Zerumbone inhibits tumor angiogenesis via NF-κB in gastric cancer

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Received September 3, 2013; Accepted October 21, 2013

DOI: 10.3892/or.2013.2842

Abstract. Zerumbone derived from a subtropical ginger, *Zingiber zerumbet* Smith, was previously reported to have antitumor growth and anti-inflammatory properties in some types of cancer. However, the effects of zerumbone against cancer angiogenesis have not been fully elucidated. In this study, we clarified the role of zerumbone in gastric cancer angiogenesis. We examined the expression of vascular endothelial growth factor (VEGF) in gastric cancer cell lines both in the basal state and following zerumbone treatment by real-time RT-PCR and enzyme-linked immunosorbent assay (ELISA). Changes in gastric cancer cell proliferation in response to zerumbone treatment were measured by WST-1 assay. Additionally, the effects of zerumbone on NF-κB activity were examined in AGS cells. Finally, the effects of zerumbone on angiogenesis in AGS cells were measured by *in vitro* angiogenesis assay in which human umbilical vein endothelial cells (HUVECs) and fibroblasts were cocultured with AGS cells. Among the 6 gastric cancer cell lines tested, AGS cells exhibited the highest expression of VEGF. Cell proliferation, VEGF expression and NF-κB activity in AGS cells were all significantly inhibited by zerumbone. Moreover, the tube formation area of HUVECs was increased by coculture with AGS cells, and this effect was inhibited by zerumbone. Both VEGF expression and NF-κB activity in AGS cells were reduced by treatment with zerumbone, thereby inhibiting angiogenesis. Thus, zerumbone may become a new anti-angiogenic and antitumor drug in the treatment of gastric cancer.

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Key words: zerumbone, angiogenesis, vascular endothelial growth factor, gastric cancer

Introduction

Gastric cancer is the fourth most frequently diagnosed cancer and the second leading cause of cancer-related mortality (1). An estimated 989,000 new cases of gastric cancer and 738,000 deaths due to gastric cancer occurred worldwide in 2008 alone, which accounted for 8% of all new cases of cancer and 10% of all cancer-related deaths (2). The highest incidence rates of gastric cancer have been reported in Eastern Asia, Eastern Europe, and South America, and the lowest rates have been reported in North America and most parts of Africa (3). In addition to surgical resection, chemotherapy constitutes an important treatment regimen for gastric cancer (4). However, despite major improvements in diagnosis and treatment regimens, gastric cancer remains one of the most lethal types of cancer, with <20% of patients surviving up to 5 years. Thus, novel agents that are nontoxic, efficacious, and can significantly enhance the effects of existing chemotherapeutic drugs are urgently required.

Angiogenesis is one of the most important factors in tumor growth and metastasis. We previously reported the critical role of angiogenesis in tumorigenesis and metastasis in a variety of gastrointestinal carcinomas (5-12). Many angiogenic factors were previously demonstrated to be involved in gastric cancer; among them, vascular endothelial growth factor (VEGF) is one of the major cytokines involved in angiogenesis in gastrointestinal tumors. In combination with its receptors, VEGF promotes endothelial cell proliferation and new blood vessel formation in *in vitro* models of angiogenesis (13). We also previously clarified the role of VEGF in gastric cancer angiogenesis using an original *in vitro* angiogenesis assay model, and revealed a correlation between VEGF expression and the metastatic potential of gastric cancer (14). Moreover, some studies have demonstrated that VEGF expression in gastric cancer correlates with patient survival (15,16). In addition, the serum concentration of VEGF has been reported to be elevated in patients with gastric cancer compared to healthy controls (17) and has been shown to correlate with patient survival (18). Therefore, we hypothesized that VEGF-targeted therapy may have some therapeutic potential in the treatment of gastric cancer.

Aberrant expression of nuclear factor-κB (NF-κB), which belongs to the rel family of transcription factors, has been associated with gastric carcinogenesis (19). Preliminary results have demonstrated that NF-κB is constitutively activated in most human gastric cancer cell lines and primary tumor specimens (20,21). Previous studies have suggested an important role for NF-κB in the regulation of apoptosis, cell adhesion, oncogenesis, and angiogenesis (22). Regulation of gene expression by NF-κB is controlled mainly by inhibitory IκB proteins, including IκBα; on stimulation, IκBα is rapidly phosphorylated and degraded via the ubiquitin-proteasome pathway, permitting activation and nuclear importation of NF-κB (23,24). Moreover, a previous study demonstrated that NF-κB plays an important role in VEGF expression and angiogenesis in gastric cancer (25). These data suggest that NF-κB may be an effective therapeutic target in the treatment of gastric cancer. Bortezomib, a proteasome inhibitor that also inhibits NF-κB activity, is already being used for the treatment of patients with multiple myeloma. In addition, bortezomib has been suggested to have potential as a novel molecular targeting drug for the treatment of unresectable advanced gastric cancer (26). However, treatment with bortezomib has been shown to elicit some critical adverse effects, such as peripheral neuropathy (27). Therefore, new and more nontoxic drugs that inhibit NF-κB activity are required.

