's modification of Eagle's medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate and antibiotics (penicillin and streptomycin) were purchased from Corning (Mediatech, Inc., Manassas, VA, USA). α-minimum essential medium (α-MEM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was from HyClone (Logan, UT, USA, USA). *p*-Hydroxycinnamic acid (HCA) (100% pure) was obtained from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). Tumor necrosis factor-α (TNF-α) was from R&D Systems (Minneapolis, MN, USA). PD98059, staurosporine, Bay K 8644, wortmannin, 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), caspase-3 inhibitor, sodium butyrate, roscovitine, sulforaphane, Alizarin red, lipopolysaccharide (LPS) and all other reagents were purchased from Sigma-Aldrich unless otherwise specified. Gemcytabine was obtained from Hospira, Inc. (Lake Forest, IL, USA). Gemcitabine and caspase-3 inhibitor were diluted in phosphate-buffered saline (PBS) and other reagents were dissolved in 100% ethanol for use in the experiments.

MDA-MB-231 cells. MDA-MB-231 human breast cancer bone metastatic cells lack estrogen, progesterone and human epithelial growth factor type 2 (HER2) receptors, and are therefore considered triple-negative (22). They express high levels of the epithelial growth factor receptor (EGFR) and activation of this receptor and its downstream signaling events enhance migration, proliferation, invasion and progression of the malignant phenotype of these cells. We used the estrogen-independent bone-seeking triple-negative human breast cancer MDA-MB-231 cells obtained from the American Type Culture Collection (Rockville, MD, USA).

Proliferation in MDA-MB-231 cells. MDA-MB-231 cells ($1x10^5$/ml/well) were cultured using a 24-well plate in DMEM containing 10% FBS and 1% P/S in the presence or absence of HCA (10, 100, 250, 500 or 1000 nM) for 1, 2, 3, 7 or 14 days in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37˚C (23). In separate experiments, MDA-MB-231 cells ($1x10^5$/ml/well) were cultured DMEM containing 10% FBS and 1% P/S in the presence of sodium butyrate (10 and 100 μM), roscovitine (10 and 100 nM), sulforaphane (1 and 10 nM), TNF-α (1 ng/ml), Bay K 8644 (1 μM), PD98059 (1 μM), staurosporin (0.1 μM), wortmannin (1 μM), DRB (1 μM) or gemcitabine (100 nM) for 3-7 days. After culture, cells were detached from each culture dish and counted.

In other experiment, MDA-MB-231 cells ($1x10^5$/ml/well) were cultured using a 24-well plate in DMEM containing 10% FBS and 1% P/S in the absence of HCA for 7 days until confluent, and then the cells were cultured in the presence of HCA (10-1000 nM) with or without caspase-3 inhibitor (5 nM) for 3 days (24). After culture, cells were detached from each culture dishe and counted.

Cell counting. After trypsinization each culture dish was treated with 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS for 2 min at 37˚C, the detached cells from the dish were collected by centrifugation (23,25). The cells were resuspended on PBS solution and stained with eosin. Cell numbers were counted under a microscope using a hemocytometer plate. For each dish, we took the average of two countings. Cell number is shown as the number per well of each plate.

Animals and bone marrow cells. Female mice (CD1-Elite, wild-type, 2 months old), which were purchased from Charles

River, were housed in a pathogen-free facility, and all procedure and protocols were approved through the Institutional Animal Care and Use Committee at Emory University. The femur and tibia tissues were removed immediately after sacrifice. Bone marrow cells were isolated with procedure of sterilization from the femoral and tibial tissues.

Mineralization in co-culture with bone marrow and breast cancer cells. To determine the effects of breast cancer cells on bone marrow osteoblastogenesis and mineralization, we used mineralization medium (MM) containing ascorbic acid (100 ng/ml) and 4 mM β-glycerophosphate in DMEM with 10% FBS and 1% P/S. Bone marrow cells ($1x10^6$ cells/1 ml/well) were cultured for 3 days at 37˚C in a humidified 5% CO_2 atmosphere, and then the cells were co-cultured with addition of breast cancer MDA-MB-231-bone metastatic cells ($1x10^4$ cells/1 ml/well) using the 12-well plates in the presence or absence of α-MEM-MM with either vehicle or HCA (10, 100 and 250 nM) for 18 days (21). The medium was changed every 3 days. After culture, cells were washed with PBS and stained with Alizarin red. For quantitation, 10% cetylpyridinium chloride solution was added to each well to elute the dye (21). After complete elution, the absorbance at 570 nm on a microtiter plate reader for the eluted solution was measured.

