Aged Garlic Extract Inhibits Angiogenesis and Proliferation of Colorectal Carcinoma Cells

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ABSTRACT Because colorectal cancer is likely to develop in many people at some point during their lives, prevention has become a high priority. Diet and nutrition play an important role during the multistep colon carcinogenic process. Garlic has been traditionally used as a spice and is well known for its medicinal properties; several studies have indicated its pharmacologic functions, including its anticarcinogenic properties. However, the mechanisms by which garlic can prevent colorectal cancer remain to be elucidated. This study investigated the effect of aged garlic extract (AGE) on the growth of colorectal cancer cells and their angiogenesis, which are important microenvironmental factors in carcinogenesis. AGE suppressed the proliferation of 3 different colorectal cancer cell lines—HT29, SW480, and SW620—in the same way, but its effects on the invasive activities of these 3 cell lines were different. The invasive activities of SW480 and SW620 cells were inhibited by AGE, whereas AGE had no effect on the invasive activity of HT29 cells. The action of AGE appears to be dependent on the type of cancer cell. On the other hand, AGE enhanced the adhesion of endothelial cells to collagen and fibronectin and suppressed cell motility and invasion. AGE also inhibited the proliferation and tube formation of endothelial cells potently. These results suggest that AGE could prevent tumor formation by inhibiting angiogenesis through the suppression of endothelial cell motility, proliferation, and tube formation. AGE would be a good chemopreventive agent for colorectal cancer because of its antiproliferative action on colorectal carcinoma cells and inhibitory activity on angiogenesis. J. Nutr. 136: 842S–846S, 2006.

KEY WORDS: • aged garlic extract • colon cancer • angiogenesis • chemoprevention

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In Western countries and Japan, the number and mortality rate of patients with colorectal cancer have been increasing, and these patients now represent the largest segment of the population with cancer (1). When it is considered that patients with gastric and uterine cancer have represented the largest proportion of individuals with cancer in Japan in previous years, and that the Japanese lifestyle has recently been quickly Westernized, lifestyle has been supposed to play an important role in cancer (2). Lifestyle factors such as diet and exercise are known to be intimately associated with colorectal cancer, which suggests that colorectal cancer can be prevented to some degree by lifestyle alterations (3).

Colorectal cancer arises from normal cells as a consequence of multistep carcinogenesis. The majority of cases occur spor-
radically, caused by noninherited factors such as diet and other environmental factors (4). The multistage carcinogenic process is actually an accumulation of multiple genetic defects in somatic cells, which may be influenced by several dietary factors. Diet and nutrition play an important role in the cause and primary prevention of colon cancer. Growing interest is now being focused on dietary changes that can contribute to the inhibition of carcinogenesis (5,6). Dietary agents contain various nutrients and/or nonnutrient compounds possessing antimutagenic and anticarcinogenic properties.

Convincing epidemiologic evidence strongly suggests that diets rich in vegetables protect against cancers of the colon and rectum (7). Garlic (Allium sativum), traditionally used as a spice in Asian and other cuisines, is well known for its medicinal properties, with varied pharmacologic functions (8). The anticarcinogenic properties of garlic have been indicated in several studies (9–11). However, the mechanisms through which garlic prevents colorectal cancer remain to be elucidated. The present study was designed to investigate the effect of aged garlic extract (AGE) on colorectal cancer cells and also on angiogenesis, a very important microenvironmental factor in carcinogenesis.

**MATERIALS AND METHODS**

**Cell culture and reagents.** Human colorectal carcinoma cell lines HT29, SW480, and SW620 were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco’s modified Eagle medium (DMEM) (Nihonseiyaku) containing 10% fetal bovine serum (FBS) (Dainippon Pharmaceutical Co., Tokyo, Japan), 1000-u/mL penicillin, and 1000-u/mL streptomycin (Invitrogen Corporation, Carlsbad, CA). For endothelial cells, the study used the ECV304 cell and the transformed rat lung endothelial cell (TRLEC). ECV304 cells were also obtained from ATCC and cultured in the same way. ECV304 cells were originally considered human transformed endothelial cells, but later they were considered bladder cancer cells with a perfect endothelial cell phenotype. TRLECs were donated by Dr. Tsufrufuji (Institute of Cytosignal Research) (12) and were maintained in DMEM supplemented with 1% FBS and penicillin-streptomycin in the same way. Because ECV304 cells and TRLECs were transformed, no growth factors such as basic fibroblast growth factor or vascular growth factor were required for the cell proliferation.

AGE was manufactured by Wakunaga Pharmaceutical Co. by slicing cloves of garlic (Allium sativum) and soaking them in a water-ethanol mixture, which was then naturally extracted and aged for >10 mo at room temperature. The AGE we used contained ~28.6% (wt/v, 286 g/L) solid material, 0.63% (6.3 g/L) arginine, and 0.1% S-allylcysteine (dry wt basis) as a marker compound for standardization (13,14). The AGE was used for the following in vitro assays.