Natural products, generally regarded as safe, have been shown to mediate anticancer activities in a variety of cell types (28). Since zerumbone is derived from a subtropical ginger (*Zingiber zerumbet* Smith) and should therefore have minimum toxicity, it is used routinely in traditional medicine (29). Moreover, zerumbone was previously reported to have antigrowth and anti-inflammatory properties in several cancer cell lines (30-38). However, to date, no studies have described such properties in gastric cancer. Treatment with zerumbone has been reported to moderate NF-κB, tumor necrosis factor-α (TNF-α), induced nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and CXC chemokine receptor 4 (CXCR4) (30,31,39). However, the effects of zerumbone on cancer angiogenesis have yet to be fully elucidated.

In the present study, we sought to determine the role of zerumbone in gastric cancer angiogenesis. Initially, we confirmed that the secretion of VEGF in AGS cells was increased compared to secretion of these factors in other gastric cancer cells. Zerumbone inhibited both the secretion of VEGF and the activity of NF-κB in AGS. Consequently, treatment with zerumbone significantly blocked gastric cancer-induced tube formation by human umbilical vein endothelial cells (HUVECs). Coculture of HUVECs and fibroblasts (FBs) for a total of 2 weeks with or without AGS cells allowed us to investigate tumor-induced angiogenesis from the viewpoint of interactions between endothelial cells and their stromal cells, and we therefore evaluated the effects of zerumbone on gastric cancer-induced angiogenesis in more detail *in vitro*. Our data demonstrated that zerumbone suppressed angiogenesis in gastric cancer by blocking NF-κB activity and subsequent production of angiogenic factors. To our knowledge, this is the first study to demonstrate the effective role of zerumbone in gastric cancer angiogenesis. Based on our results, zerumbone may be useful in treating gastric cancer.

Materials and methods

Cell lines and agents. Zerumbone was purchased from Wako, Japan, dissolved in dimethyl sulfoxide (DMSO) as a 50 mM stock, and stored at 4˚C. The following gastric cancer cell lines were used: MKN1, MKN28, MKN45, MKN74 and NUGC4 (JCRB, Japan), and AGS (ATCC, Washington, DC, USA). AGS cells were cultured in HAM-F12 (Wako) with 10% fetal bovine serum (FBS) and 1% antibiotics and antimycotics (10,000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin B per ml; Sigma, St. Louis, MO, USA) at 37˚C in an atmosphere of 5% CO_2 and 95% air. Other cells were cultured in RPMI-1640 (Sigma) with 10% FBS and 1% antibiotics and antimycotics.

WST-1 assay. We examined the proliferation of gastric cancer cells using the Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Japan). Gastric cancer cells (500 or 2,000 cells/well) were seeded into 96-well plates and incubated with different concentrations of zerumbone for 72 h. The premix WST-1 was added to the multi-well plate, and the absorbance was measured at 450 nm in each well using a SPECTRAmax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Real-time reverse transcription polymerase chain reaction (RT-PCR). The mRNA expression of VEGF in gastric cancer cells was measured with real-time RT-PCR. A total of $1x10^5$ AGS cells were cultured with 10 ml medium in 100-mm dishes, treated with zerumbone for 72 h, and collected. RNA was extracted from cell pellets using an RNeasy Plus Mini kit (Qiagen, TX, USA), and RT-PCR was performed using Superscript III First-strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA). The concentration of each cDNA was measured by NanoDrop1000 (Thermo Fisher Scientific, DE, USA) and adjusted to 40 ng/ml with diethylpyrocarbonate (DPEC) water. We performed real-time PCR with FAM-labeled TaqMan probes [*VEGF*: Hs00173625_m, *GAPDH*: Hs99999905_m1, *β2-microglobulin (β2M)*: Hs99999907_m1; Applied Biosystems, Foster City, CA, USA] and TaqMan Universal Master Mix (Applied Biosystems) using Chromo4 (Bio-Rad, Cambridge, MA, USA). PCR was carried out by an initial incubation at 50˚C for 2 min, followed by denaturation at 95˚C for 10 min and 50 cycles of 95˚C for 15 sec and 60˚C for 1 min. The expression of *VEGF* mRNA was normalized to that of *β2M* mRNA. We did not use *GAPDH* as an internal control as zerumbone treatment in gastric cancer cell lines resulted in alteration of *GAPDH* mRNA expression. Since *VEGF* mRNA expression was the highest in AGS cells compared to that in the other gastric cancer cell lines tested (Fig. 1A), we used AGS cells in subsequent experiments.