Co-culture of preosteoblastic MC3T3 cells with breast cancer cells. MC3T3 preosteoblastic cells ($2x10^5$ cells/1 ml/well) were cultured using a 12-well plate in α-MEM containing 10% FBS and 1% P/S, and 3 days later the culture medium was replaced to DMEM (containing 10% FBS and 1% P/S) in the presence or absence of mineralization medium (MM) containing ascorbic acid (100 ng/ml) and 4 mM β-glycerophosphate. After 3 days, osteoblastic cells were cocultured with addition of MDA-MB-231 cells ($1x10^3$ or $1x10^4$/ml/well) in α-MEM containing MM in the presence or absence of HCA (10, 100 and 250 nM) for 18 days (21). Medium was changed every 3 days. After culture, cells were washed with PBS and stained with Alizarin red. For quantitation of calcium deposition, after complete elution with 10% cetylpyridinium chloride solution, the absorbance at 570 nm on a microtiter plate reader for the eluted solution was measured.

Osteoclastogenesis in bone marrow cell culture. To determine the effects of breast cancer cells on bone marrow osteoclastogenesis, bone marrow cells ($2x10^5$ cells/1 ml/well) were cultured in DMEM containing 10% FBS and 1% P/S using 24-well plates (1.0 ml/well) (21). Cells were cultured with or without LPS (10 μg/ml of medium) for 3 days in the presence or absence of HCA (10, 100 and 250 nM); then 0.5 ml of the old medium was replaced with fresh medium with or without LPS (10 μg/ml of medium) in the presence or absence of HCA (10, 100 and 250 nM), and cultures were maintained for an additional 4 days. In other experiments, the cells were cultured for 3 days in medium with or without LPS (10 μg/ml of medium) in the presence or absence of HCA (10, 100 and 250 nM), and then the medium was replaced with or without LPS (10 μg/ml of medium) without HCA and cultured for additional 4 days (21). After being cultured for 7 days, cells adherent to the 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts (16,26). Briefly, cells were washed with phosphate buffered salt solution and fixed with 10% neutralized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied (16,26). The fixed cells were incubated for 90 min at room temperature in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a stain for the reaction product, in the presence of 10 mM sodium tartrate. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells. MNCs scored were mean ± SDM of six cultures.

Statistical analysis. Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparisons were performed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons post-test for parametric data. $P<0.05$ was considered statistically significant.

Results

HCA suppresses proliferation in MDA-MB-231 cells. To determine the effects of HCA on proliferation in MDA-MB-231 human breast cancer bone metastatic cells *in vitro*, the cancer cells were cultured in the presence of HCA for 1-14 days. Cell numbers increased with the time period in culture (Fig. 1). This increase was suppressed by culture with HCA (10-1000 nM) for 1 (Fig. 1A), 2 (Fig. 1B), 3 (Fig. 1C), 7 (Fig. 1D), and 14 (Fig. 1E) days. Thus, the first time, HCA was found to possess suppressive effects on proliferation of MDA-MB-231 cells *in vitro*.

Suppressive effects of HCA on proliferation in the MDA-MB-231 cells were determined in the presence of various inhibitors that induce cell cycle arrest *in vitro* (Fig. 2). Cells were cultured for 3 days in the absence (Fig. 2A) or presence (Fig. 2B) of HCA (100 nM) with or without butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM) (23,27,28). Proliferation of MDA-MB-231 cells was suppressed in the presence of these inhibitors (Fig. 2A). Suppressive effects of HCA on cell proliferation were not potentiated in the presence of these inhibitors (Fig. 2B). HCA was suggested to inhibit G1 and G2/M phase cell cycle arrest in MDA-MB-231 cells.

Next, to determine a mechanistic characterization, we examined whether suppressive effects of HCA on proliferation in MDA-MB-231 cells are modulated by various signaling factors that suppress cell proliferation. Proliferation in MDA-MB-231 cells was suppressed in the presence of TNF-α (1 ng/ml), an enhancer of NF-κB signaling (29), or Bay K 8644 (1 μM), an agonist of Ca^{2+} influx in cells (30) (Fig. 3A). Suppressive effects of HCA (100 nM) on cell proliferation were not significantly potentiated in the presence of TNF-α and Bay K 8644 (Fig. 3A). Moreover, suppressive effects of HCA (100 nM) on the proliferation in MDA-MB-231 cells were not modulated in the presence of PD98059 (1 μM), an ERK inhibitor (31), staurosporin (0.1 μM), an inhibitor of protein kinase C (32), wortmannin (1 μM), an inhibitor of PI3K (33) or DRB (1 μM), an inhibitor of transcriptional activity with RNA polymerase II inhibition (34) (Fig. 3B). Thus, suppressive effects of HCA on the proliferation in MDA-MB-231

