

[6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells

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Abstract

Gingerol (*Zingiber officinale* Roscoe, *Zingiberaceae*) is one of the most frequently and heavily consumed dietary condiments throughout the world. The oleoresin from rhizomes of ginger contains [6]-gingerol (1-[4'-hydroxy-3'-methoxyphenyl]-5-hydroxy-3-decanone) and its homologs which are pungent ingredients that have been found to possess many interesting pharmacological and physiological activities, such as anti-inflammatory, antihepatotoxic and cardiogenic effects. However, the effects of [6]-gingerol on metastatic processes in breast cancer cells are not currently well known. Therefore, in this study, we examined the effects of [6]-gingerol on adhesion, invasion, motility, activity and the amount of MMP-2 or -9 in the MDA-MB-231 human breast cancer cell line. We cultured MDA-MB-231 cells in the presence of various concentrations of [6]-gingerol (0, 2.5, 5 and 10 μ M). [6]-Gingerol had no effect on cell adhesion up to 5 μ M, but resulted in a 16% reduction at 10 μ M. Treatment of MDA-MB-231 cells with increasing concentrations of [6]-gingerol led to a concentration-dependent decrease in cell migration and motility. The activities of MMP-2 or MMP-9 in MDA-MB-231 cells were decreased by treatment with [6]-gingerol and occurred in a dose-dependent manner. The amount of MMP-2 protein was decreased in a dose-dependent manner, although there was no change in the MMP-9 protein levels following treatment with [6]-gingerol. MMP-2 and MMP-9 mRNA expression were decreased by [6]-gingerol treatment. In conclusion, we have shown that [6]-gingerol inhibits cell adhesion, invasion, motility and activities of MMP-2 and MMP-9 in MDA-MB-231 human breast cancer cell lines.

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Keywords: [6]-Gingerol; Metastasis; MMP-2; MMP-9

1. Introduction

Numerous epidemiological, biological and clinical studies indicate a strong correlation between dietary factors and prevention of human cancers [1–3]. Therefore, chemoprevention of cancers by nutraceuticals and phytochemicals has become a flourishing field of research over the past decade [4–7].

Ginger rhizome (*Zingiber officinale*), commonly known as ginger, is utilized worldwide as a spice and a flavoring agent. It has a long history of both culinary and medicinal use [8]. Ginger contains pungent phenolic substances collectively known as gingerols. [6]-Gingerol (1-[4'-hydroxy-3'-

methoxyphenyl-5-hydroxy]-3-decanone), one of the major pungent elements of ginger, has been found to exhibit antioxidant activity as determined by inhibition of phospholipid peroxidation induced by the FeCl_3 -ascorbate system [9] and confirmed in many in vitro and in vivo system [10,11]. Gingerol has also been found to inhibit platelet aggregation and formation of prostaglandin and leukotriene [12–14]. Ginger oil at a single oral dose of 33 mg/kg significantly suppressed severe chronic adjuvant arthritis in rats [15]. Ginger consumption ameliorated the pain and symptoms of rheumatic disorders [16,17] and lowered the platelet thromboxane production in humans [18].

Recently, the cancer chemopreventive potential of ginger has been determined by the inhibition of phorbol ester-induced inhibition of Epstein–Barr virus activation I Raji cells [19] and suppression of azoxymethane-induced intestinal carcinogenesis in rats and mouse skin tumor [20,21].

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Also, gingerol has been shown to exhibit anticancer activities through the induction of apoptosis [22,23].

Cancer metastasis consists of a complex cascade of events, which ultimately allow for tumor cell escape and seeding of ectopic environments [24]. For breast cancer cells to manifest their malignant potential, they must develop the ability to break through and dissolve extracellular matrix (ECM), particularly the delimitating basement membrane (BM). The degradation of the pericellular BM and ECM is catalyzed by the concerted action of several classes of ECM-degrading enzymes. One important class of ECM-degrading enzymes includes the matrix metalloproteinases (MMPs) [25]. MMPs have been implicated as possible mediators of invasion and metastasis in some cancers. Kim et al. [26] recently showed that [6]-gingerol inhibited the formation of lung metastases of B16F10 melanoma in an experimental mouse model. However, there is limited knowledge regarding the effect of [6]-gingerol in terms of metastasis and MMPs, by which it may exert its antitumor effects.

