does not produce the uncomfortable effects of raw garlic (33), and could be suitable for long-term use. Although mechanisms of the chemopreventive effect of garlic have been investigated, it has not been reported whether aging the product inhibits colon carcinogenesis in vivo. We investigated the inhibitory effect of AGE on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in rats.

MATERIALS AND METHODS

Animals, diet, and materials. Five-wk-old male Sprague-Dawley rats were obtained from Seac Yoshitomi Co. The rats were maintained according to the Institutional Animal Care Guidelines and acclimated to the diet and environment for 2 wk after arrival. They were housed 3-per-rack-mounted wire cages and given tap water ad libitum. The temperature (20–22°C), humidity (45–55%), and lighting (12 h light/dark cycle) were constantly controlled. AGE was manufactured as follows: Garlic cloves were sliced and soaked in a water-ethanol mixture solution and naturally extracted for >10 mo at room temperature (34). The extract was evaporated dry and mixed with a basal powder diet at a concentration (wt:wt) of 4%. CE-2 (CLEA Japan) was used as a basal diet throughout the study. DMH was purchased from Tokyo Kasei Industries. Monoclonal antibody MIB-5 (M7248) against Ki-67 antigen was purchased from Dako Cytomation.

Carcinogenesis protocol. The experimental protocol is shown in Figure 1. A total of 60 rats were randomly distributed into 4 groups. The rats in group 1 ($n = 12$) were fed a basal diet and received subcutaneous injection of normal saline (0.2 ml) once a week for 20 wk as control. Group 2 ($n = 12$) rats were fed a diet containing 4% AGE. Group 3 ($n = 18$) rats were fed a basal diet and received subcutaneous injection of DMH at a dose of 20 mg/kg weight, once a week for 20 wk. Group 4 ($n = 18$) rats were fed a diet containing 4% AGE and received the same DMH injections as group 3. Food intake and body weight of the rats were recorded weekly. Rats were killed at 5 wk after the last injection of DMH by ether inhalation and the weights of the liver, spleen, and kidney were measured. Venous samples were collected from hearts using a tube containing sodium citrate, centrifuged for 10 min at 3000 × g, and the supernatant fluid was stored at –20°C until analysis. At autopsies, the entire intestinal tracts were removed, flushed free of feces with saline, and cut open along the longitudinal axis. The number, size, and location of intestinal tumors were recorded.

Concentration of serum S-allylcysteine. Serum S-allylcysteine measured as a compliance marker by HPLC studies using a post-column o-phthalaldehyde derivatization ion-exchange HPLC procedure with fluorescence detection (35).

Determination of aberrant crypt foci. For the aberrant crypt foci (ACF) analysis, colons were placed flat between two filter papers and fixed in 10% formalin in 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4) for 24 h. They were briefly dipped in a 2% solution of methylene blue in distilled water for 2 min and placed on a microscope slide, with the mucosal surface up, under a dissecting microscope. At a magnification of 40, ACF were distinguished from the surrounding normal-appearing crypts by their large and elliptical luminal openings (36). The total number of ACF and the number of ACF with 4 or more aberrant crypts were counted.

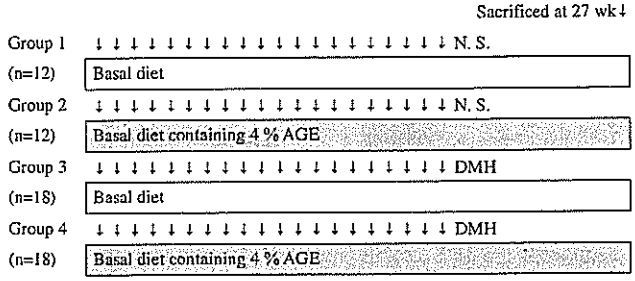

FIGURE 1 Experimental protocol. N.S., normal saline (0.2 mL/rat, s.c. injection × 1/wk); DMH, 1,2-dimethylhydrazine (20 mg/kg body wt, s.c. injection × 1/wk).

Histopathological examination. Normal-appearing colons and all protrusions were embedded in paraffin. Each specimen was cut into a 2 μm-thick section for hematoxylin and eosin and immunohistochemical staining. When the protrusion proved to be a lymph follicle by histological examination, it was excluded from the records and analysis.

Immunohistochemistry. Immunohistochemistry using monoclonal antibody MIB-5 against Ki-67 antigen was performed as a cell-proliferation marker. Each section was deparaffinized by xylene and rehydrated with graded alcohols. The sections were retrieved in a microwave oven (300W) in citrate buffer (pH 6.0) for 10 min at 100°C. Endogenous peroxidase activity was blocked by incubation of slides in absolute methanol containing 3% hydrogen peroxidase (H_2O_2) for 10 min at room temperature (RT). The sections were sequentially pre-incubated with 10% normal rabbit serum for 10 min at RT to block the possible background staining by the secondary antibody. They were then incubated with antibody MIB-5 at its working dilution of 1:20. After 60 min at RT, they were treated with EnVision reagent (Dako Cytomation) for 30 min. They were washed three times with PBS after each incubation, and 3,3'-diaminobenzidine was employed as a chromogen. The nuclei were lightly counterstained with Mayer's hematoxylin solution. The primary antibodies were replaced with PBS containing 0.1% bovine serum albumin as a negative control.

For determination of immunostaining, epithelial cells of the middle colon were counted from the lowest point to the tip of the crypt (full-length crypts) by light microscopy using 400 × magnification. The number of positively stained cells in each crypt column was recorded. The results were defined as the ratio of the number of positive-stained cells to the total number of cells counted (at least 500), and then multiplied by 100. In all cases, the counts were made by 2 independent observers who were unaware of the experimental condition or other results obtained.

Statistical analysis. Values were expressed as means ± SD All statistics were computed using a suitable program (SAS/STAT software). Unpaired Student's t test and chi-square test were used to detect statistically significant differences between groups. A P-value of < 0.05 was considered significant.

RESULTS

Food intake, body weight, organ weight, and macroscopic finding. At the end of the study, no macroscopic abnormality was observed in other intra-abdominal organs except in the intestinal tract. Body, liver, spleen, kidney, relative liver weights (g/100 g body wt), and food consumption (g/day per rat) in all groups are shown in Table 1. Average food intake did not differ significantly among the groups, which indicates that AGE administration did not suppress the calorie intake.

Concentration of serum SAC. Although SAC was not detected in the serum of rats in group 1, it was detected from all 6 evaluated rats in group 2 (range from 67 to 83 μg/L).

Number, size, and location of intestinal tumors. Intestinal tumors were only recognized in DMH-treated groups. The tumor incidence (tumor-bearing rat/rat) in the colons of group 4 (66.7%) was lower than that of group 3 (77.8%), but with no significant difference. The tumor incidence in the small intestine of group 4 (33.3%) was significantly lower than that of group 3 (93.3%) ($P = 0.0004$). The multiplicity of intestinal tumors is presented in Figure 2. The tumor multiplicity (number of tumors/rat) in the colons of group 3 was 3.7 ± 1.0 and that of group 4 was 1.0 ± 1.0, which proved lower than group 3 ($P = 0.0081$). Also, the tumor multiplicity in the small intestine of group 3 was 1.6 ± 1.3, and that of group 4 was 0.4 ± 0.7, which was lower than group 3 ($P = 0.0001$). The mean diameter of colon tumors in group 4 was smaller than that of group 3, but with no significant difference. As to the site of the tumors, the DMH-treated rats had tumors throughout the

TABLE 1

Mean Food Intake, Final Body Weight, and Organ Weight of Rats[1]

Group	Treatment	Mean Food Intake at 27 wk (g/day/rat)	Body Weight (g)	Organ Wt			
				Liver (g)	Relative Liver (%)*	Spleen (g)	Kidney (g)
1	None	31.0	767.8 ± 67.4	23.0 ± 4.5	3.0 ± 0.4	1.2 ± 0.2	2.1 ± 0.2
2	AGE	32.0	790.0 ± 65.3	23.8 ± 3.6	3.0 ± 0.3	1.2 ± 0.1	2.2 ± 0.2
3	DMH	30.7	659.3 ± 58.4	17.3 ± 3.3	2.6 ± 0.3	1.7 ± 0.4	2.1 ± 0.3
4	DMH + AGE	30.5	680.4 ± 71.4	17.9 ± 3.6	2.6 ± 0.3	1.5 ± 0.5	2.1 ± 0.3

[1] Values expressed are means ± SD.
* g/100 g body weight.

intestinal tract, from the duodenum to the distal colon. Colon tumors in group 3 were located in the proximal colon (21.8%), middle colon (52.7%), and distal colon (25.5%). Those of group 4 were located in the proximal colon (33.3%), middle colon (66.7%), and distal colon (33.3%), in which there was no significant difference between groups.

Development of ACF. Figure 3 summarizes the data on colonic ACF formation. Groups 1 and 2 showed no ACF. All DMH-treated rats in groups 3 and 4 developed ACF. In group 3, DMH induced 273.3 ± 106.0 ACF per rat, and AGE-treatment significantly reduced the number of ACF in group 4 ($P < 0.0001$). Also, the number of ACF with 4 or more aberrant crypts was reduced in group 4 as compared to group 3 ($P = 0.0002$).