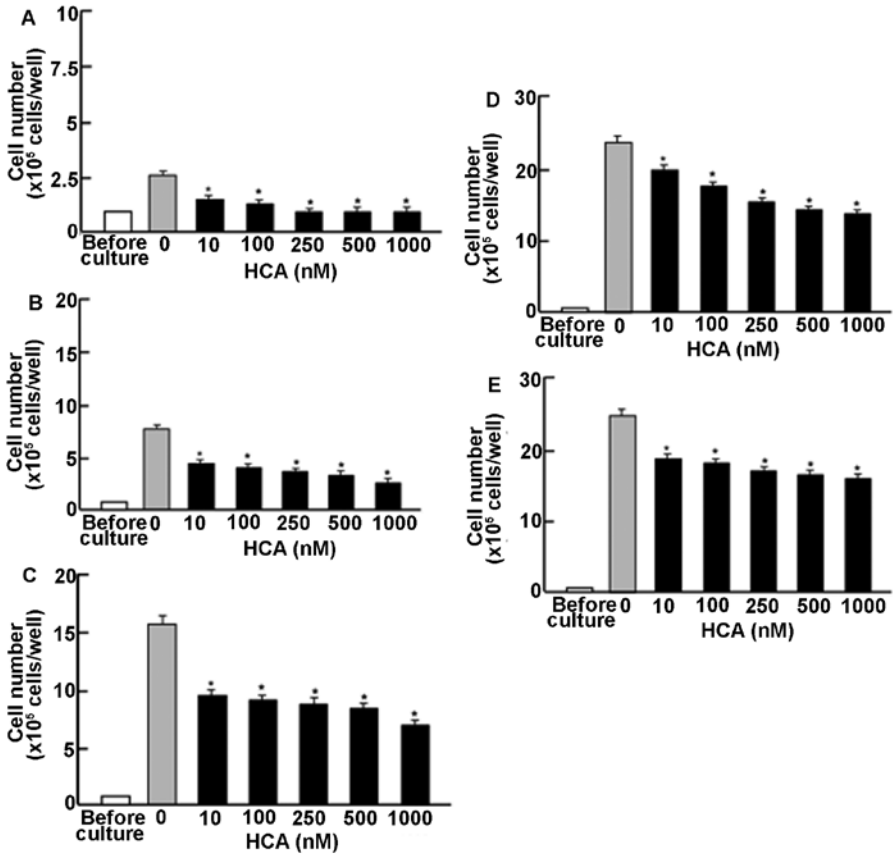

Figure 1. *p*-Hydrpxycinnamic acid (HCA) suppresses proliferation in MDA-MB-231 human breast cancer cells *in vitro*. Cells were cultured in DMEM in the presence or absence of HCA (10-1000 nM) for 1 (A), 2 (B), 3 (C), 7 (D) or 14 (E) days. After culture, the number of attached cells on a dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. *P<0.001 vs. control (grey bar). One way ANOVA, Tukey-Kramer post test.

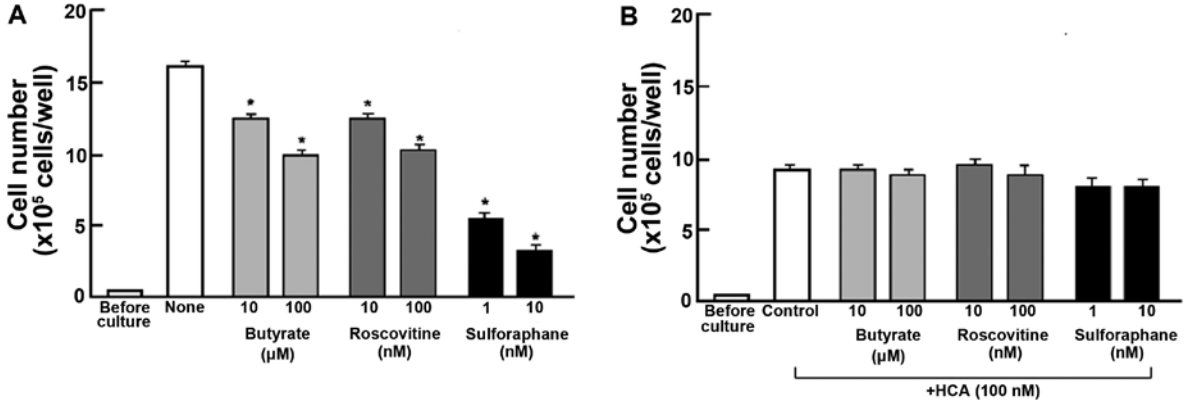

Figure 2. Effect of *p*-hydroxycinnamic acid (HCA) on proliferation in MDA-MB-231 human breast cancer cells in the presence of various inhibitors that induce cell cycle arrests *in vitro*. Cells were culture for 3 days in the absence (A) or presence (B) of HCA (100 nM) with or without butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM). After culture, the number of attached cells on a dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. *P<0.001 vs. control (none; white bar). One way ANOVA, Tukey-Kramer post test.

cells were not altered in the presence of various inhibitors that regulate intracellular signaling pathways *in vitro*.