Enzyme-linked immunosorbent assay (ELISA). The secretion of VEGF was determined using Quantikine ELISA Human VEGF Immunoassay (DVE00; R&D Systems, Minneapolis, MN, USA). A total of $1x10^5$ AGS cells were seeded in 100-mm dishes and incubated with different concentrations of zerumbone. The supernatants of gastric cancer cells were collected after 72 h of treatment with zerumbone. The supernatants were microfuged at 1,500 rpm for 5 min to remove particles and frozen at -20˚C until use in ELISA. According to the manu-

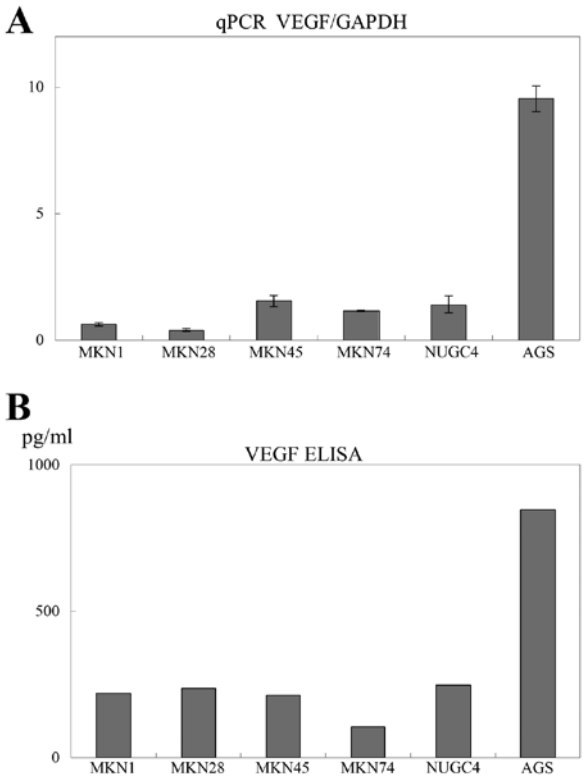

Figure 1. The expression of VEGF in gastric cancer cell lines. (A) The expression of VEGF mRNA was measured by using real-time RT-PCR. The ratios of VEGF and GAPDH were expressed as means ± SD. (B) The secretion levels of VEGF in gastric cancer cell lines were measured by using Quantikine ELISA Human VEGF Immunoassay. The supernatant of semi-confluent gastric cell lines in a 100-mm dish was collected.

facturer's instructions, supernatant samples from gastric cancer cell cultures were added to 96-well microplates coated with mouse monoclonal antibodies targeting VEGF. After washing, horseradish peroxidase (HRP)-conjugated polyclonal VEGF antibodies were added. The wells were washed again, and hydrogen peroxide and tetramethylbenzidine were added. The absorbance of each well was measured, and quantification was carried out using diluted recombinant $VEGF_{165}$ as a standard.

NF-κB (p65) transcription factor assay. A total of $5x10^5$ AGS cells were cultured with 10 ml medium in 100-mm dishes. Cells were treated with zerumbone for 12 h, and nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL, USA). The concentrations of nuclear proteins were measured using a Pierce BCA Protein Assay kit (Thermo Scientific), and protein concentrations were adjusted for equal loading. Equal amounts of nuclear proteins were then added to 96-well plates coated with a specific double-stranded DNA sequence containing the NF-κB response element. NF-κB, extracted from AGS cells, was bound with primary anti-NF-κB antibodies. After washing, secondary antibodies conjugated to HRP were added. The absorbance of each well of the multi-well plate was then measured at 450 nm. We used the transcription factor NF-κB (human p65) positive control (P.C.) from the assay kit and the positive control and transcription factor NF-κB specific competitor dsDNA as a negative control (N.C.).