MIB-5-labeling index. Monoclonal antibody MIB-5 showed clean and distinct nuclear staining of basal proliferating cells of crypts, thus showing identical patterns as reported for MIB-1 in corresponding human tissues. The mean MIB-5-labeling indices are presented in Figure 4. MIB-5-labeling index of group 4 (22.9 ± 8.3) was significantly lower than group 3 (37.8 ± 9.0) ($P < 0.0001$).

DISCUSSION

Chemoprevention has the potential to be a major component of cancer control. Accumulating evidence indicates that various food ingredients may play an essential role in colon cancer prevention (37–43). The AGE used in this study is an extract of fresh garlic that is aged over a prolonged period and contains water-soluble allyl amino acid derivatives, which account for most of its organosulfur content, stable lipid-soluble allyl sulfides, flavonoids, saponins, and essential macro- and micronutrients (44). The lipid-soluble volatile organosulfur compound allicin, which is produced enzymatically when garlic is cut or chopped, is absent in AGE. We showed that AGE given orally significantly reduced DMH-induced colon tumor and ACF without causing any apparent adverse effects. The results are mostly consistent with previous rodent chemoprevention studies using other garlic preparations or its constituents (9–13).

ACF are useful intermediate biomarkers in detecting modifying influences of natural and synthetic compounds on chemically induced colon carcinogenesis, which represents the preneoplastic lesions. Furthermore, the percentage of large ACF consisting of 4 or more aberrant crypts is considered a better intermediate biomarker of tumor occurrence than the number of ACF alone (45). Recently, Yamada et al. reported that the presence of β-catenin accumulated crypts (β-CAC), which appear soon after carcinogen exposure like ACF (46), and suggest that β-CAC is more likely to be a direct precursor of colon tumors than classical ACF in rats (47). In our study, AGE lowered the number of ACF, suggesting that AGE may inhibit the growth of ACF through suppression of cell proliferation in colonic mucosa exposed to DMH, and observations support the role of ACF as precursors to colon cancer.

Cell proliferation plays an important role in multistage carcinogenesis with multiple genetic changes (48). Modulation of cell-proliferation activity in target organs is one of the important actions of cancer chemoprevention (49). The nuclear antigen, designated as the Ki-67 protein, is exclusively expressed in the nuclei of all cells in G_1, S, and G_2 phases and mitosis, but not in the G_0 phase of the cell cycle (50), and it has therefore become useful for assessment of cell growth. MIB-1 against the Ki-67 antigen is a reliable tool for determining

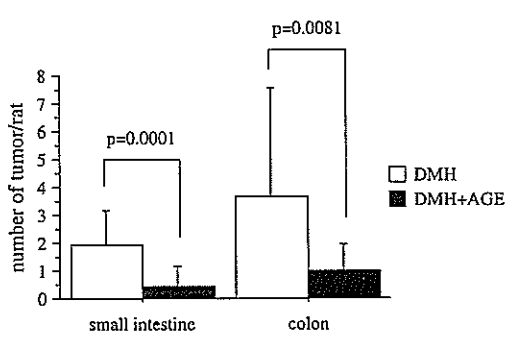

FIGURE 2 Multiplicity of intestinal tumor. The tumor multiplicity (number of tumors/rat) of the AGE-treated group was significantly lower than that of the basal diet group both in the small intestine ($P = 0.0001$) and colon ($P = 0.0081$).

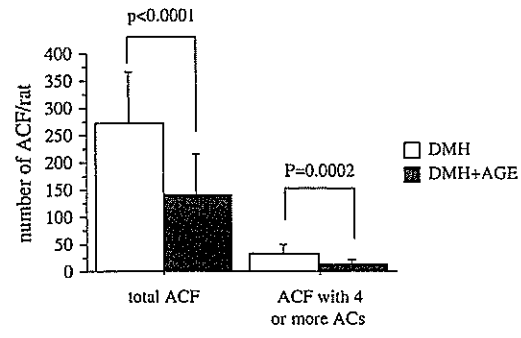

FIGURE 3 Development of DMH-induced ACF. The number of ACF was reduced in the AGE-treated group as compared to basal-diet group ($P < 0.0001$). Also, the number of ACF with 4 or more aberrant crypts (ACs) was reduced in the AGE treated group as compared with basal diet group ($P = 0.0002$).

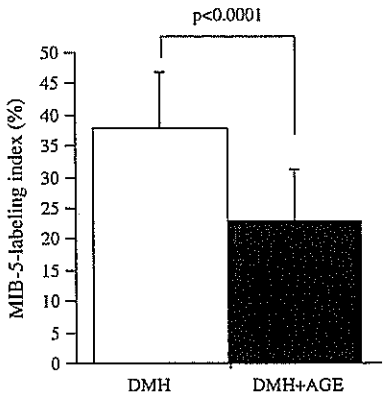

FIGURE 4 MIB-5-labeling index in normal colonic mucosa. The mean MIB-5-labeling index of the AGE-treated group was lower than that of basal diet group ($P < 0.0001$).

proliferating cells in human tissues. Recently, a novel monoclonal antibody MIB-5 was found to have the additional advantage of being able to react with the rodent-equivalent Ki-67 protein (51,52). Therefore, we used MIB-5 as an immunohistochemical proliferation marker, in which MIB-5-labeling index in normal mucosa was decreased by dietary administration of AGE; therefore, it is suggested that AGE has chemopreventive effects through inhibition of cell proliferation in the initiation period of colon carcinogenesis.

For preventive purposes, daily, long-term ingestion of product is necessary. Thus, the safety of the product should be considered seriously. Because different types of garlic preparations have different pharmacological properties, and some garlic preparations may cause undesirable effects, including gastrointestinal problems, one should be cautious about their safety as well as their effectiveness when choosing a preparation. The major unique organosulfur compounds in AGE are water-soluble SAC and S-allylmercaptocysteine, which have high content because they are produced during the process of aging (35); they are relatively non-toxic to animals when compared with other garlic volatiles, and they are likely to be more tolerable if used in human prevention studies (10).

In conclusion, this study indicates dietary AGE has chemopreventive effects on chemically induced colon carcinogenesis through modulation of cell proliferation and suggests possible applications in human clinical trials. Based on these findings, an interventional trial is being conducted in our collaborative group, in which capsules containing AGE are given to determine whether they reduce the prevalence of colon cancer or polyp.

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Significance of Garlic and Its Constituents in Cancer and Cardiovascular Disease

Aged Garlic Extract Inhibits Angiogenesis and Proliferation of Colorectal Carcinoma Cells[1–3]

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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*

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 spo-

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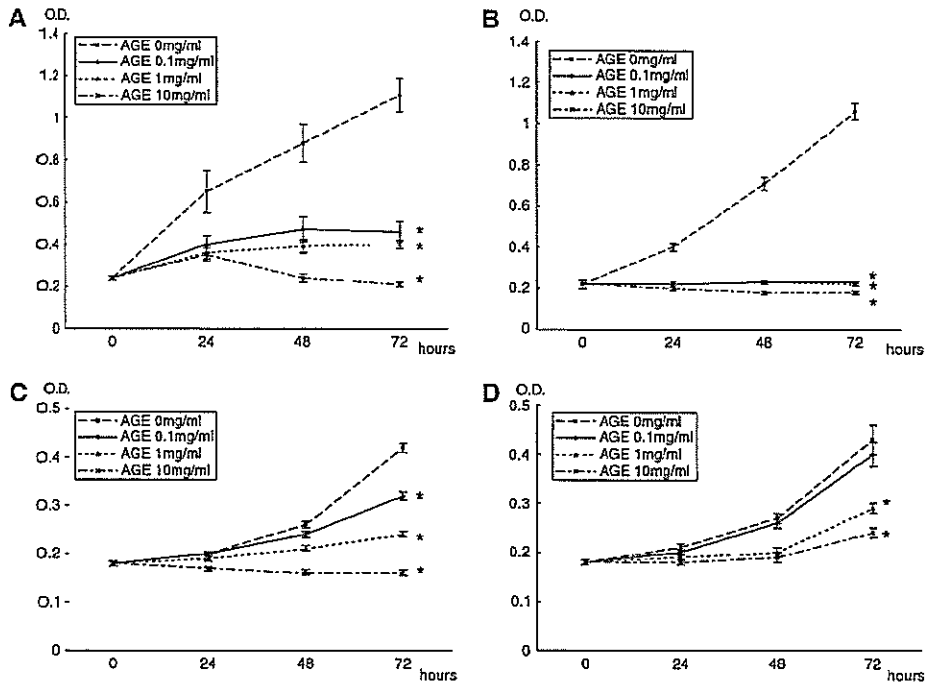

FIGURE 1 Proliferation assay (WST assay) of colorectal carcinoma cells (*A,B*) or endothelial cells (*C,D*). These graphs summarize the relative absorbance (415 nm) of the proliferation reagent WST-1 for colorectal carcinoma cell lines HT29 (*A*) and SW620 (*B*), or endothelial cell lines ECV304 (*C*) and TRLEC (*D*) at 24, 48 and 72 h in the absence or presence of AGE 0.1 g/L, 1.0 g/L, 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).