Suppressive effects of HCA on proliferation in the MDA-MB-231 cells were compared with that of gemcitabine, a strong antitumor agent, which induces nuclear DNA damage (35). Culture with gemcitabine (50-500 nM) suppressed cell proliferation (Fig. 4A). This effect was not potentiated with addition of HCA (10 nM) (Fig. 4B).

HCA stimulates cell death in confluent cultures. To determine the effects of HCA on cell death in human breast cancer MDA-MB-231 bone metastatic cells, the cells were cultured for 7 days until confluent. Confluent cells were cultured for an additional 3 days in the presence of HCA (10-1000 nM). Cell number was decreased after culture with HCA (10-1000 nM) (Fig. 5A), indicating that HCA stimulates apoptotic cell death. Such effects of HCA (10 and 100 nM) were not potentiated

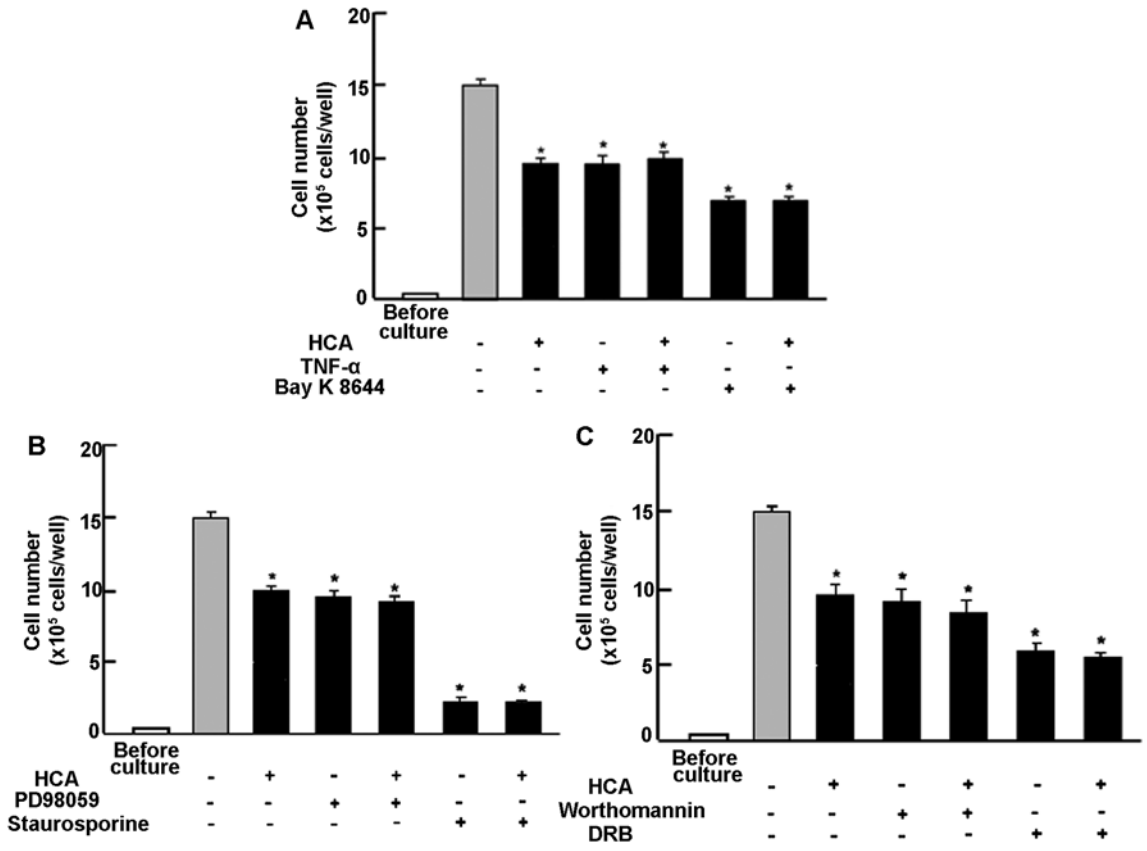

Figure 3. Effect of *p*-hydroxycinnamic acid (HCA) on proliferation in MDA-MB-231 human breast cancer cells in the presence of TNF-α, Bay K 8644, PD98059, staurosporine, wortmannin or DRB *in vitro*. (A) Cells were culture for 3 days in the presence of HCA (100 nM) with or without TNF-α (1 ng/ml) or Bay K 8644 (1 μM). (B) Cells were cultured for 3 days in the presence of HCA (100 nM) with or without PD98059 (1 μM) or staurosporin (0.1 μM). (C) Cells were cultured for 3 days in the presence of HCA (100 nM) with or without wortmannin (1 μM) or DRB (1 μM). After culture, the number of attached cells on a dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 vs. control (grey bar). One way ANOVA, Tukey-Kramer post test. **P<0.001 vs. wortmannin or DRB alone. One way ANOVA, Tukey-Kramer post test.