In this study, we investigated the effect of [6]-gingerol on tumor metastasis. Thus far, no studies have been undertaken to assess the effect of ginger on metastasis in breast cancer. Therefore, we examined the effect of [6]-gingerol against MDA-MB-231 human breast cancer cells.

2. Materials and methods

2.1. Materials and reagents

[6]-Gingerol was purchased from Biomol (USA) and was dissolved in ethanol and diluted in cell culture medium. MDA-MB-231 cells were purchased from American Type Culture Collection (Rockville, MD, USA). The following reagents and chemicals were obtained from the respective suppliers: Dulbecco's modified Eagle's medium/Nutrient Mixture Ham's F12 (DMEM/F12), streptomycin and penicillin (Gibco/BRL); RIA-grade bovine serum albumin (BSA) and transferrin (Sigma, St. Louis, MO, USA); antibodies for MMPs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents were obtained from Sigma.

2.2. Cell culture

The MDA-MB-231 human breast cancer cell line was maintained in DMEM/F12 containing 100 ml/L of fetal bovine serum (FBS) with 100,000 U/L of penicillin and 100 mg/L of streptomycin. To examine the effect of [6]-gingerol, cells were plated with DMEM/F12 containing 10% FBS. Before MDA-MB-231 human breast cancer cells were treated with [6]-gingerol, the cell monolayers were rinsed and starved of serum for 24 h, with DMEM/F12 supplemented with 5 mg/L transferrin, 1 g/L BSA and 5 µg/L selenium (serum-free medium, SFM). After serum starvation, fresh SFM with or without the indicated concentrations of [6]-gingerol was replaced. Viable cell numbers were estimated 24, 48 and 72 h after the cells were exposed to [6]-

gingerol using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously [27]. In brief, the MTT solution (0.5 mg/ml) was added to the cells and incubated at 37°C to allow cleavage of the tetrazolium ring by mitochondrial dehydrogenases and formation of blue formazan crystal. After 3 h, the residual MTT was carefully removed and crystals were dissolved with isopropanol. The absorbance at 470 nm was measured by spectrophotometry.

2.3. Adhesion assay

Ninety-six-well plates were coated with fibronectin (BD, Bioscience, Massachusetts, USA, 20 µg/1 ml PBS) and incubated for 1 h at 37°C with 5% CO₂. Coated wells were washed twice with PBS and incubated for 1 h with SFM. The wells were rewashed with PBS and dried on a clean bench, then MDA-MB-231 cells (8×10⁵ cells/well) suspended in the medium containing 0, 2.5, 5 and 10 µM [6]-gingerol were seeded into coated wells and incubated for 1 h at 37°C. Adherent cells were washed three times with PBS and reincubated in a medium containing 1 mg/ml MTT for 3 h at 37°C, and the absorbance was measured at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Experiments were performed independently three times.

2.4. Matrigel invasion assay

Wells of a matrigel chamber (BD, Bioscience) were filled with SFM and adapted at room temperature. MDA-MB-231 cells (1×10⁶ cells/ml) resuspended in the medium containing 0, 2.5, 5 and 10 µM [6]-gingerol were carefully transferred into the upper chambers. Lower chambers were filled with 10% FBS medium to attract cells. Matrigel chambers were incubated for 12 h at 37°C with 5% CO₂. Then, the cells on the upper surfaces of the filters were removed by wiping with a paper. Filters were stained with Diff-Quik stain solution (Dade Behring, Newark, NJ, USA), and the cells on the lower surface of the filter were fixed onto a glass slide. Cells in five randomly selected microscopic fields (×400) of the lower slide were counted. Experiments were performed independently three times.

2.5. Wound healing migration assay

Wound-healing migration assay is based on the repopulation of wounded cultures. The cells were seeded into 12-well culture plates at 5×10⁵ cells/ml and cultured in medium containing 10% FBS to near confluence of the cell monolayer. Confluent cell monolayers were incubated for 1 h with 1 µg/ml mytomicin C to stop cell proliferation. The monolayers were carefully wounded using a yellow pipette tip, and any cellular debris present was removed by washing with PBS. The wounded monolayers were then incubated for 48 h in SFM containing 0, 2.5, 5 and 10 µM [6]-gingerol. Photographs of the exact wound areas taken initially were again taken after 12, 24 and 48 h.