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)[5] 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), 10mg/mL penicillin, and 10000-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. Tsurufuji (Institute of Cytosignal Research) (12) and were maintained in DMEM supplemented with 10% 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-1 proliferation assay as previously described (15). Briefly, 1×10^4 cells of HT29, SW480, SW620, or ECV304 cells or TRLECs in 100 μmL 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, and 10 g/L. After 24, 48, and 72 h incubation, 10 μmL of WST-1 (Dojinsha, WAKO) solution was added to each well and incubated for 2 h at 37°C, and the absorbance at optical wavelength 415 nm was determined with a microplate reader. The experiments were performed in triplicate.

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 perlecan–extracellular matrix proteins composed of basement membrane (16). Added to the upper well, with or without AGE at concentrations of 10 g/L, were 1×10^5 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% CO_2 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

[5] Abbreviations used: AGE, aged garlic extract; ATCC, American Type Culture Collection; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; TRLEC, transformed rat lung endothelial cell.

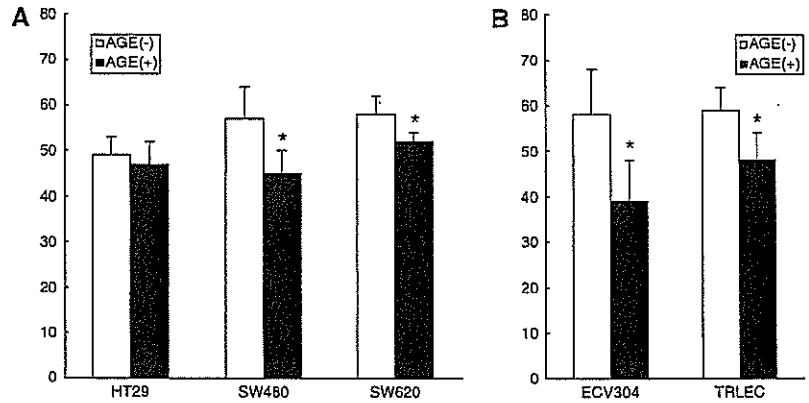

FIGURE 2 Matrigel chemoinvasion assay of colorectal carcinoma cells (*A*) or endothelial cells (*B*). These graphs summarize the invaded cell counts for colorectal carcinoma cells, HT29, SW480, and SW620, and endothelial cells, ECV304 and TRLEC at 24h in the absence or presence of AGE 10 g/L. In colorectal carcinoma cells the invasion of SW480 and SW620 cells was inhibited by AGE, whereas no invasion was induced for HT29 cells. AGE suppressed invasion of both endothelial cells. Each bar represents the mean ± SD *, $P < 0.05$; (Student's *t* test).

coated with only BSA for negative control. The ECV304 cells or TRLECs (2×10^5 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 5×10^5 cells of ECV304 or TRLECs on the Petri dish, and they were cultured in the 5% CO_2 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. 2*A*). The invasive activity of ECV304 cells or TRLECs was also suppressed by AGE (Fig. 2*B*).

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

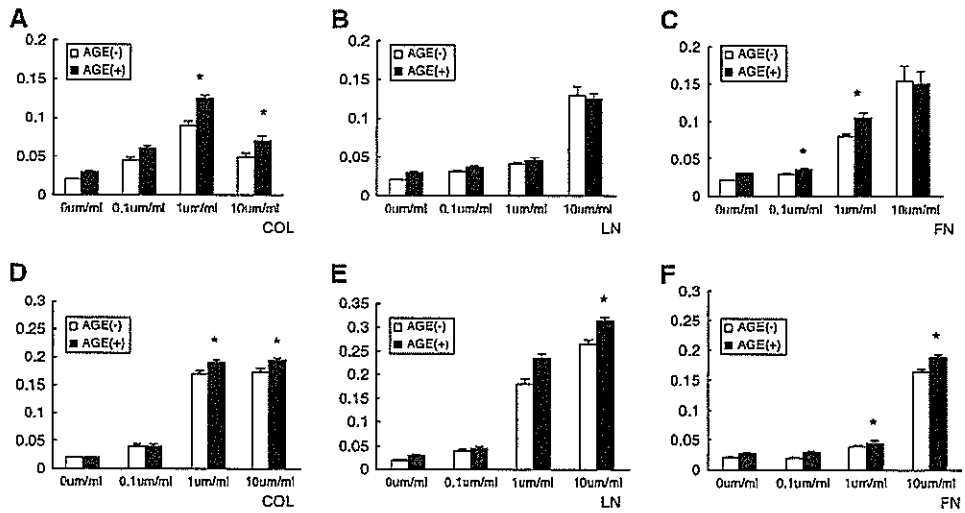

FIGURE 3 Adhesion to collagen (COL; *A, D*), laminin (LN; *B,E*), or fibronectin (FN; *C, F*) of endothelial cells. These graphs summarize the adhered cell numbers to each extracellular matrix protein of endothelial cells ECV304 (*A,B,C*) or TRLEC (*D,E,F*) for 2 h in the absence or presence of AGE 10 g/L. Adhered cell numbers were assessed for relative absorbance (540 nm) of the color reaction of 0.04% crystal violet solution. AGE significantly enhanced adhesion activities to collagen and fibronectin. Each bar represents the mean ± SD *, $P < 0.05$; (Student's *t* test).

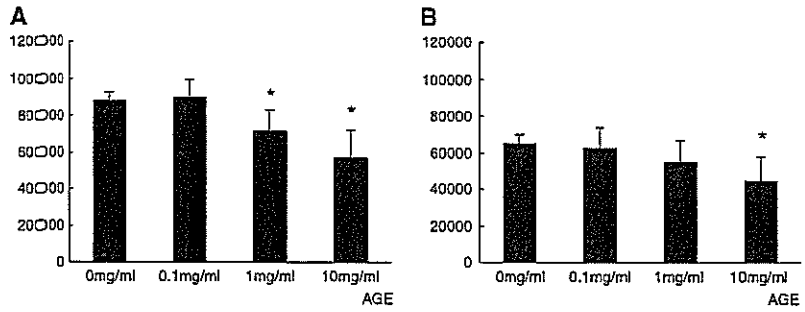

FIGURE 4 Cell migration assay (wound assay) of endothelial cells. These graphs summarize the cell migratory activity of endothelial cells ECV304 (*A*) or TRLEC (*B*) in the absence or presence of AGE 0.1 g/L, 1.0 g/L, or 10 g/L. Unhealed areas where cells were exfoliated and not migrated after 3 h were evaluated as pixel numbers in the computer analysis. AGE inhibited the cell migratory activities of both endothelial cells in dose-dependent manner. Each bar represents the mean ± SD *, $P < 0.05$; (Student's *t* test, compared with AGE absence).

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., microenvironmental 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 chemoinvasion 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

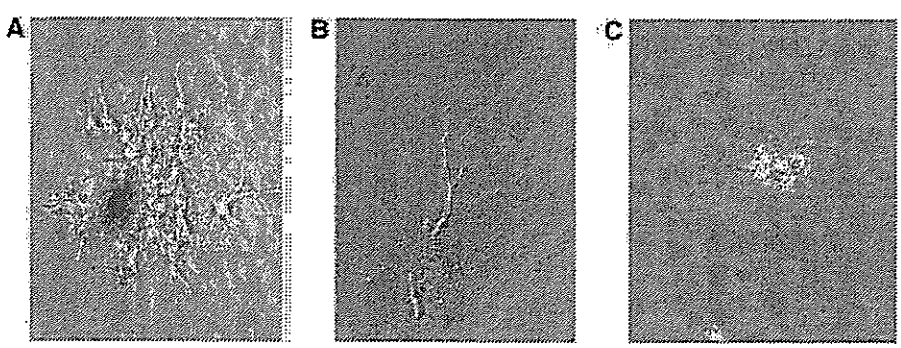

FIGURE 5 Collagen-gel sandwich tube-formation assay. TRLECs were plated on bovine collagen type 1 gel in presence of AGE 0.1 g/L (*A*), 1 g/L (*B*) or 10 g/L (*C*). Tube formation was evaluated after 48h by light microscope. Without AGE numerous tube formations were observed (*A*) whereas very few or no tubal structures were found with 1 g/L or 10 g/L AGE (*B,C*).

potential candidate for suppressing carcinogenesis through its antiangiogenic action.

A previous in vivo study demonstrated that an AGE-rich diet reduces the number of dimethylhydrazine-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.