Electrophoretic mobility shift assay (EMSA). To reconfirm the activity of NF-κB in AGS cells, EMSA was carried out using gel shift assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The following sequence was used for NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3'). Oligonucleotide probes were labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP (3,000 Ci/mmol; Amersham, Piscataway, NJ, USA) and purified by ethanol precipitation. Nuclear extracts (10 μg) were incubated with ^{32}P-labeled oligonucleotide, binding buffer, and gel loading buffer at room temperature. The samples were then loaded on nondenaturing 4% acrylamide gels in 0.5X Tris-borate-EDTA buffer and run at 350 V. The gels were dried and exposed to X-ray film.

In vitro angiogenesis assay. *In vitro* angiogenesis assay was performed using an Angiogenesis kit (Kurabo, Japan). HUVECs and neonatal normal human dermal FBs were cultured in 24-well plates with basal medium and 2% FBS Ham-F12 medium. AGS cells were cultured in the upper chamber, separated from the lower chamber with a membrane having 0.45-μm pores ($2x10^3$ cells/well). The medium was changed on the fourth, seventh, and ninth day. The upper chamber was changed on the seventh day ($2x10^3$ cells/well). HUVECs were stained with anti-CD31 antibodies on the 11th day. Tube formation areas were quantified by counting 8 random fields per sample under a microscope (magnification, x40) and pixelized with an image analyzer. We used 10 ng/ml recombinant VEGF-A as a positive control.

Statistical analysis. The data were analyzed using nonrepeated measures ANOVA and Student-Newman-Keuls test versus the vehicle-treated control (0.1% DMSO). For NF-κB and *in vitro* angiogenesis assay, we used Student-Newman-Keuls test for multiple comparisons.

Results

VEGF expression in gastric cancer cell lines. First, the mRNA expression of *VEGF* was measured in gastric cancer cell lines (MKN1, MKN28, MKN45, MKN74, NUGC4 and AGS) using real-time quantitative RT-PCR and ELISA. The mRNA expression of *VEGF* in AGS cells was higher than that in other gastric cancer cell lines (Fig. 1A). The secretion of VEGF was also higher in AGS cells than in other gastric cancer cell lines (Fig. 1B). Since AGS cells exhibited the highest expression of VEGF, we used AGS cells in subsequent experiments.

Effects of zerumbone on gastric cancer cell proliferation. The proliferation of gastric cancer cells was measured using WST-1 assay. AGS cell proliferation was inhibited by zerumbone at concentrations of ≥10 μM ($P<0.01$; Fig. 2A). Zerumbone also inhibited the proliferation of other gastric cancer cell lines in a dose-dependent manner. MKN45 cells were inhibited by zerumbone at ≥25 μM ($P<0.01$; Fig. 2B); MKN1 cells were inhibited by zerumbone at ≥100 nM ($P<0.05$) and ≥1 μM ($P<0.01$; Fig. 2C); MKN28 cells were inhibited by zerumbone at ≥1 μM ($P<0.01$; Fig. 2D); MKN74 cells were inhibited by zerumbone at ≥1 μM ($P<0.01$; Fig. 2E); and NUGC4 cells were inhibited by zerumbone at ≥10 μM ($P<0.01$; Fig. 2F).