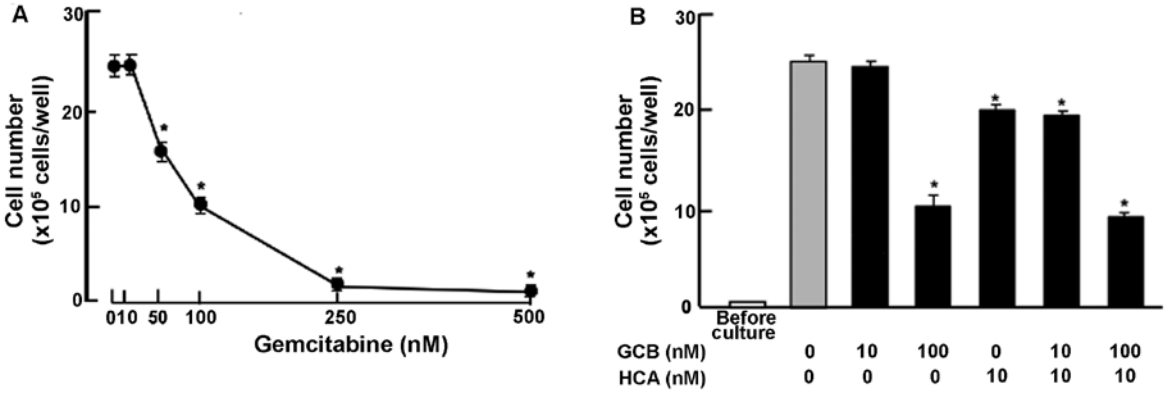

Figure 4. Effects of *p*-hydroxycinnamic acid (HCA) on proliferation in MDA-MB-231 human breast cancer cells with gemcitabine (GCB) *in vitro*. (A) Cells were cultured for 7 days in the presence of GCB (10-500 nM). (B) Cells were cultured for 7 days in the presence of HCA (10 nM) with or without GCB (10 or 100 nM). After culture, the number of attached cells on a dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 vs. control (grey bar). One way ANOVA, Tukey-Kramer post test.

in the presence of gemcitabine (100 nM) (data not shown). To determine a mechanistic characterization of the effects of HCA on apoptotic cell death, the confluent cells after culture for 7 days were further cultured in the presence of HCA (10 or 100 nM) with or without caspase-3 inhibitors (5 μM) for an additional 3 days (Fig. 5B). Stimulatory effects of HCA on cell death were completely prevented in the presence of caspase-3 inhibitors (Fig. 5B). Thus the data suggest that HCA stimulates apoptotic cell death by increasing activity of caspase-3 that activates nuclear DNA fragmentation, which induces apoptosis.

HCA suppresses the effects of MDA-MB-231 cells in bone marrow cell differentiation. To determine whether HCA prevents bone effects of human breast cancer MDA-MB-231 bone metastatic cells, we used co-culture system with

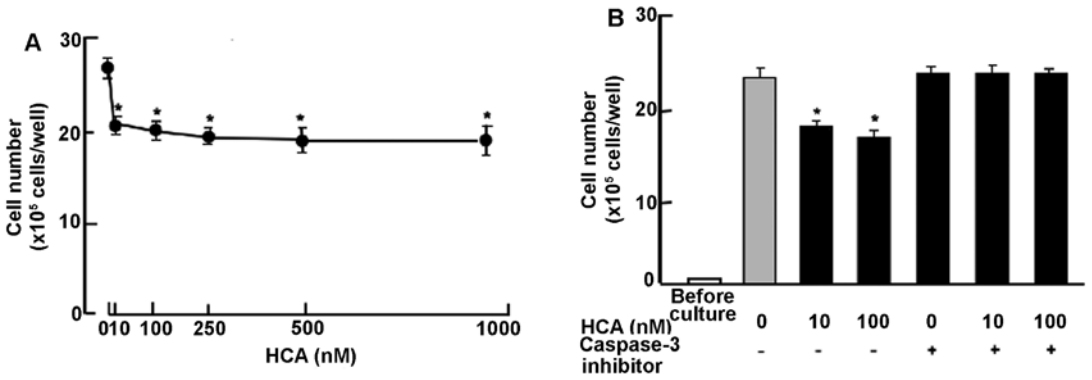

Figure 5. *p*-Hydroxycinnamic acid (HCA) stimulates apoptotic cell death in MDA-MB-231 human breast cancer cells *in vitro*. (A) Cells were cultured for 7 days until confluent, and then cultured for an additional 3 days in the presence of HCA (10-1000 nM). (B) Cells were cultured for 7 days until confluent, and then cultured for an additional 3 days in the presence of HCA (10 or 100 nM) with or without caspase-3 inhibitor (5 nM). After culture, the number of attached cells on a dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. *P<0.001 vs. control (grey bar). One way ANOVA, Tukey-Kramer post test.