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Significance of Garlic and Its Constituents in Cancer and Cardiovascular Disease

Aged Garlic Extract Prevents a Decline of NK Cell Number and Activity in Patients with Advanced Cancer[1,2]

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ABSTRACT Aged garlic extract (AGE) has manifold biological activities including immunomodulative and antioxidative effects. It is used as a major component of nonprescription tonics and cold-prevention medicines or dietary supplements. Advanced-cancer patients decline in immune functions and quality of life (QOL). The study's subjects were patients with inoperable colorectal, liver, or pancreatic cancer. In a randomized double-blind trial, AGE was administered to one group and a placebo was administered to another for 6 mo. The primary endpoint was a QOL questionnaire based on the Functional Assessment of Cancer Therapy (FACT). The subendpoints were changes in the natural-killer (NK) cell activity the salivary cortisol level from before and after administering AGE. Out of 55 patients invited to participate in the trial, 50 (91%) consented to enroll. They consisted of 42 patients with liver cancer (84%), 7 patients with pancreatic cancer (14%), and 1 patient with colon cancer (2%). Drug compliance was relatively good in both the AGE and placebo groups. Although no difference was observed in QOL, both the number of NK cells and the NK cell activity increased significantly in the AGE group. No adverse effect was observed in either group. The study showed that administering AGE to patients with advanced cancer of the digestive system improved NK cell activity, but caused no improvement in QOL. J. Nutr. 136: 816S–820S, 2006.

KEY WORDS: • *aged garlic extract* • *NK cell activity* • *advanced cancer* • *double-blind controlled trial* • *quality of life*

The prognosis of cancer patients for whom radical surgical resection is impossible is poor. In many of these patients, the quality of life (QOL)[4] deteriorates due to exacerbation of symptoms of cancer and adverse effects of chemotherapy. Also, the prognosis of the patient deteriorates because of immune dysfunction, which leads to proliferation of cancer and infection. Therefore, improvements in QOL and prevention of hypofunctions in the immune system are considered important in the care of advanced-cancer patients.

Garlic is one of the foods considered promising for improving QOL. Laboratory studies have suggested that garlic and its components suppress carcinogenesis and reduce serum lipid levels (1–7). However, garlic has been reported to cause adverse effects such as gastrointestinal disorders and anemia, in addition to its intense odor. (8,9) These adverse events are caused by allicin

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[2] Author disclosure: No relationships to disclose.

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[4] Abbreviations used: AGE, aged galic extract, FACT, Functional Assessment of Cancer Therapy; FACT-An, FACT-G plus the subscale for anemia; FACT-G, FACT general scale; NK, natural killer; PEIT, percutaneous ethanol injection therapy; QOL, quality of life; SAC, S-allylcysteine; TAE, transcatheter arterial embolization.

and lipid-soluble sulfur compounds, which are produced by a cascade of chemical reactions from allicin (9,10). Aged garlic extract (AGE), which is produced by a long-term extraction from garlic in aqueous ethanol and has no irritating odor, does not cause such adverse events and has been confirmed to be safe in preclinical trials (11–13).

AGE has been shown to have an effect against physical and mental stress (14,15), an immuno-potentiating effect (16–20), an antioxidant effect (21–24), and a peripheral blood flow–improving effect (15,25,26); it is also expected to improve QOL and prevent the decrease in immune functions in patients with advanced cancer. However, its effects in cancer patients have not been evaluated in a randomized double-blind clinical trial.

We designed a randomized double-blind clinical trial to evaluate the effects of AGE on the QOL and immune functions of patients with advanced cancer.

SUBJECTS AND METHODS

Patients. The subjects were patients with advanced colon, liver, or pancreatic cancer, aged 20 y or more, who were admitted to Osaka Medical Center for Cancer and Cardiovascular Diseases and judged by their attending physicians to be inoperable. Patients with a history of hematemesis, bloody stools, ascites, or those who have had difficulty with oral nutrition, or a history of allergy to a food or drug that contains garlic or its components, were excluded.

With the permission of the attending physicians, 2 members of our research team interviewed patients and invited them to participate in the trial. Informed consent with written confirmation was obtained from those who agreed to enroll. The subjects were recruited between 17 May and 16 November 1999.

Study design. The study was carried out as a randomized double-blind trial. It was approved by the Ethical Board of the Osaka Medical Center for Cancer and Cardiovascular Diseases, and an independent Ethical Monitoring Committee, excluding members of our research team, was established for this trial.

Data on participants who consented to the enrollment were reported anonymously to the trial statistician by fax. The trial statistician randomized the participants into 2 groups, one received AGE (AGE group) and the other received crystalline cellulose (control). Randomization was made by the block randomization method using the disease name as a factor.

Blood was sampled from the consenting participants and between 1500 and 1700 they were asked to fill out a questionnaire concerning QOL. They were then given trial capsules to be taken for the following 12 wk, starting the next day.

After 12 wk, the participants visited the Osaka Medical Center for Cancer and Cardiovascular Diseases at 1500 and met with a member of the trial team. They were questioned about their symptoms, returned the drug bottles and medication diaries, had their blood sampled, and answered a follow-up QOL questionnaire. They were then given more trial capsules and medication diaries for another 12-wk period. After 24 wk, the subjects again visited the Osaka Medical Center for Cancer and Cardiovascular Diseases at 1500, met with a member of the trial team, and answered the QOL questionnaire.

During this trial, the disease was treated ordinarily, but subjects were instructed not to take supplements containing garlic or its components other than the trial capsules.

If adverse events occurred, the information was entered onto a prescribed form and faxed to the trial statistician. The trial statistician recorded the drug that had been assigned to the patient and reported the event immediately to the chairman of the Ethical Monitoring Committee.

When the QOL questionnaire had been completed by all subjects, 6 mo after the trial began and the trial data had been stored as a computer file, the randomization codes were disclosed to all members of the trial team (after receiving approval from the Ethical Monitoring Committee).

Trial capsules. Trial capsules were prepared by Wakunaga Pharmaceutical Co. Subjects took 2 capsules after breakfast and and 2 capsules after dinner (4 capsules/d). The AGE capsules contained AGE powder that was prepared by mixing AGE with crystalline cellulose (Avicel FD-101) as an excipient. AGE is a unique garlic preparation manufactured by soaking garlic in aqueous ethanol for >10 mo. It contains water-soluble organosulfur compounds including *S*-allylcysteine (SAC), *S*-1-propenyl-L-cysteine, *A*-allylmercapto-L-cysteine and cycloalliin, steroid saponins, fructosylarginine, and 1,2,3,4-tetrahydro-β-carboline-3-caboxylic acids as biologically active components (27). The daily dose (4 capsules), contained 500 mg of AGE, 727 mg of crystalline cellulose, and 11 mg of sucrose fatty acid ester.

The placebo capsules contained 951.5 mg of crystalline cellulose and 8.5 mg of sucrose fatty acid ester per 4 capsules.

Confirmation of the compliance and blinding effect. Drug compliance was evaluated according to entries in subjects' medication diaries and by a count of remaining capsules. In addition, the blood level of a marker compound of AGE was measured. The subjects were instructed to enter the state of compliance in the medication diaries daily, and the remaining capsules were recovered and counted. Blood was sampled 3 mo after administering the capsules, and the blood SAC level was determined.

To check the blinding effect, the subjects were asked, at 3 and 6 mo after the beginning of the trial, which of the drugs they thought was assigned to them.

Observation items. At enrollment, the patients and their attending physicians were asked about the subjects' histories and the histories of their present illnesses.

Patients filled out the QOL questionnaire, Functional Assessment of Cancer Therapy (FACT) (28) before starting the treatment and again after 3 and 6 mo of receiving treatment. In principle, the subjects answered by themselves, but a member of the trial team attended the subjects and explained the contents of the questionnaire if help was desired. Entries on the questionnaire were scored according to the method determined by FACT developers and included the handling of defect values. The scores of individual domains and the total scores were calculated separately. Specifically, the score of each of the 4 domains of the general scale (FACT-G) (i.e., physical, functional, mental, and social dimensions), the score of the subscale for anemia, the total score of the FACT-G, and the total score of the FACT-G + subscale for anemia (FACT-An), were calculated.

Blood and saliva were sampled before and 3 mo after study treatment began. Blood was tested for glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, γ- glutamic-pyruvic transaminas, lactate dehydrogenase, alkaline phosphatase, tocopherol, albumin, T-Chol., TG, UA, BUN, Cr, Na, K, Cl, Ca, IP, T-Bil, FBS, blood cell counts, blood-homocystein concentration, NK cell activity, NK cell count (measured as the lymphocyte subset 2-color CD16+/CD56+), CD4 and CD8-positive cell count, and SAC concentration. The number of immunocytes was determined by flow cytometry. NK cell activity was determined by ^{51}Cr-release assay according to the method described by Domzig et al. (32). The effector cell (mononuclear cell) and target cell (K562 cell) ratio is 20:1.

The blood SAC concentration was measured at Healthcare Institute of Wakunaga Pharmaceuticals using the method of Kodera et al. (33). Saliva was collected using Salivette sampling devices (Sarstadt) (34). A Salivette includes a small cotton swab and, when chewed, stimulates saliva flow to allow for the collection of a sufficient amount of saliva within 1 min. After centrifugation at 3,000 × *g* for 10 min, saliva was stored at −80°C until assay. Salivary cortisol levels were determined with a commercial enzyme immunoassay kit (Ciron) (35) at the Department of Social and Environmental Medicine, Osaka University.

