Preventive Effect of Green Tea Catechins on Experimental Tumor Metastasis in Senescence-Accelerated Mice

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Successful avoidance of the immune surveillance system is critical for the development of a blood–borne metastasis. Previous findings suggest that experimental tumor metastasis was enhanced in senescence-accelerated mice prone 10 (SAMP10) due to a reduction in immune surveillance potential with age. In the present study, water containing green tea (GT)-catechins was freely given to SAMP10 mice, and the chemopreventive effect of GT-catechin intake on tumor metastasis was examined. Natural killer cell activity, which is an indicator of immune surveillance potential and is reduced in control mice with age, was maintained by GT-catechin intake. The early accumulation of lung-metastatic K1735M2 melanoma cells in lungs after intravenous injection of the cells and subsequent experimental lung metastasis was investigated in mice given GT-catechins. The accumulation at 6 and 24 h after injection of K1735M2 cells was significantly suppressed, and the number of lung-metastatic colonies was significantly reduced, in comparison with those in control mice. The results suggest that GT-catechin intake prevented the experimental tumor metastasis in aged SAMP10 mice *via* its inhibition of a reduction in immune surveillance potential with age.

Key words aging; tumor metastasis; green tea catechin; immune surveillance

Tumor metastasis is a key step in the development of a tumor and becomes a critical trigger for the death of a patient. Hematogenous metastasis is established by a series of steps, which begin with the dissociation from primary sites and culminate with the formation of metastatic colonization.[1] This process is greatly dependent on surrounding host factors such as host resistance to cancerous cells.[2,3] In a previous study, we demonstrated that the early accumulation of metastatic tumor cells in a target organ after intravenous injection of the cells and following tumor metastasis were enhanced by reduction of the host immune surveillance potential,[4,5] suggesting that avoidance of the surveillance associated with immune cells, such as natural killer (NK) cells, was a critical step for the completion of an experimental tumor metastasis.

The senescence-accelerated mice prone (SAMP) strain has been established as a mouse model for aging research[6] and exhibits a more accelerated senescence process than normal mice.[7,8] By using this model, we found that the immune surveillance potential of 8-month-old aged SAMP10 mice was lower than that of 2-month-old young mice, and that the experimental lung metastasis was significantly induced in aged mice. These data suggest that the aging process produces an environment susceptible to metastatic tumor cells in the bloodstream to complete metastasis, and that such an environment is produced with age due to the reduced immune surveillance potential.[9]

Green tea (GT)-catechins are functional polyphenols, and are known to have various actions, such as antibiotic, anti-inflammatory, antioxidative, and anti-cancer effects. It is anticipated they will become useful as a functional food that promotes human health and longevity. Although animal studies have shown that GT-catechin treatment suppressed tumorigenesis, tumor growth, and tumor angiogenesis,[10,11] the crucial mechanism of the action of GT-catechin against cancer has not been fully elucidated. Our previous study demonstrated that (−)-epigallocatechin gallate (EGCG, a major component of GT-catechins) suppressed tumor angiogenesis through the inhibition of membrane type-1 matrix metalloproteinase (MT-1MMP) activity and subsequent induction of dormancy of solid tumor growth.[12,13] Tachibana *et al.* also reported that 67-kDa laminin receptors are a receptor for EGCG and that EGCG treatment inhibited the receptor-mediated signaling pathway and subsequent tumor cell growth.[14,15] Several reports have described various such approaches to treat and prevent cancer using GT-catechins. In the present study, we hypothesized that GT-catechin intake modulates the immune surveillance potential in aged mice, and enhances the susceptibility to experimental tumor metastasis. To prove this, we exposed SAMP10 mice to GT-catechin in their drinking water for an extended period of time (aged/catechin mice) and examined their NK activity as an indicator of immune surveillance potential. We then also examined the accumulation of K1735M2 melanoma cells in the lungs, the target organ, after intravenous injection of the cells as well as the preventive effect of GT-catechin intake on experimental tumor metastasis.

MATERIALS AND METHODS

Animals SAMP10 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). They began to show several senescence symptoms such as brain atrophy and dehairing from about 6 months-old. Since their immune surveillance potential was adequately reduced at 8 months-old,[9] we used 8-month-old or older SAMP10 mice as an appropriate model for the cancer prone age (aged SAMP10 mice). In another group of aged mice (aged/catechin SAMP10 mice), water containing 0.02% (w/v) GT-catechins (Polyphenon70S, Mitsui Norin Co., Ltd., Tokyo) was given *ad libitum* beginning

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at the age of 1-month-old. Polyphenon70S is a crude extract from green tea and contains various kinds of catechins such as (−)-epigallocatechin gallate (EGCG), which is the main component, and (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and epicatechin (EC). The contents of the various catechins in Polyphenon70S are shown in Table 1 (Information was provided by Mitsui Norin Co., Ltd.). Animal care and experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka.