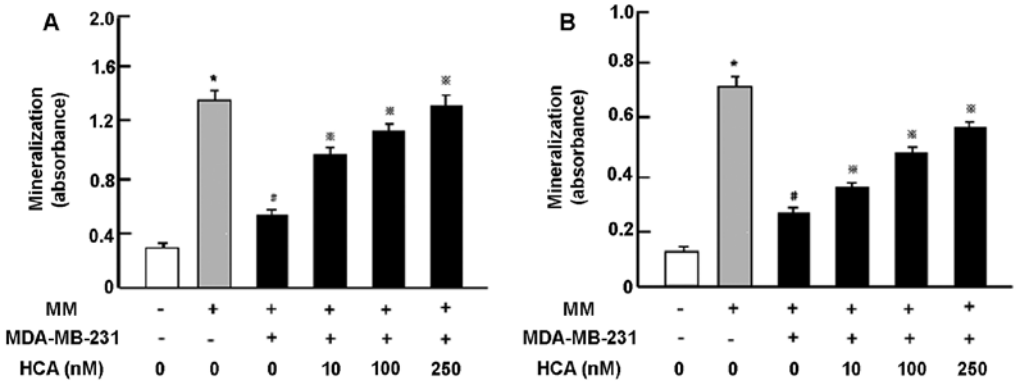

Figure 6. *p*-Hydroxycinnamic acid (HCA) prevents the suppression of mineralization in bone marrow cells (A) and preosteoblastic MC3T3 cells (B) induced by co-culture with MDA-MB-231 human breast cancer cells *in vitro*. (A) Bone marrow cells were cultured in DMEM in the presence or absence of mineralization medium (MM). After 3 days, the cells were cocultured with addition of MDA-MB-231 cells in DMEM containing MM in the presence or absence of HCA (10, 100 or 250 nM) for 18 days. (B) Osteoblastic cells were cultured in the presence or absence of MM for 3 days, and then the cells were co-cultured with addition of MDA-MB-231 in medium containing MM in the presence or absence of HCA (10, 100 or 250 nM) for additional 18 days. After culture, the cells were stained with Alizarin red. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 relative to control without MM (white bar) or ※P<0.001 vs. control with MM (grey bar). ※P<0.001 vs. MM without HCA (black bar). One way ANOVA, Tukey-Kramer post test.

MDA-MB-231 cells and mouse bone marrow cells *in vitro* (21). We firstly examined change in the mineralizations in bone marrow cells and preosteoblastic MC3T3 cells cocultured with MDA-MB-231 cells *in vitro* (Fig. 6A). Bone marrow cells were cultured in the presence or absence of mineralization medium (MM) (Fig. 6A). After 3 days, the cells were cocultured with addition of MDA-MB-231 cells in the presence or absence of HCA (10, 100 or 250 nM) for 18 days that reveal mineralization. Mineralization in bone marrow cells was suppressed by coculture with MDA-MB-231 cells. This suppression was prevented in the presence of HCA (10-250 nM) (Fig. 6A). Then, preosteoblastic MC3T3 cells were cultured in the presence or absence of HCA for 3 days, and then the cells were co-cultured with addition of MDA-MB-231 cells in medium containing MM in the presence or absence of HCA (10, 100 or 250 nM) for additional 18 days (Fig. 6B). Co-culture with MDA-MB-231 cells suppressed mineralization in osteoblastic cells. This suppression was prevented by the presence of HCA (10-250 nM) (Fig. 6B).

Moreover, we determined suppressive effects of HCA on osteoclastogenesis *in vitro* (Fig. 7). Mouse bone marrow cells were cultured in the presence of LPS, which induces osteoclastogenesis in bone marrow cells, with or without HCA (10-250 nM) for 7 days (Fig. 7A). Culture with LPS caused a remarkable increase in osteoclastogenesis in bone marrow cells. This increase was prevented in the presence of HCA (10-250 nM) (Fig. 7A). Thus, HCA was confirmed to possess suppressive effects on osteoclastogenesis induced by LPS in bone marrow culture *in vitro*. Moreover, bone marrow cells with co-culture of MDA-MB-231 cells were cultured in the presence or absence of HCA (10-250 nM) without LPS for 7 days (Fig. 7B). Osteoclastogenesis was markedly enhanced by co-culture with MDA-MB-231 cells. This enhancement was prevented in the presence of HCA (10-250 nM) (Fig. 7B).