If chemotherapy was performed at the beginning of the study, blood sampling and completion of the questionnaire were carried out 6 or more d after anticancer drug treatment was completed.

Endpoints. The primary endpoint was whether, after 6 mo of treatment, any subjects had a FACT-G or FACT-An score that deteriorated by 1 SD. The SD of the FACT-G was set at 15.9 (n = 466, mean = 82.0), based on Cella (29), and the SD of the FACT-An at 26.8 (n = 47, mean = 141.6), also based on Cella (28). Data of the subjects who died during the trial were regarded as defect values. Subendpoints were changes in the NK cell activity and salivary cortisol level from before and after treatment.

Statistical procedures. The target number of subjects to be recruited was 40 (20 in each group) because a statistical difference

could be established with this number of subjects at a P-value of 5% and a power of 80% if the QOL score after treatment deteriorated to 76% (compared with that before treatment) in the placebo group and to 38% in the AGE group. During the registration period, invitation to participate was limited to a maximum of 60 patients.

Two-sample t tests were performed for comparisons between the 2 groups: paired t tests were performed for comparisons from before and after the treatments, and Fisher's exact test was performed for the analysis of nonparametric data. The data were represented as means ± SD unless otherwise indicated. The results were analyzed by interation-to-treat analysis.

RESULTS

Enrollment and randomization. During the entry period, 55 patients were invited to enroll and 50 (91%) of them consented. The characteristics of the 50 patients enrolled are listed in Table 1. They consisted of 42 patients with liver cancer (84%), 7 patients with pancreatic cancer (14%), and 1 patient with colon cancer (2%). These patients were determined to be inoperable because of their advanced cancer although performance status of the patients was good. In the patients with liver cancer, 38 patients bore multiple liver tumors and 4 showed hepatic failure. In the pancreatic cancer patients, 4 showed vascular invasion, 2 showed direct invasion, and 1 showed liver metastasis. One patient with colon cancer showed liver metastasis. All of the patients with liver cancer were treated with transcatheter arterial embolization (TAE) and/or percutaneous ethanol injection therapy (PEIT), and 6 of them underwent hepatectomy before starting the study. The patients with pancreatic cancer did not undergo treatment before the study except for 1 who was treated with cisplatin and fluorouracil. No difference was observed in age, gender, and clinical stage between the AGE and control groups.

After randomization, 3 patients of the AGE group requested to be withdrawn from the trial without taking AGE and were lost to the trial. Another patient of the AGE group developed angina pectoris on day 83 of the study and was lost. In the control group, 1 patient wanted to withdraw from the trial due to diarrhea on day 7 of the study and was lost.

Ten patients with liver cancer underwent TAE and/or PEIT treatment, of whom 1 took fluorouracil, but the rest were not treated during the study. Fluorouracil was administered for all of the pancreatic cancer patients, of whom 5 were treated with radiation and 1 took irinotecan along with fluorouracil. During the study, a significant difference in the treatments was not observed between the 2 cohorts.

Four patients in the AGE group died due to cancer on days 55, 85, 99, and 106 of the study, and 5 patients of the control group died due to cancer on days 45, 143, 153, 168, and 170. Data collected up to the loss or death of patients was adopted for analyses of the results.

After completing the 6-mo trial, most of the subjects voluntarily started taking AGE.

Confirmation of compliance and blinding effect. Compliance was relatively good in both groups.

Changes in the blood SAC concentration are recorded in Table 2. Before beginning treatment, the blood SAC concentration in many subjects was low. After 3 mo, the SAC concentration was significantly increased to 10 μg/L or above in 14 (78%) of the subjects in the AGE group ($P = 0.01$). SAC concentration increased as well in the control group though less markedly than in the AGE group ($P = 0.19$). Values were therefore higher in both groups.

According to the questionnaire, entries concerning the blinding effect, 4 (24%) in the AGE group and 5 (27%) in the control group, believed that they were taking AGE capsules after 3 mo, and 5 (29%) in the AGE group and 4 (21%) in the control group believed they were taking AGE capsules after 6 mo, so blinding was judged successful.

QOL. No difference was observed in QOL between the AGE and control groups not only before but also at 3 and 6 mo after the study began. No particular change was observed in QOL at 3 and 6 mo after administering treatment compared with before.

Indices of cell-mediated immunity. Changes in the peripheral blood NK cell count and NK cell activity are listed in Table 3. Analysis was performed by excluding the data of 1 patient from the AGE group for whom blood could not be sampled for measuring indices of cell-mediated immunity.

The NK cell count was not different between the AGE group and control group before or 3 mo after study treatment began. It increased significantly in the AGE group, and, while it also increased in the control group, the increase was not significant.

The NK cell activity was not different between the 2 groups before or 3 mo after the administering treatment. The NK cell activity increased significantly in the AGE group. It also increased in the control group, but the increase was not significant. The NK cell activity appeared to decrease rapidly in the control group compared with the AGE group. Five subjects (22%) in the control group showed >25% decrease in the NK cell activity but none did in the AGE group ($P = 0.051$) (Table 3, Fig. 1). Only 1 (3%) of the 35 patients in whom the NK cell activity did not decrease by 25% or more 3 mo after administering treatment died within the following 3 mo, but 3 (60%) of the 5 patients in whom the NK cell activity decreased by 25% or more died within the following 3 mo ($P = 0.04$).

TABLE 1

Baseline characteristics of subjects

Characteristic	AGE group ($n = 25$)	Control group ($n = 25$)
Age,[1] *y*	63.6 ± 8.3	65.8 ± 6.3
Male sex, %	21 (84)	18 (72)
Cancer, %		
Liver	21 (84)	21 (84)
Pancreas	4 (16)	3 (12)
Colon	0 (0)	1 (4)
Dropped out, %	4 (16)	1 (4)
The death (exam. period), %	4 (16)	5 (20)

[1] Values are means ± SD.

TABLE 2

Changes in blood S-allylcysteine concentration[1]

	AGE group ($n = 18$)		Control group ($n = 23$)	
	Pretreatment	After 3 mo[2,3]	Pretreatment	After 3 mo[4]
<10 *ng/mL*	16 (89)	4 (22)	20 (87)	15 (65)
10–14 μg/L	2 (11)	10 (56)	1 (4)	3 (13)
15–19 μg/L	0 (0)	3 (17)	2 (9)	2 (9)
≤20 μg/L	0 (0)	1 (6)	0 (0)	3 (13)

[1] Values indicate number and (%) of patients.
[2] $P = 0.01$.
[3] 17 (94%) had increased concentration compared with before treatment and 1 (6%) was changed or reduced.
[4] 17 (74%) had increased concentration compared with before treatment and 6 (26%) were changed or reduced.

TABLE 3

Changes in index values of cell-mediated immunity[1]

	AGE group (n = 17)		Control group (n = 23)	
	Pretreatment	After 3 mo[2]	Pretreatment	After 3 mo[3]
WBC, */μL*	4433 ± 1623	4517 ± 1143	4873 ± 2081	5283 ± 2211
$CD4^+$, *cells/μL*	650 ± 271	667 ± 228	746 ± 356	866 ± 475[4]
$CD8^+$, *cells/μL*	409 ± 155	457 ± 181	403 ± 312	533 ± 418
NK, *cells/μL*	207 ± 142	277 ± 150[4]	231 ± 207	288 ± 222
NK activity, %	27.2 ± 15.9	36.0 ± 13.2[5]	32.6 ± 16.3	39.1 ± 15.7
NK activity/100 cells	19.0 ± 17.2	17.4 ± 12.9	19.9 ± 14.1	24.0 ± 21.9

[1] Values are means ± SD.
[2] 17 (100%) did not show a ≥25% decrease in NK cell activity compared with before treatment.
[3] 18 (78%) did not show a ≥25% decrease in NK cell activity compared with before treatment and 5 (22%) did show this change.
[4] $P < 0.05$ after 3 mo compared with the value before treatment.
[5] $P < 0.01$ after 3 mo compared with the value before treatment.
NK cells = CD16+/CD56+.

There was no significant change in the number of CD8+ cells in peripheral blood but CD4+ cells significantly increased in the control group.

Other markers. No significant difference was observed in the salivary cortisol concentration or the values of blood biochemical parameters between the AGE group and control group, either before or 3 mo after administering treatment.

While the salivary cortisol concentration showed no change from before to after administering the treatment in the AGE group, it increased significantly after 3 mo in the control group.

The serum total protein, albumin, total cholesterol, and HDL cholesterol levels were increased 3 mo after administering treatment compared with the values before the study began in both groups.

Adverse events. In the AGE group, 4 died due to cancer on days 55, 85, 99, and 106 of the study. One developed a duodenal ulcer, and 2 developed severe acute gastritis. In the control group, 5 died due to cancer on days 45, 143, 153, 168, and 170 of the study. One developed a gastric ulcer, and 1 suffered severe acute gastritis. There was no difference in the occurrence of adverse events between the 2 groups.