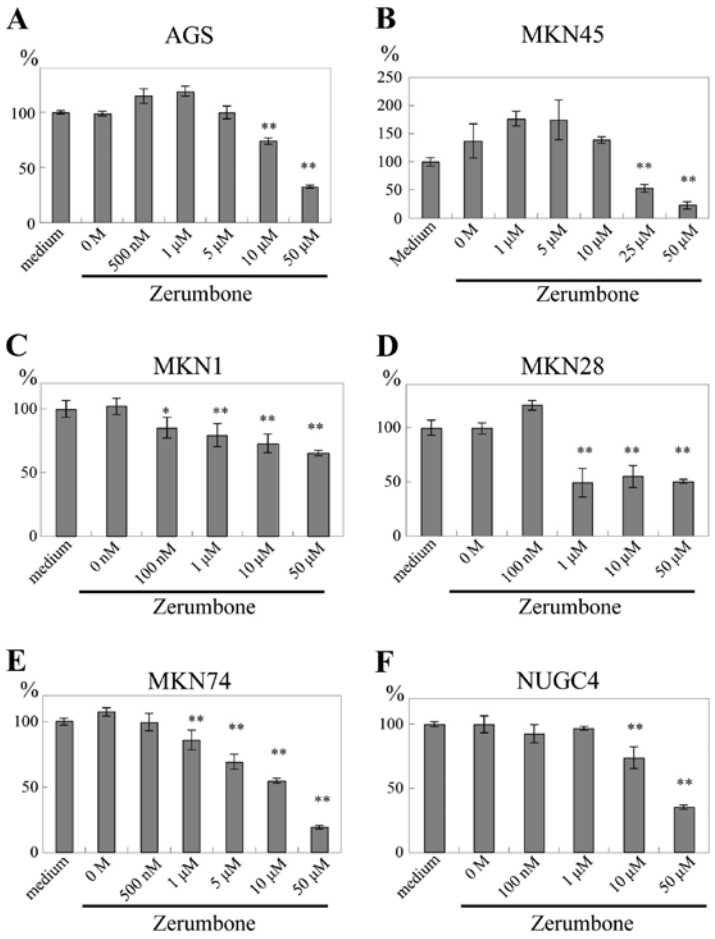

Figure 2. The effects of zerumbone on gastric cancer cell proliferation. The proliferation of gastric cancer cell lines was measured using WST-1 assay. The percentages compared with medium were expressed as means ± SD. Statistical significance was analyzed using one-way ANOVA followed by Student-Newman-Keuls test. **P<0.05 and *P<0.01 vs. control. (A) AGS, (B) MKN45, (C) MKN1, (D) MKN28, (E) MN74, and (F) NUGC4.

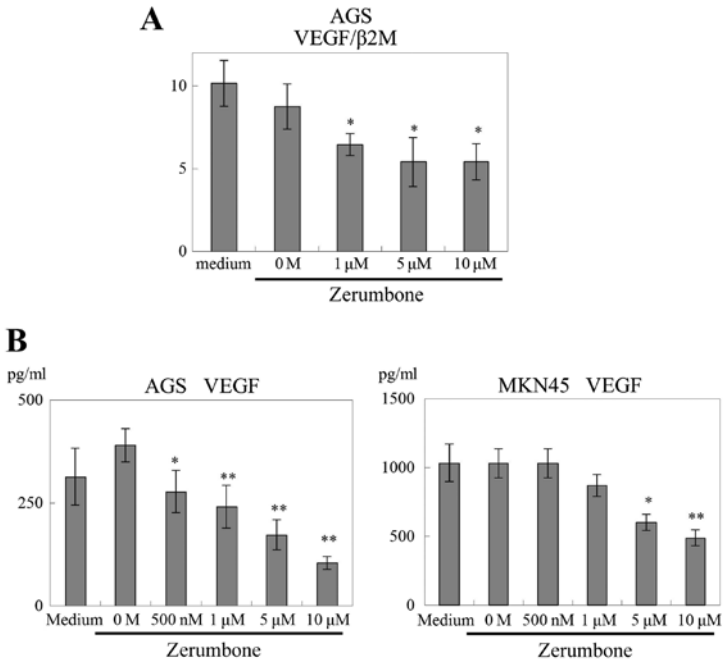

Figure 3. The alteration of VEGF expression by zerumbone. (A) The alteration of VEGF mRNA expression in AGS by zerumbone. For standardization we used β2-microglobulin (β2M). The ratios of VEGF and β2M were expressed as means ± SD. Statistical significance was analyzed by one-way ANOVA followed by Student-Newman-Keuls test. **P<0.05 and *P<0.01 vs. control. (B) The alteration of VEGF secretion levels from gastric cancer cell lines by zerumbone. The concentration of VEGF in the supernatant was measured using ELISA kit and expressed as means ± SD. The supernatants of MKN45 were collected and VEGF concentration was measured by ELISA. Statistical significance was analyzed by one-way ANOVA followed by Student-Newman-Keuls test. **P<0.05 and *P<0.01 vs. control.