Discussion

The present study demonstrates that the flavonoid HCA mediates a suppressive effect on the proliferation in MDA-MB-231 human breast cancer bone metastatic cells, and that HCA prevents the suppressed osteoblastogenesis and enhanced osteoclastogenesis induced by coculture with MDA-MB-231

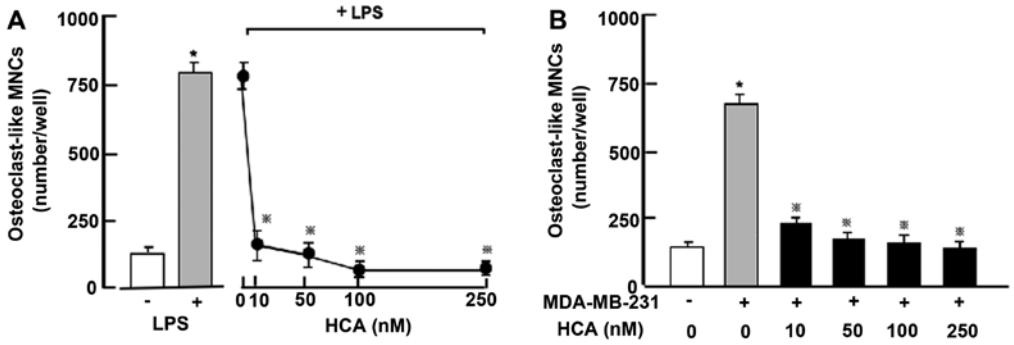

Figure 7. *p*-Hydroxycinnamic acid (HCA) suppresses osteoclastogenesis enhanced by coculture with mouse bone marrow cells and MDA-MB-231 human breast cancer cells *in vitro*. (A) Bone marrow cells were cultured in the presence of LPS (1 μg/ml) with or without HCA (10-250 nM) for 7 days. (B) Bone marrow cells were cultured in the presence or absence of HCA (10-250 nM) and/or addition of MDA-MB-231 cells without LPS for 7 days. After culture, cells were stained with TRAP. TRAP$^+$ multinucleated cells (3 or more nuclei) were quantitated and averaged for 8 independent wells for each data point. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 relative to control without LPS (white bar). #P<0.001 vs. LPS without HCA. One way ANOVA, Tukey-Kramer post test.

cells and bone marrow cells *in vitro* models. Thus, HCA was found to possess anticancer effects and anti-bone metastatic effects in human breast cancer cells *in vitro*.

Suppressive effects of HCA on the proliferation of MDA-MB-231 cells were not seen in the presence of butyrate, roscovitine or sulphoraphan that induce cell cycle arrest. Roscovitine is a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2m and cdk5 (27). Sulforaphane induces G2/M phase cell cycle arrest (28). Butyrate induces an inhibition of G1 progression (23). The data suggest that HCA induces G1 and G2/M cell cycle arrest in MDA-MB-231 cells.

Next, to investigate a mechanistic characterization of the suppressive effects of HCA on cell proliferation, we used various factors that regulate intracellular signaling processes. Suppressive effects of HCA on the proliferation in MDA-MB-231 cells were not potentiated in the presence of TNF-α, an enhancer of NF-κB signaling (29), Bay K 8644, an agonist of Ca^{2+} entry in cells (30), PD98059, an inhibitor of ERK/mitogen-activated protein kinase signaling pathway (31), staurosporin, an inhibitor of calcium-dependent protein kinase C signaling pathway (32) and wortmannin, an inhibitor of PI3/Akt signaling pathway (33). These findings suggest that HCA mediates suppressive effects that are mediated through the inhibition of various signaling pathways related to NF-κB, ERK, protein kinase C, calcium signaling, or PI3K in breast cancer MDA-MB-231 cells. Moreover, suppressive effects of HCA on cell proliferation were not potentiated by the presence of DRB, an inhibitor of transcriptional activity that targets RNA polymerase II (34). Thus, we speculate that HCA suppresses proliferation by inhibiting various signaling processes in MDA-MB 231 cells. Further studies are needed to determine its molecular mechanism.

HCA was found to stimulate cell death in MDA-MB-231 cells *in vitro*. This effect was not seen in the presence of caspase-3 inhibitor. HCA may stimulate apoptotic cell death through the mechanism by which it increases the activity of caspase-3 that activates nuclear DNA fragmentation, which induces apoptosis. It is possible that HCA directly activates caspase-3. However, the suppressive effects of HCA on apoptotic cell death remains to be elucidated.