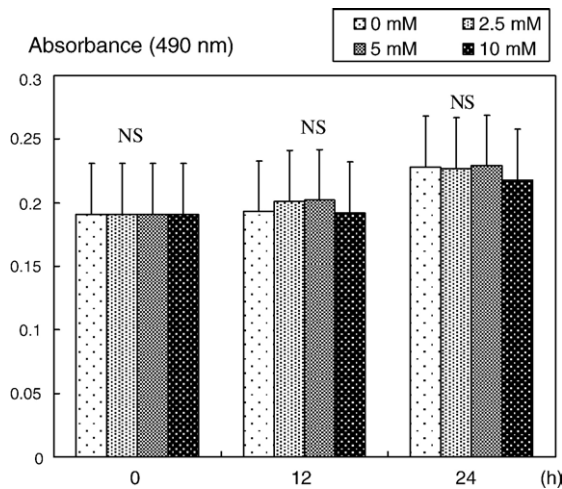


Fig. 1. Effect of [6]-gingerol on cell proliferation in MDA-MB-231 cells. MDA-MB-231 cells were plated at a density of 2.5×10^4 cells/ml in a 24-well plate with DMEM/F12 supplemented with 10% FBS for 48 h. The monolayers were serum starved with DMEM/F12 supplemented with 5 μ g/ml transferrin, 5 ng/ml selenium and 1 mg/ml bovine serum albumin for 24 h. After serum starvation, the monolayers were incubated in serum-free medium with 0, 2.5, 5, 10 μ M [6]-gingerol. Viable cell numbers were estimated by the MTT assay. Each bar represents the mean \pm S.D. calculated from three independent experiments.

2.6. Boyden chamber motility assay

PVPF filters (8 μ m diameter pore size) were coated with 0.01% gelatin solution for 16 h at room temperature. The lower chamber was filled with 10% FBS medium to attract cells. MDA-MB-231 cells (2×10^6 cells/ml) resuspended in the medium containing 0, 2.5, 5 and 10 μ M [6]-gingerol were carefully transferred into the upper chambers. The lower chamber was filled with 10% FBS medium to attract cells. The Boyden chamber was incubated at 37°C with 5% CO₂ for 22 h. After gentle removal of the filter from the chamber, the cells on the upper side of the filter were removed by wiping with a paper. The filter was stained with Diff-Quik stain solution (Dade Behring), and the cells on the lower surface of the filter, which penetrated the pore of gelatin-coated filter, were fixed onto a glass slide. Cells in five randomly selected microscopic fields ($\times 400$) of the lower slide were counted. Experiments were performed independently three times.

2.7. Matrix metalloproteinase activity (gelatin zymography)

Cells were seeded into a six-well plate at 1×10^3 cells/ml and incubated in a medium containing 10% FBS for 48 h. Supernatants were collected and concentrated using Centricon, and MMP activity of the supernatant was investigated using gelatin zymography. The supernatant was mixed with 2 \times sample buffer (Invitrogen), and Zymography was performed using gels (10% polyacrylamide, 1% gelatin). MMP activities were visualized by staining with Coomassie blue.

2.8. Matrix metalloproteinase amount assay

Cells were seeded into a six-well plate at 1×10^3 cells/ml and were incubated in medium containing 10% FBS for 48 h. Supernatants were collected and concentrated using Centricon. Next, 96-well plates were coated with a 200- μ l coating buffer solution (1.59 mg Na₂CO₃, 2.93 mg NaHCO₃/1 ml DDW, pH 9.6) at 37°C for 1 h. Coated wells were incubated with diluted primary monoclonal antibody (1:200). Prepared supernatant was then added into wells, which were incubated at room temperature for 48 h. Wells were washed twice with 0.02 M PBS (pH 7.4) containing 0.15 M NaCl and were blocked with 1% BSA solution for 3 h and then washed. Wells were incubated with secondary mouse antibody (1:1000) for 2 h at 37°C and then washed. TBM substrate solution (TBM 1 tablet/DMSO 100 ml) was added into wells, and wells were incubated overnight in the dark. The reaction was stopped by the addition of 1 M H₂SO₄. The absorbance was measured at 450 nm in a microplate reader (Molecular Devices).