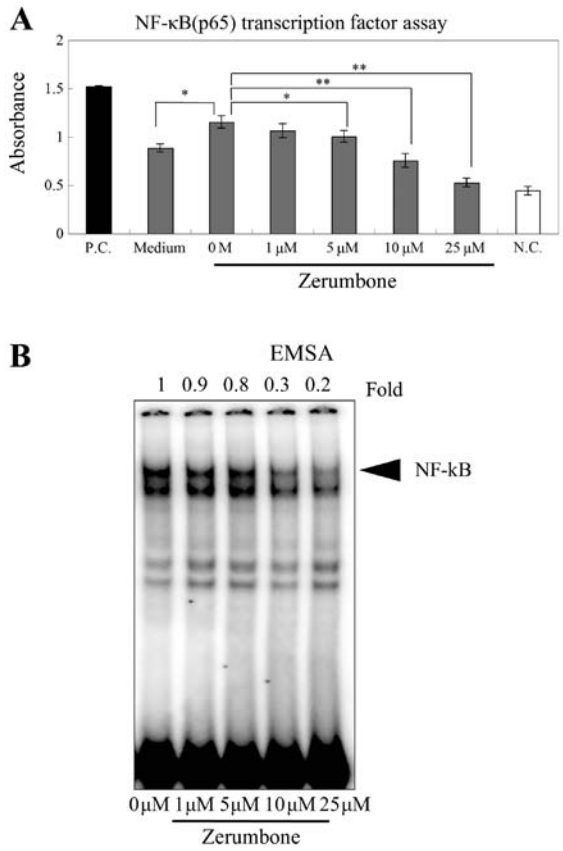

Figure 4. The activity of NF-κB in the nuclear proteins of AGS cells. (A) NF-κB (p65) transcription factor assay. The nuclear proteins were extracted and measured using NF-κB transcription factor assay. We used transcription factor NF-κB (human p65) positive control in the assay kit as positive control (P.C.). In addition, we used both positive control and transcription factor NF-κB specific competitor dsDNA in the assay kit as negative control (N.C.). Statistical significance was analyzed by one-way ANOVA followed by Student-Newman-Keuls test for multiple comparison. **P<0.05 and *P<0.01 vs. control. (B) Electrophoretic mobility shift assay (EMSA). AGS cells were treated with zerumbone for 12 h. Oligonucleotide probes were labeled using T4 polynucleotide kinase and (γ-^{32}P) ATP (3,000 Ci/mmol) and purified by ethanol precipitation. Nuclear extracts (10 μg) were incubated with ^{32}P-labeled oligonucleotide, binding buffer, and gel loading buffer at room temperature. Then the samples were loaded on a nondenaturing 4% acrylamide gel in 0.5X Tris-borate-EDTA buffer and run at 350 V. The gels were then dried and exposed to X-ray film.

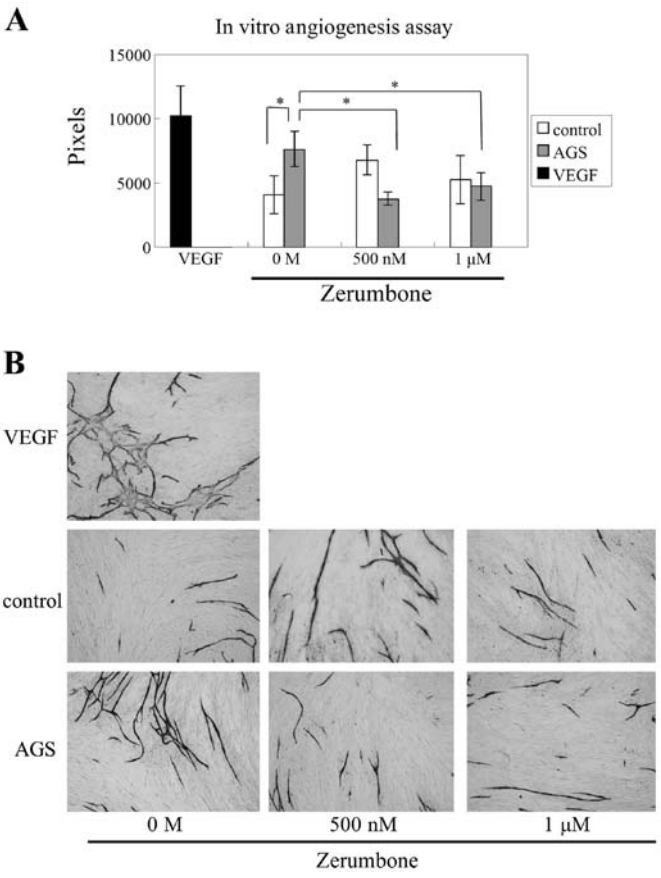

Figure 5. *In vitro* angiogenesis assay. (A) Human umbilical vein endothelial cells (HUVECs) and neonatal normal human dermal fibroblasts (FBs) were cultured in a 24-well plate. The tube formation areas were quantified by counting 8 random fields/sample under the microscope (x40) and pixelized with an image analyzer. The white column shows control, the tube formation of HUVECs cocultured with FBs only. The gray column shows the tube formation of HUVECs cocultured with both FBs and AGS using double chamber methods. The black column shows positive control, the tube formation of HUVECs cocultured with FBs stimulated with 10 ng/ml recombinant VEGF-A. The pixels of tube formation areas were expressed as means ± SD. Statistical significance was analyzed using one-way ANOVA followed by Student-Newman-Keuls test for multiple comparison. **P<0.05 and *P<0.01 vs. control. (B) Microscopic images of angiogenesis assays. These images are representative from three independent analyses of HUVEC tube formation followed by staining with CD31 antibody. The upper column shows the tube formation of HUVECs and FBs with 10 ng/ml recombinant VEGF-A. The middle columns show the control, HUVECs were cocultured with FBs stimulated with different concentrations of zerumbone (0-1 μM). The lower columns show the tube formation of HUVECs cocultured with both FB and AGS cells using double chamber methods stimulated with different concentrations of zerumbone (0-1 μM).

