Allicin Inhibits Blood Vessel Growth and Downregulates Akt Phosphorylation and Actin Polymerization

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INTRODUCTION
Angiogenesis is a multistep cellular process in which new vessels emerge from preexisting endothelial vasculature in the presence of various growth factors and extracellular matrix (ECM) proteins (1). This process includes movement of endothelial cells (EC) to the site of future vessel development, proliferation, and formation of tube-like structures followed by recruitment of pericytes and smooth muscle cells, that is, vessel maturation (1). Evidently, initial stages of vessel growth are dependent on the integrity of cellular cytoskeleton in ECs and the ability of intracellular monomeric actin to polymerize (2).

Angiogenesis underlies a wide spectrum of both physiologic and pathologic processes including primary tumor growth, invasion, and metastases (3,4). In addition, inflammation and angiogenesis are linked together (5), and blood vessel growth constitutes an essential part in the pathogenesis of a number of inflammatory-mediated diseases including inflammatory bowel disease and rheumatoid arthritis (6,7). Therefore, inhibition of angiogenesis is a challenge that modern biology faces, and the development of novel antiangiogenic agents is highly important.

Allicin (diallyl-thiosulfinate) is a major ingredient of fresh garlic extract. It is produced during the crushing of garlic cloves by the chemical interaction between the nonprotein amino acid allin and the enzyme alliinase, which is also present in the cloves (8). Allicin has a variety of therapeutic properties. For example, it exhibits antimicrobial activity against various Gram-negative and Gram-positive bacteria and even acid-fast bacteria (9,10). In addition, allicin reduces serum cholesterol and lowers the low-density lipoprotein (LDL) level and LDL oxidation (11), thereby reducing the risk of atherosclerosis and the formation of fatty streaks (atherosclerosis) in hyperlipidemic mice (12). Allicin inhibits the proliferation of human tumor cells (13). Moreover, we have recently demonstrated that allicin affects the immune system via the inhibition of T-cell adhesion to and migration through one of the ECM glycoproteins, fibronectin, and through the human umbilical vein EC layer (14).

Lately, different garlic extract components other than allicin have been reported to affect angiogenesis-related properties of ECs. For example, diallyl trisulfide inhibits Akt phosphorylation and suppresses the expression of vascular endothelial growth factor (VEGF) receptors on the cell membrane (15,16). However, effects of allicin, a major component of garlic, on angiogenesis remain poorly investigated. In this study, we demonstrate that allicin inhibited basic steps of the angiogenic process such as proliferation of ECs and their ability to form tube-like structures. Furthermore, we show the downregulation of actin polymerization and Akt phosphorylation in ECs as potential molecular mechanisms underlying the effects of allicin.
MATERIALS AND METHODS

Reagents and Antibodies

Allicin (kindly provided by Dr. T. Miron, Weizmann Institute of Science) was prepared by applying synthetic allicin onto an immobilized alliinase column as previously described (17). The concentration of allicin was determined according to Miron et al. (18). Matrigel and tissue culture supplements were purchased from Biological Industries, Beit-Haemek, Israel. Tissue culture plates were from Becton Dickinson Labware (Franklin Lakes, NJ). Antiphosphorylated Akt (pAKT) and anti-Akt was obtained from Cell Signaling Technology (Beverly, MA). VEGF and basic fibroblast growth factor (bFGF) were from Peprotech (Rocky Hill, NJ). All other reagents were from Sigma, Israel.

Rat Aortic Ring Assay

Aortic rings were prepared using a protocol first described by Nicosia and Ottinetti (19) with several modifications (20). Aorta was obtained from male SABRA rats (weight 170 to 200 g; obtained from Harlan laboratories, Indianapolis, IN). Rats were sacrificed; aortas were incised, cleaned of surrounding connective tissue, and cut into 1-mm rings. The rings were then maintained in Bio-MPM medium (Biological Industries, Kibbutz, Beit-Haemek, Israel) supplemented with glutamine and antibiotics overnight (37°C, 8% CO2, humidified atmosphere).