2.9. Reverse transcriptase polymerase chain reaction

Total RNA was isolated using Tri-reagent (Sigma), and cDNA was synthesized using 2 μ g of total RNA with SuperScript II reverse transcriptase (Invitrogen). For amplification of cDNA, primers for MMP-2 (upstream primer, 5'-CAGGCTCTTCTCCTTTCSCAAC-3'; downstream primer, 5'-AAGCCACGGCTTGTTTTCCTC-3') and primers for MMP-9 (upstream primer, 5'-TGGGCTACGTGACCTATGACCAT-3'; downstream primer, 5'-GCCAGCC-CACCTCCACTCCTC-3', annealing at 55°C for 1 min with 35 cycles) were used. The expression of human β -actin transcripts was examined as an internal control, as described previously [28]. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. Bands corresponding to each specific PCR product were quantified by densitometric scanning of the exposed film using the Bio-profile Bio-IL application (Vilber-Lourmat).

2.10. Statistical analysis

Statistical analysis was performed using the Statistical Analysis System software (SAS Institute, Cary, NC, USA). Data were expressed as means with standard deviations and analyzed via analysis of variance (ANOVA). Statistically significant differences among the means of the groups were tested at $\alpha = .05$ using Duncan's multiple range test.

3. Results

3.1. Effects of [6]-gingerol on growth and adhesion

The viable MDA-MB-231 cell numbers did not differ with [6]-gingerol treatment from 0 to 10 μ M within 24 h (Fig. 1). To elucidate the effects of [6]-gingerol on metastasis, we first examined the effects of [6]-gingerol on cell adhesion. Fig. 2 shows the effect of [6]-gingerol on the

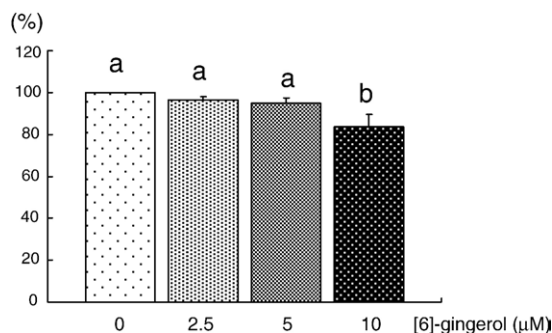


Fig. 2. Effect of [6]-gingerol on adhesion in MDA-MB-231 cells. After 8×10^5 cells/ml suspended in DMEM/F12 containing 0, 2.5, 5, 10 μ M [6]-gingerol had been plated in each well of 96-well fibronectin-coated plates for 1 h, the medium was gently removed, and attached cells were assayed using MTT assay. Each bar represents the mean \pm S.D. calculated from three independent experiments. Comparisons among different concentrations of the [6]-gingerol that yielded statistically significant difference ($P < .05$) are indicated by different letters above each bar.

cell adhesion of MDA-MB-231 cells. [6]-Gingerol had no effect on cell adhesion at 5 μ M, but resulted in a 16% reduction at 10 μ M compared to that at 0 μ M.

3.2. Effects of [6]-gingerol on invasion and motility

The invasion and motility of the MDA-MB-231 human breast carcinoma cell line were examined. [6]-Gingerol showed a dose-dependent inhibitory effect on cell invasion through the Matrigel chamber (Fig. 3). Cell migration (motility) is a critical process of invasion allowing primary