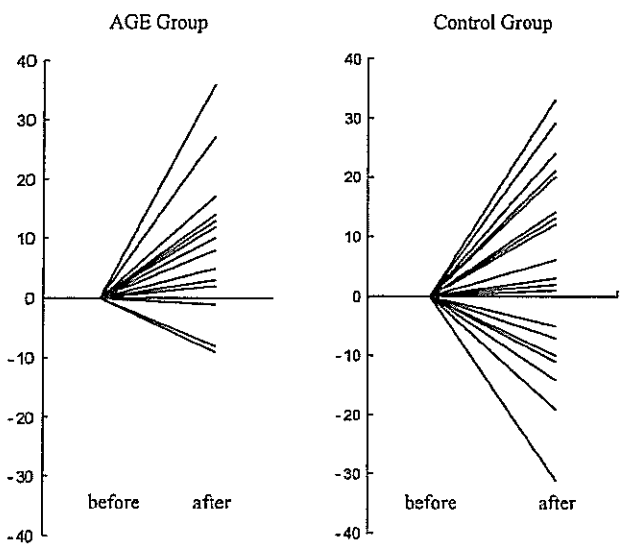

FIGURE 1 NK cell activity before and after the study. Changes of NK cell activity of patients 3 mo after the beginning of the study are shown as a percentage.

DISCUSSION

Administering AGE did not improve QOL but caused improvements in the NK cell activity in patients with advanced cancer of the digestive system.

Many patients who undergo chemotherapy receive alternative treatments in addition to ordinary treatments such as anticancer agents. However, the effectiveness of few alternative treatments for patients with advanced cancer has been confirmed by scientific assessment methods such as the randomized double-blind comparative study.

Garlic is used widely all over the world as an alternative treatment. Although epidemiological studies (36) have suggested that garlic prevents carcinogenesis, its therapeutic effects or its effects on QOL in patients with advanced cancer have scarcely been evaluated. Double-blind studies have been considered difficult because of the characteristic odor of garlic, but blinding was successful in our study using AGE. Therefore, AGE is expected to facilitate the execution of double-blind clinical trials in the future.

No effect of AGE was noted in QOL, which was the primary endpoint of this study, probably because QOL hardly deteriorated in the control group. This may be explained by the fact that most of the subjects had liver cancer, which causes relatively mild symptoms even in an advanced stage. Moreover, the blood SAC concentration tended to increase also in the control group, possibly because all subjects ingested garlic more than usual from meals after having been informed of the effectiveness of garlic during the process of informed consent. This may also have been a factor of the small deterioration of QOL in the control group. The shortness of the trial period may have been another reason. As a food, garlic has mild effects and an increased difference might have been revealed over a longer trial period.

In this study, improvements were observed in various serum nutritional parameters 3 mo after treatment began compared with before treatment in both the AGE and control groups. These improvements may be related to the fact that most of the patients were hospitalized before the beginning of the study but were treated on an outpatient basis and were eating at home after 3 mo.

Although the salivary cortisol concentration increased significantly in the control group, the values varied widely within the group, so that the increase may well have been accidental.

Administering AGE significantly increased the NK cell count in peripheral blood 3 mo after the study began, as can be seen in

Tab le 3 The NK cell activity increased in the AGE group but the specific activity (activities in 100 cells) did not change. In addition, no differences were observed in chemotherapy received by patients in the AGE and control group. These facts indicate that AGE increases the number of NK cells, resulting in the increase of NK cell activities. The mechanism of the increase in NK cell numbers remains unclear, but these findings were in agreement with the results of laboratory studies (16–20). Moreover, while many patients in whom the NK cell activity decreased rapidly died within the following 3 mo, none of the subjects in the AGE group showed such a rapid decrease, which suggests that AGE may prevent death due to cancer.

A significant increase in number of $CD4^+$ T cells, which represent helper T cells, was observed in the control group. The precise reason is unclear, but we think inflammation reaction accompanied by cancer progression is a possible reason, because helper T cells are involved in antibody production and the stimulation of cellular immune functions in regions of inflammation. To confirm this hypothesis, we should investigate the relation between an increase in the number of $CD4^+$ T cells and cancer progression.

We realize that an understanding of the effect of AGE is incomplete because we did not prohibit treatments such as TAE, PEIT, radiation, and the administering of anticancer drugs during the course of the study, which probably affected NK cell activity. Furthermore, it is possible that patients in the control group might have taken garlic and/or garlic products because SAC concentration in the serum of some patients in the control group increased after the study. However, these facts could underestimate but never overestimate the effect of AGE. Despite these facts, we think our conclusion that AGE increased NK cell activity is reasonable.

In this study, some patients in the AGE group dropped out before the treatment began, but the loss is considered to have been accidental. No difference was observed between the 2 groups in adverse events during the trial period, including deaths due to cancer, and AGE is considered safe to administer to patients with advanced cancer.

After the 6-mo trial period, most of the subjects started taking AGE. For this reason, effects of the study treatment on the remote outcome, including death due to cancer after the trial period, could not be evaluated. Clinical trials with primary endpoints of death due to cancer and enlargement of cancer need to be performed in the future.

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特集：消化管の“前癌病変”

〔大腸〕

大腸癌好発疾患とその取り扱い

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要旨　大腸癌好発疾患である消化管ポリポーシスの家族性大腸腺腫症(FAP)，Cronkhite-Canada症候群，Peutz-Jeghers症候群と遺伝性非ポリポーシス大腸癌(HNPCC)の特徴や取り扱い方法などを説明した。

FAPは，常染色体優性遺伝疾患であり，大腸に多数の腺腫を発生する。第一に選ばれる治療法は大腸全摘回腸肛門吻合術(IAA)，または結腸全摘回腸直腸吻合術(IRA)であるが，散在型で癌がなくポリープが比較的小さければ内視鏡的に大きめのポリープを摘除しながら厳重な経過観察を行うことも可能かもしれない。大腸全摘をしない場合には，経過観察が中断しないように，組織的なサポートが重要である。

key words: 家族性大腸腺腫症，遺伝性非ポリポーシス大腸癌，大腸癌

Ⅰ．大腸癌好発疾患

大腸癌の発生が一般集団よりも高頻度な疾患として，消化管ポリポーシスや遺伝性非ポリポーシス(hereditary non-polyposis colorectal cancer: HNPCC)，炎症性腸疾患などがあり，肥満症などの生活習慣病との関係も指摘されている。

本稿では，消化管ポリポーシスとHNPCCについて紹介する。

Ⅱ．消化管ポリポーシス

消化管ポリポーシスは，消化管に多数のポリープが存在する状態の総称であり，ポリープの組織像や随伴疾患により，幾つかの疾患に分けられる(表1)。腺腫性，過誤腫性の消化管ポリポーシスではほとんどが遺伝性であること，悪性化しやすいことが臨床的に重要である。

家族性大腸腺腫症(familial adenomatous polyposis: FAP)とGardner症候群は，別の疾患と考えられていたが，FAPも詳細に観察すると消化管以外にも骨腫や線維腫などの病変が認められることが多く，現在は同一疾患と考えられている。本項では，消化管ポリポーシスのなかで重要な疾患であるFAP，Cronkhite-Canada症候群，Peutz-Jeghers症候群について説明する。

1．家族性大腸腺腫症(familial adenomatous polyposis: FAP)

A．FAPの特徴と内視鏡所見

FAPの診断基準は，大腸に腺腫を100個以上認めることである。常染色体優性遺伝疾患であり，原因遺伝子として*APC*遺伝子が見出されている。しかし，最近になり*APC*遺伝子とは別の*MYH*遺伝子の変異により発症するFAPが報告されている。*MYH*遺伝子異常による発病は，常染色体劣性遺伝形式と考えられているが，詳細はまだ不明である。

大腸腺腫は必ず存在し(図1)，数百～1万個を超えるものまである。大腸腺腫は，盲腸から直腸の大腸全体に発生するが，若年期は直腸や下行結腸下部，横行結腸右側などに限局してみられることもある。

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表 1　消化管ポリポーシス

ポリープの組織像	疾患名	ポリープの分布	ポリープの数	悪性化	遺伝性	消化管以外の随伴性病変
腺腫性	家族性大腸腺腫症(FAP)(Gardner症候群)	大腸＞胃，小腸	数百～数万個	多	あり	骨腫，デスモイド腫瘍，表皮嚢胞甲状腺腫瘍，網膜色素上皮肥大
	Turcot症候群		比較的少ない	多	あり	中枢神経系腫瘍
過誤腫性	Peutz-Jeghers症候群	小腸＞胃，大腸	数個～数百個	中	あり	口唇・口腔・手掌・足底の斑状色素沈着
	若年性ポリポーシス	主に大腸	数十～数百個	中	あり	稀に先天性奇形
	Cowden病	全消化管 主に食道・胃	数百～数千個	少	あり	顔面・四肢末端の多発性丘疹，口腔内粘膜の乳頭腫症，乳腺・甲状腺・卵巣腫瘍など
	結節性硬化症	主に直腸	数十～数百個	稀	あり	全身の過形成性・過誤腫性病変(脳内結節，顔面血管線維腫，爪囲線維腫，腎病変など)
過形成性	過形成性ポリポーシス	大腸	数十以上	不明	不明	特になし
炎症性	炎症性ポリポーシス	炎症罹患部位	数十～数百個	稀	なし	特になし
	Cronkhite-Canada症候群	胃・大腸＞小腸	数百～数千個	中	なし	脱毛，皮膚色素沈着，爪甲異常，味覚異常