Type I collagen obtained as described elsewhere (21) was mixed with modified eagle medium (MEM) ×10 and a 0.3 M solution of NaHCO3 in a ratio of 7:1:2. The resulting mixture (400 μl) was immediately transferred in separate wells of 24-well cell culture dishes and incubated for 15 min (37°C) to obtain a gel. Aortic rings were placed in the middle of the wells (1 ring per well) and covered with another 200 μl of the collagen mixture. After a short incubation period (15 min, 37°C), Bio-MPM medium (500 μl) supplemented with glutamine and antibiotics was put into wells, and allicin was added to the appropriate samples. The medium was changed every 3 days. After 6 days of incubation, the developed capillary network was fixed with 4% buffered formalin and stained with 0.02% crystal violet solution in ethanol. These samples were photographed with an inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan) equipped with a digital camera [Digital Video Camera Co. (DVC); Austin, TX]. ImageJ version 1.330 software for PC by Wayne Rasband was used for image analysis of total tubule length.

Endothelial Cell Proliferation

Bovine aortic endothelial cells (BAEC; kindly provided by Professor N. Savion, Tel Aviv University) were used as reference. BAEC were generally stored in liquid nitrogen. Before starting experiments, cells were defrosted and grown in Dulbecco’s (D)-MEM, supplemented with 10% foetal calf serum, L-glutamine, and 1% penicillin/streptomycin (8% CO2, 37°C, humidified atmosphere) to 90% confluence and then passaged. Cells were used between passages 10 through 15. Proliferation of ECs was estimated by measuring the ability of the cells to metabolize (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, Israel) to formazan. Cells were seeded onto 96-well plates (1 × 104 cells per well), and various concentrations of allicin with or without bFGF were added. After 48 h incubation, cell monolayers were rinsed with phosphate-buffered solution (PBS) and a 1 mg/ml MTT reagent was added (4 h, 37°C). The formed formazan crystals were dissolvd in dimethyl sulfoxide, and absorbance was measured at 570 nm using an enzyme-linked immunoabsorbent assay reader.

Tubule Formation Assay

Twenty-four well tissue culture plates were coated with a thin layer (50 μl/well) of matrigel. BAEC (5 × 104) were seeded in 0.5 ml of serum-free D-MEM. Endothelial cells were allowed to adhere to the surface for a period of 2 h after which allicin in various concentrations was added. After 18 h of incubation (37°C, 8% CO2, humidified atmosphere), medium was withdrawn, and detached cells were removed by 3 washings with PBS. Samples were then fixed (ice-cold 80% ethanol, 1 h) and stained with 0.02% crystal violet solution in ethanol. These samples were photographed with an inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan) equipped with a digital camera [Digital Video Camera Co. (DVC); Austin, TX]. ImageJ version 1.330 software for PC by Wayne Rasband was used for image analysis of total tubule length.

Actin Polymerization

ECs that were grown to confluence on coverslips were incubated with various concentration of allicin (2 h, 37°C in a 8% CO2, humidified atmosphere). Cells were then washed, treated with 50 ng/ml of VEGF for 15 min at 37°C and fixed by 3.7% paraformaldehyde for 30 min at 22°C. Next, cell membranes were permeabilized (5 min) in a solution containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (20 mM), sucrose (300 mM), NaCl (50 mM), MgCl2 (3 mM), and TritonX-100 (0.5%), and cells were stained (30 min) with fluorescein isothiocyanate (FITC)-phalloidin (2 μg/ml) and 4′-6-Diamidino-2-phenylindole (DAPI; 2 μg/ml, 5 min). The slides were photographed using an upright fluorescent microscope Olympus (Nikon, Tokyo, Japan) coupled with a charge-coupled device camera (DVC, Austin, TX). ImageJ version 1.330 software for PC by Wayne Rasband was used for image analysis. F-actin polymerization was quantified by calculating the average fluorescence intensity of the cell population after background subtraction as previously described (22), and to depict the resulted cell morphological changes, we used the cytoplasmic to nuclear ratio as previously described (23).