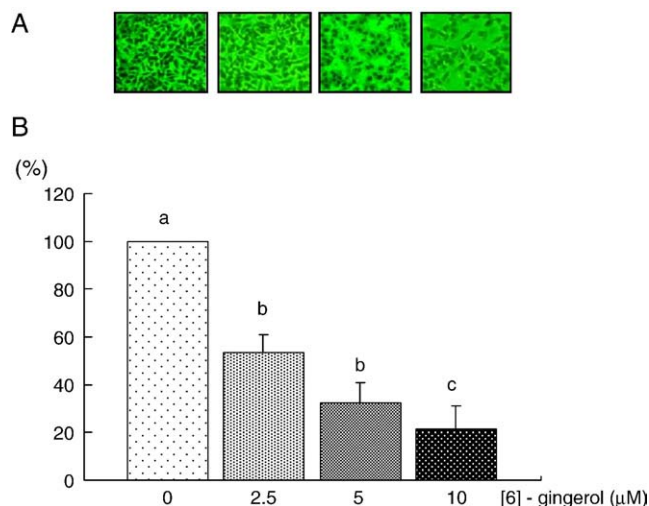


Fig. 3. Effects of [6]-gingerol on invasion in MDA-MB-231 cells. The cells were cultured in the presence of various concentrations of [6]-gingerol for 12 h within a Matrigel invasion chamber. Microphotographs of filters (A) and quantitative analysis of the Matrigel chamber invasion assay (B) are shown. Invasion was expressed as a percentage of control (0 μ M). Each bar represents the mean \pm S.D. calculated from three independent experiments. Comparisons among different concentrations of the [6]-gingerol that yielded statistically significant difference ($P < .05$) are indicated by different letters above each bar.

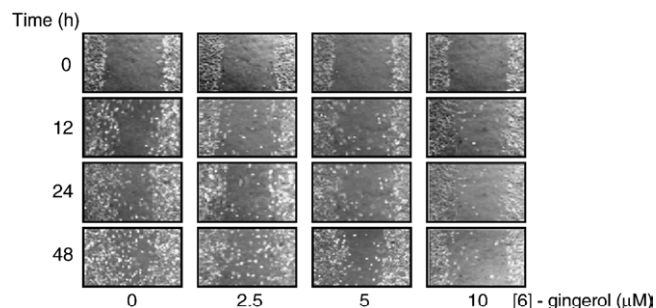


Fig. 4. Effects of [6]-gingerol on migration in MDA-MB-231 cells. The cells were plated in a 12-well plate at a density of 0.5×10^5 cells/well with DMEM/F12 supplemented with 10% FBS. Confluent monolayers were wounded and then incubated in serum-free medium in the presence of 0, 2.5, 5, 10 μ M [6]-gingerol. At 0, 12, 24 and 48 h after wounding, the cells were photographed under a phase contrast microscope.

tumors to metastasize. The treatment of MDA-MB-231 cells with increasing concentrations of [6]-gingerol led to a concentration-dependent decrease in wound healing cell migration (Fig. 4) and cell motility by Boyden chamber assay (Fig. 5).

3.3. Effects of [6]-gingerol on activities, amount and mRNA expression of MMPs

Invasion of BMs is principally mediated by the gelatinase matrix metalloproteases, MMP-2 and MMP-9. To determine whether [6]-gingerol affects the MMP-2 and MMP-9, their activities, amounts and mRNA expressions were tested. Fig. 6 shows that the activity of MMP-2 or MMP-9 in MDA-MB-231 cells was decreased by [6]-gingerol in a dose-dependent manner. The amount of MMP-2 protein in culture

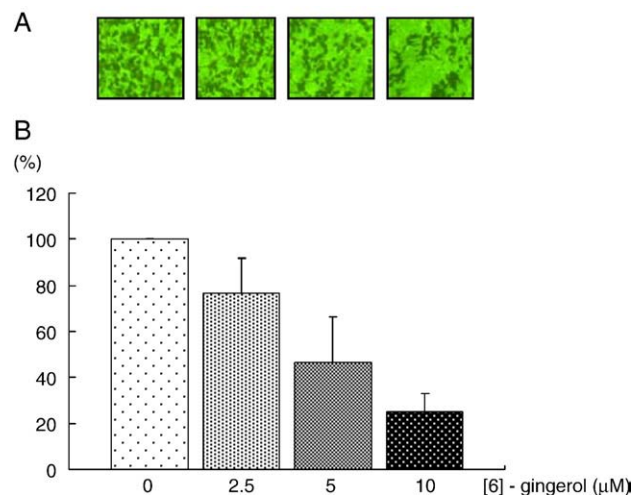


Fig. 5. Effects of [6]-gingerol on cell motility in MDA-MB-231 cells. The cells were cultured in the presence of various concentrations of [6]-gingerol for 22 h within a Boyden chamber. Microphotographs of filters (A) and quantitative analysis of the Boyden chamber assay (B) are shown. Motility was expressed as a percentage of control (0 μ M). Each bar represents the mean \pm S.D. calculated from three independent experiments.