Effects of zerumbone on VEGF mRNA expression in gastric cancer. The effects of zerumbone on *VEGF* mRNA expression in AGS cells were examined. The expression of *VEGF* mRNA (*VEGF/β2M*) in AGS cells was significantly inhibited by the addition of zerumbone in a dose-dependent manner (≥1 μM, P<0.05; Fig. 3A).

Subsequently, the secretion of VEGF protein from gastric cancer cells was measured using VEGF ELISA. Treatment with zerumbone significantly reduced the secretion of VEGF from AGS cells (500 nM, P<0.05; and ≥1 μM, P<0.01; Fig. 3B). In addition, the secretion of VEGF from MKN45 cells was decreased by zerumbone (5 μM, P<0.05; and 1 μM, P<0.01; Fig. 3B).

Effects of zerumbone on the activation of NF-κB in gastric cancer cells. The activity of NF-κB was measured using NF-κB (p65) transcription factor assay. The activity of NF-κB was increased by 0.1% DMSO, the solvent used to dissolve zerumbone. This increased activity was significantly inhibited by ≥5 μM concentrations of zerumbone (≥5 μM, P<0.05; ≥10 μM, P<0.01; Fig. 4A). We also performed EMSA to examine changes in NF-κB expression in response to zerumbone treatment in AGS cells. Consistent with our previous results, the activity of NF-κB was also inhibited by zerumbone in a dose-dependent manner (Fig. 4B).

Effects of zerumbone on HUVEC tube formation. In order to estimate the effects of zerumbone on angiogenesis, we used *in vitro* angiogenesis assay. HUVECs and FBs were cocultured with AGS cells, and the effects of zerumbone treatment were examined. Tube formation by HUVECs was

significantly enhanced by coculture with AGS cells ($P<0.05$; Fig. 5A). Moreover, the enhancement of tube formation by AGS cells was inhibited by zerumbone (500 nM or 1 μM, $P<0.05$; Fig. 5A and B).

Discussion

The aim of the present study was to determine whether zerumbone, a component of shampoo ginger that has been linked to anticancer activities, could suppress NF-κB activity and consequently reduce the production of VEGF in gastric cancer cells. Almost all types of gastric cancer cells produced VEGF, and zerumbone downregulated VEGF expression in various gastric cancer cell lines. In addition to VEGF production, zerumbone suppressed NF-κB expression in gastric cancer cells. Thus, our results showed, for the first time, that zerumbone inhibited the production of VEGF in gastric cancer cells via downregulation of NF-κB.

Angiogenesis is one of the most important factors in tumor metastasis. Along with its receptors, VEGF promotes endothelial cell proliferation and new blood vessel formation in *in vitro* models of cancer associated-angiogenesis (13). VEGF expression in gastric cancer is correlated with survival prognosis (15,16). Moreover, serum VEGF has been reported to be elevated in gastric cancer patients as compared with healthy individuals (17) and has also been correlated with patient survival (18). Therefore, these data support that VEGF-targeted therapy may have some therapeutic potential.

Aberrant expression of NF-κB, which belongs to the rel family of transcription factors, has been associated with gastric carcinogenesis (19). Previous studies have suggested an important role for NF-κB in the regulation of apoptosis, cell adhesion, oncogenesis and angiogenesis (22). In addition, another study demonstrated that NF-κB plays an important role in VEGF expression and angiogenesis in gastric cancer (25). Therefore, NF-κB may be a therapeutic target in the treatment of gastric cancer. NF-κB inhibitors, such as bortezomib, are already being used for patients with multiple myeloma; however, these inhibitors commonly have adverse side-effects that limit their widespread use (40). Thus, new and more nontoxic drugs that inhibit NF-κB activity are needed.

In this study, we focused on the effects of natural products, which are generally regarded as safe and nontoxic. Some natural products have been reported to have anticancer effects. Natural products, such as curcumin (41), baicalin (42), sesamin (43), and zerumbone (29), have been reported to regulate cell survival, proliferation and invasion in some types of cancer. Additionally, some studies have demonstrated that the effects of these natural products on cancer are derived from the inhibition of NF-κB activity (41).