Western Blot Analysis of EC Cell Lysates

ECs that were grown on Petri dishes until 90% confluence were maintained in serum-free D-MEM for 12 h. Next,
cells were incubated with various concentration of allicin (1 h, 37°C in 8% CO₂, humidified atmosphere), washed, and activated with VEGF (50 ng/ml, 15 min, 37°C). Thereafter, cells were lysed (60 min, 4°C) in a buffer containing ethylendiamine tetraacetate acid (0.5 mM), NaCl (150 mM), NaF (10 nM), Tris (pH 7.5, 25 mM), Triton X-100 (1%), phenylmethylsulphonyl fluoride (200 µg/ml), and a phosphatase inhibitor cocktail (1%; Sigma), cleared by centrifugation (30 min, 14 × 10³ rpm), and the supernatants were analyzed for protein content. A sample buffer was then added, and after boiling, samples were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membranes. The membranes were blocked [Tris-Buffered Saline Tween buffer containing low-fat milk (5%), Tris (pH 7.5, 20 mM), NaCl (135 mM), and Tween 20 (0.1%)], and probed with antiphosphorylated Akt (pAkt) and antitotal Akt2 antibodies. Immunoreactive protein bands were visualized using labeled secondary Abs and the enhanced ECL system. The results of densitometry (performed using the NIH image program) were normalized to the total protein bands.

RESULTS

Allicin Downregulated VEGF/bFGF-Induced Angiogenesis in the Aortic Ring Model

The area covered with sprouting vessels in the absence of any additional stimuli was 0.3 ± 0.5 mm² (Fig. 1). The addition of VEGF together with bFGF resulted in marked sprouting (5.1 ± 3.1 mm²) that remained unaffected in the presence of a low concentration of allicin (0.2 µM; vessel area 4.7 ± 2.2 mm², P > 0.2 vs. VEGF/bFGF). In contrast, increased amounts of allicin significantly inhibited the angiogenic response in a dose-dependent fashion (vessel area 1.8 ± 1 mm² and 0.5 ± 0.6 mm² for 0.5 µM and 1 µM of allicin, respectively). It is noteworthy that the highest dose of allicin used in this set of experiments (1 µM) actually abrogated the effect of proangiogenic cytokines to the level of unstimulated rings (P > 0.5).

Effect of Allicin on Endothelial Cell Proliferation

The ability of ECs to proliferate is one of the basic properties necessary for angiogenesis. Therefore, we next evaluated the effect of allicin on EC proliferation. As shown in Fig. 2, allicin alone did not affect this process. As expected, bFGF induced a substantial proliferation that was reversed by increasing amounts of allicin in a dose-dependent manner. The minimal allicin concentration that significantly inhibited EC proliferation was 0.5 µM. Maximal inhibitory effect was achieved already with 1 µM of allicin, and further increases in allicin concentration did not result in additional inhibition (P = 0.1 between 1 mM and 10 mM of allicin).

Allicin Inhibited the Formation of Tube-Like Structures by ECs

The ability of ECs to organize in tubules is essential for successful vessel development at the later stages of angiogenesis. When seeded on a matrigel layer, BAEC spontaneously formed tubules in the absence of any additional stimulation (Fig. 3). Incubation with allicin strongly prevented this naturally occurring process in a dose-dependent manner. This effect started at an allicin concentration of 1 µM. At higher doses of allicin, no tubules developed, and EC grew in a regular monolayer, although no dead cells were observed in the medium.

Influence of Allicin on VEGF-Induced Actin Polymerization in ECs

Taking into account the importance of cell cytoskeleton rearrangement for most angiogenic stages, we next addressed the effect of allicin on actin polymerization by staining cells for F-actin. The addition of VEGF induced the appearance of actin threads inside ECs (Fig. 4A1–A2) that was accompanied by a significant increase in cell fluorescence intensity. It was accompanied by morphological changes because the cytoplasmic to nuclear ratio was also increased (Fig. 4B and 4C). Concomitant incubation with allicin induced a significant downregulation of both parameters in a dose-dependent manner. The minimal allicin concentration that significantly inhibited EC fluorescence intensity and cytoplasmic to a nuclear ratio was 0.5 µM. At the allicin concentration of 4 µM, inhibition of both parameters reached a level even lower than observed in intact ECs.