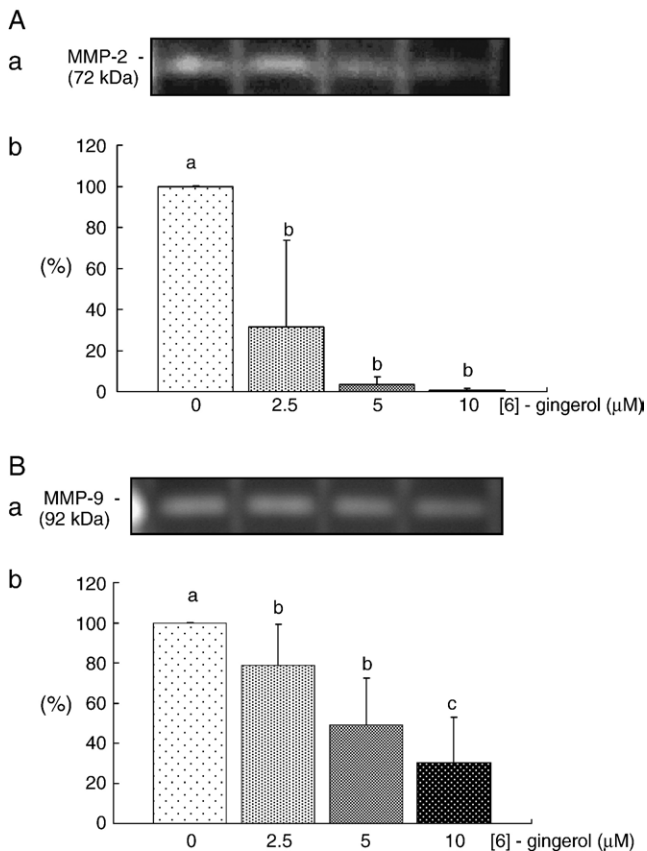


Fig. 6. Effect of [6]-gingerol on MMP-2 and MMP-9 activity in MDA-MB-231 cells. MDA-MB-231 cells were plated in six-well plates at a density of 1×10^5 cells/well with DMEM/F12 supplemented with 10% FBS for 48 h; the monolayers were incubated in serum-free medium in the absence or presence of 0, 2.5, 5, 10 μ M [6]-gingerol for 22 h. Medium was collected, and the activities of MMP-2 (A) and MMP-9 (B) were measured by zymography. (a) Photograph of the MMP bands, which is representative of three independent experiments, is shown. (b) Quantitative analysis of the bands. Each bar represents the mean \pm S.D. calculated from three independent experiments. Comparisons among different concentrations of the [6]-gingerol that yielded statistically significant difference ($P < .05$) are indicated by different letters above each bar.

supernatants was detected to decrease in a dose-dependent manner as a result of MDA-MB-231 cell culture with various concentrations of [6]-gingerol (Fig. 7). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of MDA-MB-231 cell mRNA suggested that the expression of MMP-2 and MMP-9 was decreased in these cells upon treatment with [6]-gingerol (Fig. 8). Although there was no change in the MMP-9 protein levels, the mRNA expression for MMP-9 was decreased by [6]-gingerol treatment.

4. Discussion

This investigation attempted to address the role of [6]-gingerol on metastasis, by which it may exert its antitumor effects. Metastasis is a complex multi-step process involving cell adhesion, invasion and motility. Hence, interruption of

one or more of these steps is one approach for anti-metastatic therapy. To date, there is no direct evidence to show that [6]-gingerol exerts such an effect on the development of breast cancer metastasis. The present study is the first to demonstrate that [6]-gingerol can significantly inhibit the metastatic process of MDA-MB-231 human breast cancer cells.

The initial invasive action of metastatic cells involves interaction of tumor cells with the ECM, through the process of cell matrix adhesion. Once malignant cells have detached from the primary tumor, they bombard the surrounding BMs and adhere to its meshwork of Type IV collagen, laminin and fibronectin [29]. As shown in Fig. 2 of this manuscript, [6]-gingerol inhibits attachment to the fibronectin, which is one of the major components of BM, dose dependently.