**Cell proliferation assay.** Cell proliferation was evaluated by use of a WST assay as previously described (15). Briefly, 1 × 10⁴ cells of HT29, SW480, SW620, or ECV304 cells or TRLECs in 100 µL of DMEM containing 1% FBS were plated into each well of a 96-well plate. The cells were cultured in the absence or presence of AGE concentrations of 0.1, 1.0, or 10 g/L. AGE inhibited proliferation of both colorectal carcinoma cells and endothelial cells in a dose-dependent manner. Each bar represents the mean ± SD *, P < 0.05; (Student’s t-test, compared with AGE absence).

**Matrigel chemoinvasion assay.** Invasive activity through basement membrane components was assessed by measuring the invasion of colorectal carcinoma cells or endothelial cells through transwell inserts with 8-µm pores coated with Matrigel (Becton Dickinson Bioscience), which includes laminin, type IV collagen, and perlecancellular matrix proteins composed of basement membrane (16). Added to the upper well, with or without AGE at concentrations of 10 g/L, were 1 × 10⁴ cells of HT29, SW480, SW620, or ECV304 cells or TRLECs. DMEM supplement with 10% FBS (700 µL) as a chemoattractant was added to the lower well. After incubation in 10% CO₂ for 24 h at 37°C, the number of cells that had invaded to the lower surface of the Matrigel-coated membrane was counted in four random fields under a microscope.

**Cell adhesion assay.** 96-well plastic plates were coated with 0.1, 1, or 10 mg/L of collagen, laminin, or fibronectin (Iwaki Glass Co.) in phosphate-buffered saline (Invitrogen) for 2 h at 37°C and then treated with 3% bovine serum albumin (BSA) for 1 h at 37°C, or were
coated with only BSA for negative control. The ECV304 cells or TRLECs (2 x 10⁵ cells/mL) in serum-free DMEM containing 0.1% BSA were plated and incubated for 2 h at 37°C with or without AGE at the concentration of 10 g/L. After removal of the medium, a 0.04% crystal violet solution was added, and incubation was conducted for 10 min at room temperature. The wells were washed three times with phosphate-buffered saline, and 20 μL of Triton X-100 was added for permeabilization. Finally, distilled water was then added for a total quantity of 100 μL, and the number of adherent cells was assessed with a microplate reader (measurement wavelength 550 nm; reference wavelength 630 nm).

Migration assay. The effect of AGE on endothelial cell migratory activity was examined by wound assay (17). We seeded 5 x 10⁵ cells of ECV304 or TRLECs on the Petri dish, and they were cultured in the 5% CO₂ incubator for 10 h until they were completely confluent. The medium was then replaced with serum-free DMEM. One linear scar was drawn in the monolayer by a yellow tip. A set of digital photos was taken at the time of scarring, and the denuded area was marked by use of NIH image analysis software. The dishes were washed, and fresh serum-free medium containing 0.1% BSA in the absence or presence of AGE concentrations of 0.1, 1.0, and 10 g/L were added. After 3 h, a second set of photos was taken. These photos were superimposed on the first photo set to measure the migration of the cells. Cell migration activity was evaluated by the unhealed wound area without migratory cells, which was measured with pixel units in the computer analysis. Each condition was tested in duplicate in two independent experiments.

Tube formation assay. Tube formation was evaluated by three-dimensional collagen gel assay (18). A suspension of ECV304 cells or TRLECs in collagen gel was placed as a middle layer, between a collagen layer at the bottom and a culture medium layer with AGE at concentrations of 0.1, 1.0, and 10 g/L at the top. The tube formation ability of endothelial cells in the middle layer was evaluated after 14 d by a light microscope (Nikon Diaphot 200).

Statistical Analysis. All data are expressed as means ± SD. Comparisons between groups were performed by use of Student’s t test and the Mann-Whitney U test. Differences were considered to be significant at P < 0.05.

RESULTS

Effect of AGE on proliferation of colorectal carcinoma cells and endothelial cells. In the WST-1 cell proliferation assay, the proliferation of HT29, SW480, and SW620 cells was significantly suppressed by AGE solutions (Fig. 1 and, B). AGE also inhibited the growth of ECV304 cells and TRLECs (Fig. 1C and D). Colorectal carcinoma cells seemed to be suppressed at slightly lower concentrations of AGE than were endothelial cells.

Effect of AGE on invasive activity of colorectal carcinoma cells and endothelial cells. The Matrigel chemoinvasion assay showed that the invasive activity of SW480 and SW620 cells was inhibited by AGE significantly, whereas no suppressive activity was observed on HT29 cell invasion (Fig. 2A). The invasive activity of ECV304 cells or TRLECs was also suppressed by AGE (Fig. 2B).