Allicin Inhibits VEGF-Induced Akt Phosphorylation

The previous experiments indicated that allicin might serve as an inhibitor of vessel sprouting by downregulating EC proliferation, tubule formation, and VEGF-induced actin-polymerization. Akt is a kinase that participates in signaling cascade leading to the activation of the cytoskeleton and therefore to actin polymerization (24). This kinase is phosphorylated following stimulation of EC with VEGF (25). Therefore, we next studied the effect of allicin on VEGF-induced Akt phosphorylation. Figure 5 demonstrates that whereas VEGF stimulation increased Akt phosphorylation, preincubation with allicin downregulated the VEGF-induced phosphorylation of this kinase. Significant inhibition was achieved already at 1 µM of allicin, and this inhibitory effect reached a maximum (of the concentrations used) at 4 µM. Thus, the suppression of VEGF-induced actin polymerization is accompanied by decreased VEGF-induced Akt phosphorylation.

DISCUSSION

Consumption of garlic and its derivatives is widely common. These substances possess various biological activities and a potential for treating several pathological conditions (9–13). Herein, we demonstrated a strong antiangiogenic effect of
Allicin and inhibition of angiogenesis

Fig. 1. Allicin inhibits vessel growth in the aortic ring model. Rat aortic rings were isolated and grown as described in the Material and Methods. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF; both 50 ng/ml) with or without different concentrations of allicin were added on Day 1 of the experiment, and samples were maintained for 6 days. A: Representative experiment out of 3 performed in duplicate. B: Area covered with sprouts (mean ± SD) calculated from 3 experiments performed. *, P < 0.05.

Allicin, a major component of garlic. In particular, allicin reduced angiogenesis in the aortic ring model as well as basic stages of vessel growth including EC proliferation and tubule formation. These effects were accompanied by downregulation of intracellular actin polymerization and Akt phosphorylation. These findings might suggest a potential use of garlic or its major ingredient, allicin, in the complex treatment of angiogenesis-based diseases.
FIG. 2. Allicin downregulates basic fibroblast growth factor (bFGF)-induced endothelial cell proliferation. Bovine aortic endothelial cells were grown in serum-free medium in the presence of different concentrations of allicin with or without bFGF (50 ng/ml) for 2 days. Cell proliferation was analyzed using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test. Representative experiment out of 3 performed in quadruplicate. *, $P < 0.05$.

Although the ability of ECs to proliferate is by itself insufficient for successful angiogenesis, EC proliferation is one of the essential stages of vessel development (1). There is a correlation between the ability of an agent to reduce EC proliferation and its antiangiogenic potential (26). Whereas allicin markedly suppressed bFGF-induced EC proliferation in a dose-dependent manner, it did not affect intact EC. This suggests that the observed effect did not result from any toxic properties of allicin but rather interference in certain intracellular signaling pathways through which bFGF operates. These results are in agreement with previously reported inhibition of cancer cell proliferation by allicin (13). The fact that allicin exerted its effect only on actively proliferating cells may suggest a potential use of the substance for treating pathological conditions in which intensive cell proliferation is a prerequisite for their further development.

Whereas EC proliferation predominates at the early stages of angiogenesis, subsequently ECs organize into tubule-like shapes. This property of EC is often considered as representative of the whole angiogenic process (27). We showed that allicin prevented the formation of these primitive vessels resulting in cell growth in a monolayer but without leading to cell death and detachment. This effect may be considered as avoidance of EC differentiation (28).