The process of tumor cell invasion and metastasis requires the degradation of connective tissue associated with vascular BMs and interstitial connective tissue [30,31]. The BM is the largest barrier between a free malignant cell and the bloodstream, and it must be traversed before malignant cells can enter circulating blood [32]. Therefore, invasion through a BM is a critical step in metastasis [33]. Breast cancer cell invasiveness was

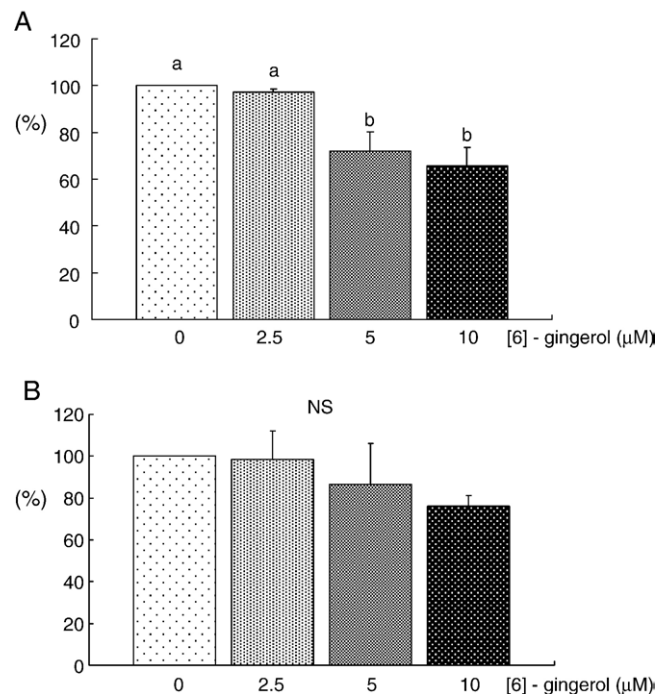


Fig. 7. Effect of [6]-gingerol on MMP-2 and MMP-9 amount in MDA-MB-231 cells. MDA-MB-231 cells were plated in six-well plates at a density of 1×10^6 cells/well with DMEM/F12 supplemented with 10% FBS for 48 h. The monolayers were incubated in serum-free medium in the absence or presence of 0, 2.5, 5, 10 μ M [6]-gingerol for 22 h. Medium was collected and the protein amount of MMP-2 (A) and MMP-9 (B) was estimated by ELISA. Each bar represents the mean \pm S.D. calculated from three independent experiments. Comparisons among different concentrations of the [6]-gingerol that yielded statistically significant difference ($P < .05$) are indicated by different letters above each bar.