Gemcitabine is an antitumor agent that induces nuclear DNA damage (35). This agent suppresses cell proliferation and stimulates apoptotic cell death in various types of cancer cells. Suppressive effects of HCA on cell number were not potentiated in the presence of gemcitabine in MDA-MB-231 cells, suggesting that HCA partly acts on processes involved in action mode of gemcitabine. However, HCA revealed suppressive effects on the cell number with lower concentrations rather than gemcitabine, indicating that HCA has lower toxicity. HCA may provide a useful tool as a new antitumor agent. This remains to be elucidated *in vivo* experiments.

HCA has been shown to stimulate osteoblastic mineralization and suppress osteoclastogenesis and adipogenesis in mouse bone marrow cells (20). Bone marrow mesenchymal stem cells are multipotent cells, which among other cell lineages give rise to adipocytes and osteoblasts (36). This occurs through crosstalk between complex signaling pathways including those derived from bone morphogenic proteins, wingless-type MMTV integration site (Wnt) proteins, hedgehogs, delta/jagged proteins, transcriptional regulators including peroxisome proliferator-activated receptor-γ (PPARγ) and runt-related transcription factor 2 (Runx2), and MAPK/ERK signaling pathway. HCA has not been identified to target specific molecules in signaling pathways of a differentiation process in bone marrow cells. However, HCA was found to stimulate the differentiation process of preosteoblasts due to suppressing differentiation to preadipocytes by inhibiting MAPK/ERK signaling pathway (20). Moreover, HCA was shown to directly stimulate mineralization in preosteoblastic MC3T3 cells *in vitro* (15,17).

We determined whether HCA mediates preventive effects on bone metastatic activity of breast cancer cells using co-culture system with bone marrow cells. Osteoblastic mineralization in mouse bone marrow cells was markedly suppressed after coculture with MDA-MB-231 cells. Such an effect was also observed in preosteoblastic MC3T3 cells *in vitro*. Thus, MDA-MB-231 cells were confirmed to directly suppress osteoblastic mineralization *in vitro* models. TNF-α, which is produced in breast cancer cells (9,10), suppresses osteoblastic mineralization that is mediated through activation of NF-κB signaling (17,29). MDA-MB-231 cell-induced suppression of osteoblastic mineralization may be partly related to TNF-α, which is produced by the bone metastatic cells. Culture with HCA was found to

prevent the suppression of osteoblastic mineralization in bone marrow cells and preosteoblastic MC3T3 cells, which were induced by co-culture with MDA-MB-231 cells. HCA has been shown to prevent suppression of osteoblastic mineralization induced by TNF-α in preosteoblastic MC3T3 *in vitro*, and it suppressed potently TNF-α-enhanced NF-κB-luciferase activity in preosteoblastic MC3T3 *in vitro* (17). HCA may prevent suppressive effects of TNF-α on osteoblastic mineralization by depressing TNF-α-induced activation of NF-κB signaling in osteoblastic cells that were co-cultured with MDA-MB-231 cells.

Osteoclasts are differentiated from hematopoietic precursors of the monocyte/macrophage lineage by stimulation with the TNF family cytokines RANKL and M-CSF (12). Osteoclastogenesis in mouse bone marrow culture in the absence of bone resorbing-factors was enhanced by co-culture with MDA-MB-231 cells *in vitro*. Breast cancer cells are known to produce RANKL, which plays a pivotal role in formation from preosteoclastic cells to mature osteoclasts (7-13). Stimulatory effects of MDA-MB-231 cells on osteoclastogenesis in bone marrow culture may be due to RANKL, possibly produced in the breast cancer cells. HCA was found to suppress osteoclastogenesis, which was enhanced by stimulation with LPS and coculture with MDA-MB-231 cells, in bone marrow culture *in vitro*. HCA has been shown to suppress osteoclastogenesis through antagonizing RANKL-enhanced NF-κB-luciferase activity in preosteoclastic RAW267.4 cells *in vitro* (17). Suppressive effects of HCA on osteoclastogenesis enhanced by coculture with MDA-MB-231 cells and bone marrow culture *in vitro* may be related to antagonizing activation of NF-κB signaling induced by RANKL.

In conclusion, the present study demonstrates that the flavonoid HCA mediates anticancer effects on MDA-MB-231 human breast cancer bone metastatic cells *in vitro*, and that the flavonoid possesses preventive effects on the suppressed osteoblastogenesis and stimulated osteoclastogenesis in bone marrow cells induced by coculture with MDA-MB-231 cells. Suppressive effects of HCA on bone metastasis may partly be based on its anticancer cell effects. Moreover, HCA directly activates osteoblastogenesis and suppresses osteoclastogenesis to prevent bone metastasis. Thus, HCA was found to reveal both effects on anticancer cells and anti-bone metastasis in MDA-MB-231 human breast cancer bone metastatic cells. HCA may be a new useful tool in the prevention and therapy in breast cancer bone metastasis *in vivo*.

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