Although both proliferation and tube formation are pivotal steps in angiogenesis, this process is complex and depends on many different factors. Therefore, we explored the net effect of allicin on blood vessel growth. Recently it was reported that aged garlic extract affects several angiogenesis-related properties of EC and attenuates cancer angiogenesis (16,29). In this study, by using the aortic ring model as a reference approach reflecting angiogenesis in toto, we demonstrated for the first time that allicin abolished vessel sprouting induced by VEGF and bFGF. This finding suggests that regardless of allicin effects on separate stages of vessel sprouting, the net effect of allicin on the total angiogenic process is its inhibition.

VEGF operates via different signaling routes regulating cytoskeletal rearrangement and actin polymerization (2). We demonstrated that allicin suppressed VEGF-induced actin polymerization, resulting in a decline of the cytoplasmic:nuclear ratio in ECs. This finding can be at least one of the possible explanations for the antiangiogenic effect of the substance. Indeed, polymerization of actin constitutes a pivotal process underlying cytoskeleton rearrangement that is obligatory to cell movement, attachment, and proliferation (30). In turn, EC migration and adhesion at the site of future vessels with further propagation are features necessary for angiogenesis (1). Therefore, by inhibiting intracellular cytoskeleton organization, allicin could downregulate the whole process of vessel growth. In addition, it has been reported that within somatic cells with a normal ratio between the cytoplasm and the nucleus, a well-developed network of cytoskeletal elements (predominantly actin) protects the nucleus against various types of stress (31). Therefore, the observed decrease in the relative cytoplasmic volume might indicate elevated susceptibility of cells to stress stimuli that develops under the influence of very high doses of allicin.

Akt is one of the central kinases involved in cell proliferation and survival associated with the antiapoptotic effect (32,33). Due to its role in these vital processes, Akt is important for angiogenesis (34). We demonstrated that allicin suppressed Akt phosphorylation. This could explain the inhibitory effect of allicin on actin polymerization because Akt is known to mediate intracellular events leading to cytoskeletal rearrangement. In addition, suppressing phosphatidylinositol 3 kinase/Akt pathway results in the inhibition of EC proliferation (32). Another potential mechanism of allicin-induced downregulation of angiogenesis via Akt is the substantial inhibition of cell survival that could suppress the angiogenic process already at its early stages.

The beneficial biological effect of allicin is attributed to its rapid interaction with thiol-containing proteins (35). Allicin, which has high membrane permeability, can penetrate very
Fig. 3. Allicin suppresses tubule formation by endothelial cells. Bovine aortic endothelial cells were seeded on a matrigel layer, allowed to adhere for 2 h, and then allicin in different concentrations was added. Cells were maintained for 18 h and then fixed, stained, and photographed. Pictures were analyzed and the total tubule length was measured. A: Representative experiment out of 3 performed in duplicate. B: Tubule length (mean ± SD) calculated from all experiments performed.
Allicin inhibits actin polymerization in endothelial cells (EC). Bovine aortic endothelial cells were incubated with various concentrations of allicin (2 h), washed, and further incubated with vascular endothelial growth factor (VEGF; 50 ng/ml, 15 min). Thereafter, cells were fixed and stained (30 min) with fluorescein isothiocyanate-phalloidin and 4′-6-diamidino-2-phenylindole. Then samples were photographed and calculated for cell population fluorescence intensity (B), expressed as % normalized to non-VEGF induced EC and for cytoplasmic to nuclear ratio (C). A: Representative experiment out of 4 performed in duplicate. B and C: Calculated from all experiments performed (expressed as mean ± SEM), *, P < 0.05.

Allicin, being a major active component of fresh garlic extract, strongly suppresses angiogenesis by affecting central steps of this process. These results encourage future efforts to apply allicin in the complex treatment of angiogenesis-dependent diseases such as cancer growth. Due to its short life span in the blood, allicin could potentially be given using intravenous dripper, although recent studies report in vivo efficacy...
of allicin given per os (38). Potential mechanisms of the antiangiogenic effect might include inhibition of cytoskeleton reorganization and Akt phosphorylation, although other pathways can not be excluded and their elucidation requires further studies.

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REFERENCES