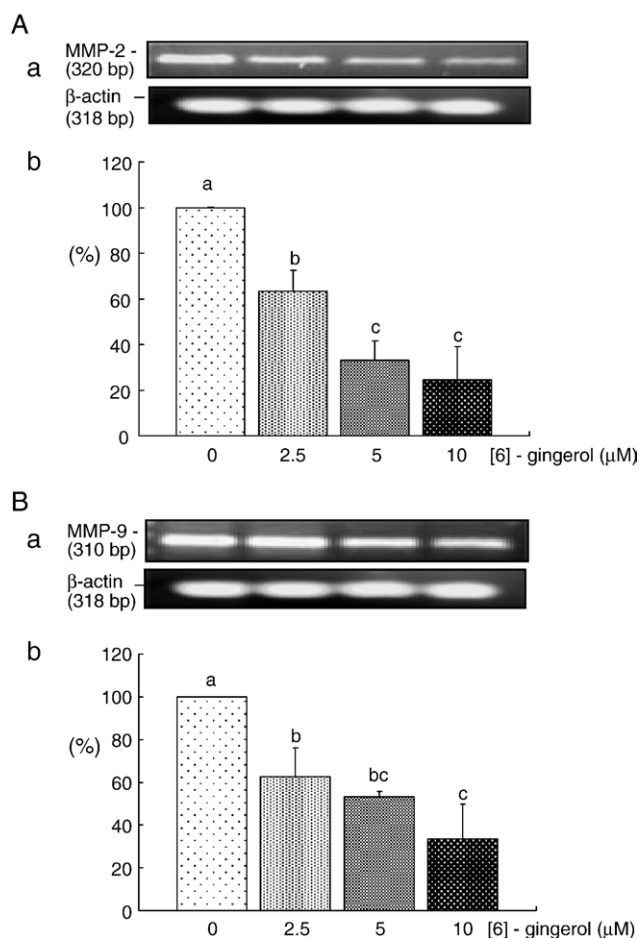


Fig. 8. Effect of [6]-gingerol on MMP-2 and MMP-9 mRNA expression in MDA-MB-231 cells. For RT-PCR, MDA-MB-231 cells were treated with [6]-gingerol. Total RNA was isolated and RT-PCR was performed to investigate the mRNA expression of MMP-2 (A) and MMP-9 (B). (a) Photographs of ethidium bromide-stained gel, which were representative of three independent experiments, are shown. (b) Quantitative analysis of RT-PCR. Relative abundance of each band was estimated by densitometric analysis. Each bar represents the mean \pm S.D. calculated from three independent experiments. Comparisons among different concentrations of the [6]-gingerol that yielded statistically significant difference ($P < .05$) are indicated by different letters above each bar.

investigated in this study using a transwell chamber system. To successfully penetrate the filter membrane, cells must successfully adhere, degrade and traverse the Matrigel-coated insert. The present study showed that [6]-gingerol inhibited the invasion of MDA-MB-231 breast cancer cells in a dose-dependent manner. However, in this study, cell proliferation was not affected by 2.5–10 μ M [6]-gingerol treatment until 24 h (Fig. 1), although cell invasion was decreased during this period. Therefore, these data suggest that the effect on inhibition of cell invasion takes priority over the decrease in cell proliferation.

Motility is another property of cancer cells that is needed for migration from the primary site to a secondary organ. Any alteration of this property would interrupt the metastatic cascade [33]. The present study showed that treatment with

various concentrations of [6]-gingerol could reduce the motility of MDA-MB-231 cells.

The mechanism by which [6]-gingerol inhibits cell adhesion, invasion and migration is not yet clear. Several lines of evidence strongly implicate MMPs, particularly MMP-2, in the process of tumor cell invasion and metastasis. These include a positive correlation between MMP-2 expression and invasive potential, and the inhibition of metastasis formation by MMP inhibitors in vivo [30,34,35].

MMPs are a family of zinc-dependent enzymes consisting of propeptide, catalytic, hinge and COOH-terminal domains. All MMPs are produced in latent forms that require catalytic removal of the propeptide domain for function. MMP-2 and MMP-9 are unique because of the inclusion of three fibronectin Type II repeats within their catalytic domains [36]. Saad et al. [37] demonstrated that MMP-2 significantly contributes to the invasion of bone marrow fibroblasts by MDA-MB-231 cells. These investigators excluded the possibility that contact between breast cancer cells and bone marrow fibroblasts results in the rapid release of inactive membrane-associated MMP-2. Once released, MMP-2 may associate with breast cancer cell-associated membrane Type 1 MMP/tissue inhibitor of MMP-2 complexes, facilitating the activation and subsequent invasion of normal tissue by the malignant cells. Because most malignant cells contain surface-associated fibronectin, this is likely to be an important mechanism for the development of metastases in many cancers. Somiari et al. [38] reported that plasma concentration and activity of MMP-2 were significantly lower in controls compared to those in cancer, benign and high-risk patients. And, MMP-9 expression has been described as a positive prognostic marker in node-negative breast cancer [39].

Here, we show that the activities of MMP-2 and MMP-9 were decreased (Fig. 6) by [6]-gingerol treatment and suggest that the levels of mRNA for MMP-2 and MMP-9 were decreased in MDA-MB-231 cells treated with [6]-gingerol (Fig. 8). Additionally, [6]-gingerol was found to decrease the amount of MMP-2 protein in culture supernatant as a result of treatment of MDA-MB-231 cells with [6]-gingerol at levels above 5 μ M, but the level of MMP-9 remained unchanged.

In conclusion, we have described here for the first time that [6]-gingerol inhibits metastasis of the MDA-MB-231 breast cancer cell line. As such, we suggest that the anticancer effect of [6]-gingerol may contribute to the inhibition of metastasis by decreasing the activities and expressions of MMP-2 and MMP-9. Ginger can be ingested in considerable amounts in the human diet (250 mg–1 g/day). Considering that ginger contains 1.0–3.0% [6]-gingerol and its derivatives [40], the concentration of [6]-gingerol used in this study (i.e., 10 μ M) should be achieved in vivo. So, [6]-gingerol may be useful for preventing or treating some cancer.

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