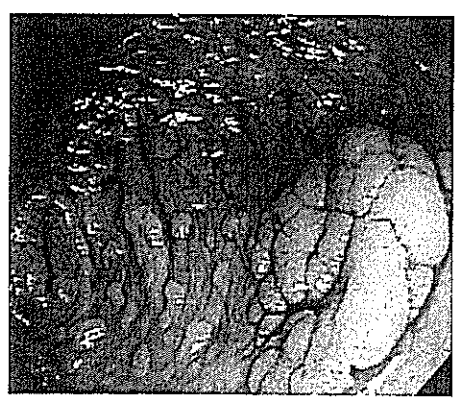

図 1　典型的なFAPの大腸内視鏡像

図 2　若年期にみられる微小なポリープ

若年期には，通常観察でほとんどポリープを認めず，色素散布にてわずかに小ポリープを認めることもあり(図2)，詳細な観察が必要である。

胃には55～74%の確率で，主に胃体部から胃底部領域に発生する胃底腺ポリープや，主に前庭部に発生する胃腺腫，胃癌を認める。胃腺腫は多発傾向があり，平盤状隆起で，隆起の中央に陥凹を認めることが多い。通常観察では病変がはっきりしないこともあるため，色素散布による詳細な観察が必要である。

十二指腸には86～100%の確率で腺腫を認める。十二指腸の腺腫は，広基性で数mm大のことが多く，乳頭部近傍に好発する。内視鏡的には白色を呈することが多い。十二指腸乳頭開口部に腺腫が発生することも多い。通常内視鏡の観察では異常を認めなくても生検にて組織学的に腺腫を認めることもあり，側視型の内視鏡を用いるなどして，乳頭部の詳細な観察が必要である。

FAPの亜型として，attenuated FAPがある。これは，大腸腫瘍は100個以下で右側大腸に好発し，発症年

表 2　家族性大腸腺腫症(FAP)の一次予防と二次予防

	一次予防	二次予防
大腸癌	予防的大腸摘出術，内視鏡的ポリープ摘除 適度な運動 赤身肉，アルコールの摂取制限 野菜の摂取促進	16歳頃からの定期的な大腸内視鏡検査
胃癌	胃腺腫の摘除，禁煙	20歳頃からの定期的な上部消化管内視鏡検査
十二指腸癌	十二指腸腺腫の摘除	20歳頃からの定期的な上部消化管内視鏡検査
甲状腺癌	不明	触診，甲状腺超音波検査
デスモイド	術直後の妊娠を避ける	腹部超音波検査

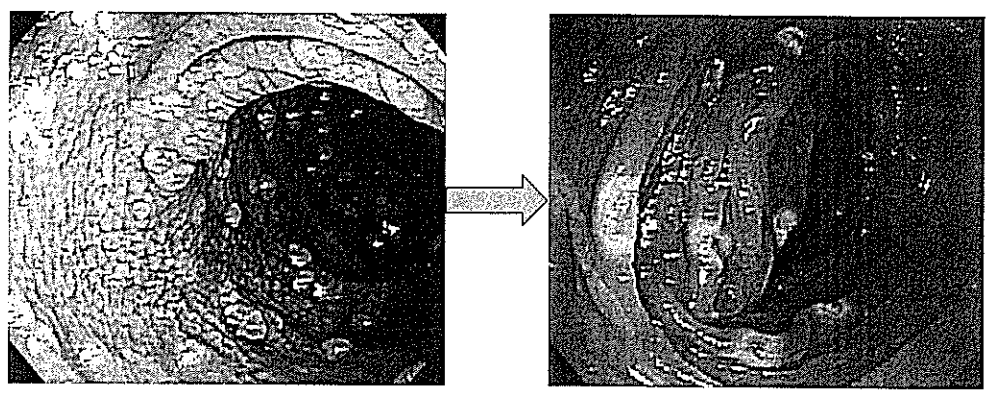

図 3　内視鏡的摘除の様子

齢は通常のFAPより15年ほど遅い。*APC*遺伝子には病的変異を認めることが多い。

*APC*遺伝子の病的変異の95%は点変異によるナンセンス変異か，延期の挿入や欠失によるフレームシフト変異であり，stop codonを生じるため，不完全な長さの変異蛋白質が産生される。この特性を用いた病的変異検出法(protein truncation test: PTT法)が，簡便で安価なため広く用いられている。

B．FAPの大腸発癌予防(一次予防)(表2)

大腸癌の一次予防として，評価が定まっているのは，予防的大腸摘出術である。大腸を全摘すれば大腸癌は発生しないが，術後の下痢などによる生活の質(QOL)の低下や，デスモイドや術後イレウスの発生などの問題がある。

大腸癌の母地と考えられる比較的大きな(おおよそ7 mm以上)ポリープを内視鏡的に摘除することで大腸癌の発生が予防できる可能性も考えられる(図3)。筆者らは十分な説明を行い，内視鏡による経過観察のリスクについて理解され経過観察を希望された患者に対しては，図4に示す方針により経過観察をしている。筆者の施設では，これまでに経過観察中に進行癌が発生した症例はない。しかし，厳重な経過観察をしていても進行癌が発生した報告もあること，頻繁に大きい腺腫を内視鏡的に摘除することにより手術を回避できるか否かについての知見がまだ得られていないことより，大腸摘出術を行わず内視鏡的に経過観察する場合には，慎重な対応が必要である。また，20歳頃の多感な時期に負担の多い大腸内視鏡検査を頻繁に行うため，中断することなく確実に経過観察を行うためには，医師だけでなく看護師やカウンセラーなどを含めた組織的な受診勧告のシステムを整えることが重要である。

FAPの同一家系でも，発癌時期やポリープの大きさに差がみられることから，大腸腫瘍の増大や癌化

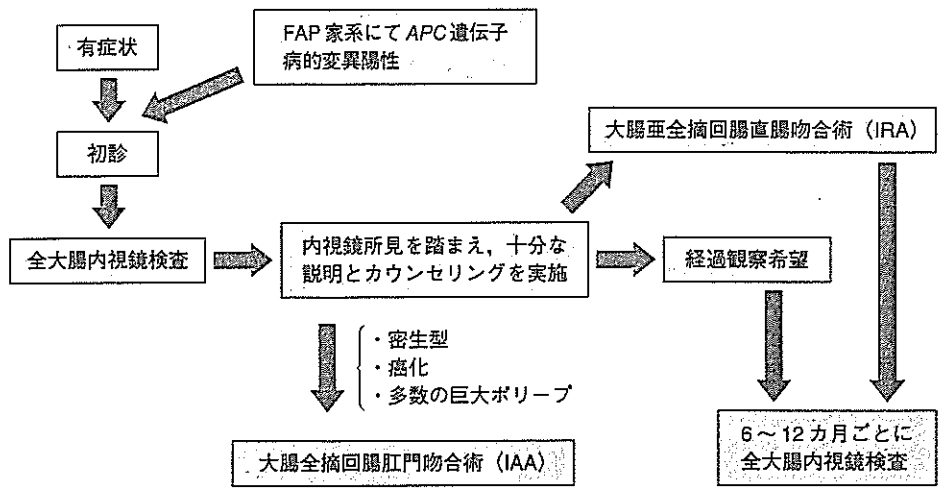

図 4　家族性大腸腺腫症(FAP)の治療方針

には，遺伝子変異だけではなく，運動や食事，喫煙などの環境要因も関与していると考えられる。これまでに大腸癌を予防することが判明している生活習慣は，適度な運動，赤身肉を1日80 g以下，野菜の十分な摂取，禁煙，アルコールは1日に男性は1合，女性は0.5合までなどがある。大腸を摘出していない患者には，このような食生活指導を勧めるべきであろう。大腸腺腫の縮小を目的とした薬物治療は，非ステロイド系抗炎症剤のスリンダクやcyclooxygenase-2(COX2)選択的阻害剤などが試みられているが，有効性や副作用の面から，いまだ臨床応用できるところまでは至っていない[1]。

C．FAPにおける癌の早期発見(二次予防)(表2)

FAPの診断がついたら，大腸摘出術を検討するが，小さい腺腫のみの例，大きい腺腫の数が少ない例などでは，比較的大きな腺腫を内視鏡的に摘除しながら，厳重な経過観察(6～8カ月に1回の全大腸内視鏡検査)を行う場合もある。