Effect of AGE on cell adhesion of endothelial cells to extracellular matrix. In the cell adhesion assay to collagen, laminin, and fibronectin, AGE significantly enhanced the
binding activity of both ECV304 cells and TRLECs to collagen and fibronectin (Fig. 3). Adhesion to laminin was also increased, but mostly not significantly. These enhanced effects of AGE on adhesion to collagen and fibronectin might be associated with suppression of invasion or cell migration activity.

**Effect of AGE on migration of endothelial cells.** The wound assay showed that AGE inhibited the migration activity of both ECV304 cells and TRLECs in a dose-dependent manner (Fig. 4).

**Effect of AGE on tube formation of endothelial cells.** Tube formation of ECV304 cells or TRLECs was observed in the three-dimensional collagen assay for 2 wk. However, AGE suppressed tube formation of endothelial cells effectively at concentrations of 1 and 10 g/L (Fig. 5). No apparent suppression of tube formation was induced by AGE at the concentration of 0.1 g/L.

**DISCUSSION**

The mechanisms of cancer chemopreventive agents include direct action on cancer cells, through suppression of proliferation or induction of apoptosis, and indirect action (i.e., micro-environmental factors) through inhibition of angiogenesis and potentiation of immunologic reaction. This study focused on elucidating the mechanisms of garlic in the chemoprevention of colorectal cancer. We demonstrated that AGE has not only direct antiproliferative effects on colorectal cancer cells but also an inhibitory effect on angiogenesis and that both actions would effectively suppress the generation of colorectal cancer.

Three different cell lines—HT29, SW480, and SW620—were used as colorectal cancer cells, and AGE suppressed the proliferation of all 3 in the same way. Yet, the effects of AGE on invasive activities of the 3 cell lines were different. AGE inhibited the invasive activities of SW480, and SW620 cells were inhibited by AGE, but it had no effect on the invasive activity of Ht29 cells. Interestingly, the antiproliferative actions of AGE were also different in details among 3 colorectal carcinoma cells. The exposure of AGE to HT29 cells was found to result in cell apoptosis, whereas by contrast, SW480 and SW620 cells did not progress to apoptosis, but G1 arrest in the cell cycle was induced in these cells when they were exposed to AGE (data not shown). It can be concluded that in HT29 cells, growth inhibition is induced mainly through apoptosis and that there is no effect with regard to cell invasion. In both SW480 and SW620 cells, growth inhibition was due to cell-cycle arrest, and cell invasion was evident in those cells but not in HT29 cells. The mechanism of AGE action appears to be dependent on the type of cancer cell.

Angiogenesis is essential for tumor growth. Cells obtain oxygen and nutrition from blood vessels. Tumors cannot grow to >1–2 mm in size without neovascularization. Once a state of neovascularization occurs, unlimited tumor growth can result (19,20). Therefore, angiogenesis is a good target for cancer chemoprevention. This study tested the effect of AGE on cell biologic functions associated with angiogenesis. The functions examined were cell adhesion to the extracellular matrix, cell motility, cell proliferation, and tube formation. AGE enhanced the adhesion of endothelial cells to collagen and fibronectin, and it suppressed cell motility and invasion in the wound assay and the Matrigel chemo-invasion assay, respectively. Stronger cell adhesion to an extracellular matrix might be associated with lower cell motility. AGE also potently inhibited the proliferation and tube formation of endothelial cells. These results suggest that AGE could prevent tumor formation by inhibiting angiogenesis through the suppression of endothelial cell motility, proliferation, and tube formation. Without angiogenesis, cancer cells cannot grow, and they remain dormant. In autopsies of older people who died of diseases other than cancer, occult thyroid cancer or latent prostate cancer was frequently found in a dormant state without angiogenesis. Although angiogenesis inhibitors are now given attention as therapeutic drugs for cancer (21), the most effective use of angiogenesis inhibitors should be directed to chemoprevention. AGE might be a good...
potential candidate for suppressing carcinogenesis through its antiangiogenic action. A previous in vivo study demonstrated that an AGE-rich diet reduces the number of dimethylyhydrazine-induced colon tumors in rats, as well as aberrant cryptic foci (22). In addition, the proliferation index of normal colonic mucosa decreased in the animals administered AGE diets. We demonstrated that AGE has an antiproliferative action on colorectal carcinoma cells and an inhibitory activity on angiogenesis, and that both could contribute to chemoprevention of colorectal cancer. Other work has shown that AGE administration to humans with advanced cancer induces immunomodulatory effects, including the number and activity of natural killer cells, and immunopotentiation of AGE can play a role in cancer chemoprevention (23). Because AGE contains multiple substances, a single factor might be responsible for the anticarcinogenic action, or AGE as a whole, like Chinese herbal extract in traditional Chinese medicine (24), might be effective. Biologic responses to AGE, including antitumor, cholesterol-lowering, and depressed platelet aggregation effects, have been reported in various model systems and in some investigations in humans (25).

AGE is potentially a good agent for chemoprevention of colorectal cancer, and clinical trials should be considered.

LITERATURE CITED