Zerumbone is derived from a subtropical ginger (*Z. zerumbet* Smith) and has a molecular weight of 218.33 Da. *Z. zerumbet* is commonly known as the pinecone or shampoo ginger and has several names in various countries, including 'Lempoyang' (Malaysia and Indonesia), 'Awapuhi' (Hawaii) (29,36,44-47), 'Hana shoga' and 'White ukon' (Japan). The white rhizome of *Z. zerumbet* is traditionally used as a botanical medicine for the treatment of several conditions and was previously reported to have anti-inflammatory (45,46,48,49), antinociceptive (45,49,50), antimicrobial (51-54), and anti-allergic effects (55). It was also found to have anticancer effects in colon cancer (30,32-34), leukemia (35), myeloid cancer (36), liver cancer (37), breast cancer (31,38), pancreatic cancer (31), and lung cancer (34). However, to date, no studies have reported the inhibition of gastric cancer by zerumbone. The molecule targets of zerumbone include cyclooxygenase 2 (COX2) (56,57), free radical generation (30), NF-κB (34,38,39), iNOS (56), and CXCR4 (31), among others. However, the effects of zerumbone on cancer angiogenesis have not been elucidated. Therefore, in this study, we investigated the effects of zerumbone on angiogenesis in gastric cancer cells.

Based on this premise, we first examined the expression of VEGF in several gastric cancer cell lines. mRNA and protein expression of VEGF were observed in all tested gastric cancer cells. Since AGS cells exhibited the highest levels of VEGF expression, we primarily used these cells in our subsequent experiments to determine the anticancer effects of zerumbone. Zerumbone significantly inhibited the proliferation of both AGS and MKN45 cells. Additionally, we provided evidence supporting that zerumbone markedly altered VEGF expression in gastric cancer cells, including secretion of VEGF protein from both AGS and MKN45 cells. Based on these results, we concluded that zerumbone has not only direct pro-apoptotic effects on gastric cancer cells (at higher concentrations), but also anti-angiogenic effects, mediated through reduction of VEGF production by gastric cancer cells (at lower concentration). Our data demonstrated that zerumbone partially induced apoptosis in AGS cells at concentrations greater than 10 μM, but significantly decreased VEGF production in AGS cells at 500 nM. We did not observe any cytotoxicity (by WST-1 assay) in AGS cells at this low concentration, indicating that zerumbone may have potential use as a nontoxic agent in the treatment of gastric cancer.

To clarify the molecular signaling mechanisms through which zerumbone inhibited the production of VEGF, we examined the effects of zerumbone on NF-κB activity. Our data revealed that the activity of NF-κB was significantly inhibited by zerumbone in a dose-dependent manner. In gastric cancer, many studies have demonstrated that NF-κB is constitutively activated and promotes tumorigenesis (19-21). Additionally, some studies have revealed that *Helicobacter pylori* activates NF-κB and consequently enhances carcinogenesis (58,59). NF-κB activity has also been shown to stimulate VEGF production from gastric cancer cells (60). These previous studies are consistent with our results demonstrating that zerumbone inhibited NF-κB and consequently decreased VEGF production in gastric cancer cells.

Finally, we examined whether zerumbone regulated the vascularization of gastric cancer using *in vitro* angiogenesis assay. Tube formation by HUVECs was significantly enhanced by coculture with AGS cells, and this effect was significantly inhibited by zerumbone. To our knowledge, this is the first study to demonstrate the marked effects of zerumbone on gastric cancer-induced angiogenesis. Since zerumbone did not decrease HUVEC tube formation during coculture with FBs only (i.e. without gastric cancer cells), we concluded that zerumbone inhibited gastric cancer-induced angiogenesis by affecting the gastric cancer cells directly. Even low concentrations of zerumbone decreased VEGF production from gastric cancer cells. Therefore, the natural product zerumbone may

have the potential to become an anti-angiogenic drug in the treatment of gastric cancer.

In conclusion, our results indicated that zerumbone inhibited both proliferation and angiogenesis in gastric cancer, and these effects were correlated with the suppression of NF-κB. Notably, gastric cancer-induced angiogenesis was inhibited by zerumbone, even at low concentrations. Since low-dose zerumbone did not block angiogenesis associated with normal physiological function, use of the natural product zerumbone may be safer and may show reduced toxicity compared to other available treatments. Therefore, zerumbone has potential use as a new anti-angiogenic drug for the treatment of gastric cancer.

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