直腸を残す結腸全摘回腸直腸吻合術(ileorectal anastomosis: IRA)を受けた患者には，直腸の癌を早期に発見するために，6カ月～1年ごとに大腸内視鏡検査を行い，大きな腺腫を認めれば内視鏡的に摘除する。大腸全摘回腸肛門吻合術(ileoanal anastomosis: IAA)や大腸全摘回腸肛門管吻合術(ileoanalcanal anastomosis: IACA)でも，肛門にわずかに直腸粘膜が残存し，そこから大腸癌が発生する可能性もあり，術後長期間経過するとJ型回腸嚢に腺腫が発生することもあるため，IAAの術後でも数年ごとの肛門からの内視鏡検査を行うことが望ましい。

胃癌，十二指腸癌に対する二次予防については，20歳頃からの定期的な上部消化管内視鏡検査を行う。

2．Cronkhite-Canada症候群(Cronkhite-Canada syndrome: CCS)

CCSは，消化管，特に胃と大腸にポリープが多発する原因不明の非遺伝性疾患である。組織像は若年性ポリープに類似した非腫瘍性病変であり，炎症性変化が目立つ。経過とともに縮小，消失することが多い。胃や大腸では腺腫や癌の合併も認められる。蛋白漏出性胃腸症による低栄養状態，皮膚色素沈着，爪の萎縮，脱毛などを伴う。中年以降に発症し，男性に多い。1999年までに306例の報告があり，そのうち70%以上は本邦からの報告である。

病因は不明である。感染，栄養素欠乏，免疫低下，身体的・精神的ストレスなどが原因として疑われている。症状は下痢が最も多い。それ以外に腹痛，体重減少，食思不振，味覚異常などを認めることがある。低蛋白血症や低アルブミン血症があれば，それによる症状も伴う。

消化管ポリープは胃と大腸に多く，小腸には少ない。食道には稀である。胃，大腸には内視鏡的に発

赤の強いポリープがみられる。ポリープの密度，大きさはさまざまであるが，密生することが多い。介在粘膜にも浮腫状で発赤を伴う炎症所見がみられる。組織所見では，腺管の過形成・拡張がみられ，粘膜固有層の間質には高度の炎症性細胞浸潤と浮腫がみられる。胃，大腸の腺腫，癌の合併は比較的多い。

消化管病変のほかに，外胚葉の病変として，脱毛，皮膚色素沈着，爪の萎縮，白濁などがみられる。低蛋白血症，低アルブミン血症は多い。血清電解質異常がみられることもある。

副腎皮質ステロイドが奏効することがある。栄養改善のために完全静脈栄養法や成分栄養法が行われる。癌を認めた場合には，通常の癌と同様の治療を行う。予後は比較的良好である。経過とともに比較的短期間に縮小・消失するものが多い。

3．Peutz-Jeghers 症候群(Peutz-Jeghers syndrome: PJS)

口腔や唇，指趾に特有な色素沈着と消化管に多発性の過誤腫性ポリープを合併する常染色体優性遺伝疾患である。原因遺伝子の一つとして，第19染色体の*LKB1*遺伝子の異常が指摘されている。原因遺伝子の一つと考えられている*LKB1*遺伝子は，キナーゼ機能をもつ蛋白であると推測され，その不活化が腫瘍発生につながる癌抑制遺伝子と考えられている。

比較的若年期に発症する。イレウス症状や腹痛，血便，ポリープの肛門脱などがみられる。約半数で腸重積を合併する。黒褐色ないし茶褐色の色素沈着は4～5歳頃から出現する。口腔，唇，指趾の特徴的な色素沈着を皮膚科医に指摘されて診断されることもある。

消化管ポリープは，食道を除く胃，小腸，大腸に発生し，特に小腸に多い。数十個程度のことが多い。大きさはさまざまで，数mmから5cmを超えるような大きいものもある。

消化管ポリープの組織像は，粘膜筋板が樹枝状に延長し，正常腺管と同様の腺管が増生している。胃ポリープでは，上記所見に加え，腺窩上皮の増殖と嚢胞状拡張を認める。できるだけ保存的に行い，大きなポリープは内視鏡的または外科的に切除する。小腸では穿孔に注意が必要である。

消化管癌の合併率は20～25%で，大腸癌が多い。ポリープから癌が発生すると考えられている。卵巣癌，乳癌，膵癌，肺癌など他臓器癌の合併も高率であるため，全身の定期的な検査が必要である。

Ⅲ．遺伝性非ポリポーシス大腸癌(HNPCC)

1．HNPCCの特徴

大腸癌の発生には，発癌抑制遺伝子の機能異常による発癌とミスマッチ修復遺伝子の機能異常による発癌が知られている。このミスマッチ修復遺伝子に変異をもつ疾患として，HNPCCがある。HNPCCは以前にはLynch症候群とよばれていたが，最近はHNPCCと称している。

家族歴からの診断基準として，アムステルダム診断基準Ⅱが広く用いられている。アムステルダム診断基準Ⅱは，血縁者に3名以上の組織学的に証明されたHNPCC関連癌(大腸癌，子宮内膜癌，小腸癌，腎盂・尿管癌)に罹患しており，かつ，以下のすべての条件に合致していることである。

1)罹患の1名は他の2名の第一度近親者であること，2)少なくとも継続する2世代にわたり罹患者がいること，3)罹患者の1名は50歳未満で診断されていること，4)FAPが除外されていること。

本邦ではHNPCC家系から胃癌の発生を多く認めるため，HNPCC関連癌に胃癌を加えることが多い。このようにHNPCCは病歴や家族歴による診断基準により診断していたが，ミスマッチ修復遺伝子異常の発見により，遺伝子変異による診断も試みられるようになってきている。

職域検診における家族歴の聞き取り研究より，本邦では6万人程度のHNPCCの体質をもつ患者がいる可能性が考えられている。

2．HNPCCの治療と予防(表3)

HNPCCでは，まだ発癌前の大腸全摘術は行われていない。しかし，大腸癌を認めたときに，残存する大腸を少なくするため，大きく大腸を切除したり，併せて子宮を摘除することが検討されている。まだ，確立した早期発見プログラムはないが，ICG-HNPCCのガイドライン[2]では，大腸癌に対して20～25歳から2年に1回の大腸内視鏡検査，子宮内膜癌

表 3　遺伝性非ポリポーシス大腸癌(HNPCC)の一次予防と二次予防

	一次予防	二次予防
大腸癌	適度な運動 赤身肉，アルコールの摂取制限 野菜の摂取促進	20歳前半から1～2年に1回の大腸内視鏡検査
胃癌	禁煙	30歳前半から1～2年に1回の上部消化管内視鏡検査
子宮内膜癌	肥満予防	30歳前半から1～2年に1回の婦人科検診，細胞診，CA-125，超音波検査
腎盂・尿管癌	禁煙	30歳前半から1～2年に1回の尿検査，超音波検査 血尿を認めたらすぐに精査
小腸癌	不明	便潜血検査

に対して30～35歳から1～2年ごとの婦人科検診，経腟的超音波検査，腫瘍マーカーであるCA-125の血液検査，胃癌に対して30～35歳から1～2年ごとの胃内視鏡検査，尿路系癌に対して30～35歳から1～2年ごとの腹部超音波検査と尿検査を勧めている。

2年ごとの大腸内視鏡検査にて進行した大腸癌が発見された例があり，2年ごとより毎年の大腸内視鏡検査が望ましいが，長期間にわたる毎年の大腸内視鏡検査は患者に多大な負担になり，経過観察が完遂されない危険性がある。

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Current Concepts: Gastrointestinal Polyposis and Hereditary Non-polyposis Colorectal Cancer

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This report reviews the state of the art in cancer prevention of gastrointestinal polyposis (familial adenomatous polyposis (FAP), Cronkhite-Canada syndrome, Peutz-Jeghers syndrome) and hereditary non-polyposis colorectal cancer.

FAP is a hereditary disease characterized by the development of multiple adenomatous polyps in the gastrointestinal tract. The timing of colectomy depends on the size and number of adenomatous polyps. The two types of colectomy are total colorectomy with ileoanal anastomosis (IAA) and subtotal colectomy with ileorectal anastomosis (IRA). If subtotal colectomy is performed, at least annual surveillance of the remaining rectum is recommen-ded. For individuals with mild polyposis, colectomy is recommended, but it may be deferred until polyps are difficult to control.

Endoscopic or surgical removal of duodenal adenomas is advisable if polyps show severe atypia and exceed one centimeter. Upper-gastrointestinalscopy should be begun when colonic polyposis is detected or by age 20 years and repeated every year.

key words: familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC), colorectal cancer

Legends to Figures and Tables

Figure 1　Colonoscopic image of a typical familial adenomatous polyposis.
Figure 2　Colonoscopic image of a young patient with familial adenomatous polyposis.
Figure 3　Endoscopic resection of polyposis.
Figure 4　Surveillance policy of familial adenomatous polyposis.
Table 1　Classification of gastrointestinal polyposis syndrome.
Table 2　Surveillance and management of familial adenomatous polyposis.
Table 3　Surveillance and management of hereditary non-polyposis colorectal cancer.