Cells Highly lung-metastatic, murine K1735M2 melanoma cells were kindly provided by Dr. Jun Yokota of the National Cancer Center (Tokyo, Japan). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Japan Bio Serum Co., Ltd., Hiroshima, Japan) at 37 °C in the presence of 5% CO_2 in a humid atmosphere. NK-sensitive YAC-1 cells were purchased from the RIKEN Bioresource Center Cell Bank (Ibaraki, Japan) and cultured in RPMI1640 medium containing 10% FBS at 37 °C in the presence of 5% CO_2.

Experimental Lung Metastatic Model K1735M2 cells (5×10^4 cells/mouse) were intravenously injected into 2-month-old, 8-month-old, or 13-month-old SAMP10 mice *via* a tail vein and metastatic colonies were allowed to form in their lungs. In the chemoprevention experiment, K1735M2 cells were injected into 8-month-old aged or 8-month-old aged/catechin SAMP10 mice. Twenty-one days after injection of the tumor cells, the animals were sacrificed under diethylether anesthesia, and their perfused lungs were isolated. Tumor metastasis was evaluated by counting the number of metastatic colonies on the surface of the lungs.

NK Activity Assay To measure the NK activity, spleen cells were used as effector cells. Spleen of aged or aged/catechin SAMP10 mice was harvested, and the splenocytes were carefully flushed several times with an 18 G needle-tipped syringe and passed through nylon mesh to prepare the single cell suspension. The resultant cells were washed twice after removal of erythrocytes, and the number of cells was adjusted to 1.25, 2.5, and 5×10^6 cells/ml. On the other hand, to prepare target cells for ^{51}Cr release assay, mouse NK-sensitive lymphoma YAC-1 cells were used. The cells were incubated with $Na_2{}^{51}CrO_4$ solution (GE Healthcare U.K., Ltd., England) for 60 min at 37 °C for ^{51}Cr-radiolabeling. After removal of free ^{51}Cr, the number of radiolabeled cells was adjusted to 1×10^5 cells/ml. NK activity of aged or aged/catechin SAMP10 mice was measured by ^{51}Cr release assay as follows: Both the effector and the target cells were mixed in 96-well-plates and the plates were incubated for 4 h at 37 °C. After centrifugation of the cell suspension, radioactivity in the supernatant was measured with a gamma counter. Spontaneous release and maximum release were determined by adding RPMI medium containing 10% FBS or 0.1% TritonX-100 solution to the target cell suspension. Specific cytotoxicity of NK cells was determined using the following formula:

$$\%\ \text{lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

Distribution of Metastatic Tumor Cells K1735M2 cells were radiolabeled with 5-[^{125}I]iodo-2′-deoxyuridine ([^{125}I]IUdR, GE Healthcare U.K., Ltd.) as described previously.[16] Briefly, these cells were incubated overnight with [^{125}I]IUdR at 37 °C in the presence of 5% CO_2. After incubation, the cells were collected and centrifuged to remove unincorporated [^{125}I]IUdR, and the number of cells was adjusted to 1×10^4 cells/mouse for injection. These cells were intravenously injected into 8-month-old aged and 8-month-old aged/catechin SAMP10 mice. At 6 and 24 h after the injection, the mice were sacrificed under anesthesia with diethylether to collect blood and organ (heart, lung, liver, spleen, and kidney) samples. The amount of accumulation of tumor cells in each organ was determined by radioactivity. Distribution data are presented as % injected dose per tissue. The total weight of blood was assumed to be 7.56% of the body weight.

Table 1. Composition of Green Tea Catechins

GT-catechins	Content (w/w, %)
Epigallocatechin (EGC)	16.5
Epicatechin (EC)	5.7
Epigallocatechin gallate (EGCG)	34.5
Epicatechin gallate (ECG)	10.1
Gallocatechin gallate (GCG)	2.9
Catechin gallate (CG)	0.6

Information of the content was provided from Mitsui Norin Co., Ltd.

Statistic Analysis The variance in a group was evaluated using the *F*-test, and the differences among each group were evaluated using Student's *t*-test.

RESULTS

Enhanced Experimental Tumor Metastasis with Age in SAMP10 Mice We first investigated whether this enhancement of tumor metastasis is dependent on the degree of aging or not. Therefore, 2-month-old, 8-month-old, and 13-month-old SAMP10 mice were prepared and an experimental tumor metastasis assay was performed using K1735M2 melanoma cells. As in a previous report, the number of metastatic colonies in the lungs of 8-month-old aged mice was larger than that in 2-month-old young mice. In 13-month-old aged SAMP10 mice, the number of colonies was further increased (Fig. 1). This result suggested that the susceptibility to experimental tumor metastasis in SAMP10 mice increased in an age-dependent manner.

Effect of GT-Catechin Intake on NK Activity in Aged SAMP10 Mice We assumed that GT-catechin intake could suppress the reduction of immune surveillance potential with aging. Two groups of SAMP10 mice were grown in the same environment except that aged/catechin SAMP10 mice were given water containing 0.02% Polyphenon70S instead of normal water beginning at 1-month-old. When these mice reached 8 months-old, their NK activity was examined. The results of ^{51}Cr release assay showed that NK activity in aged/catechin SAMP10 mice was significantly higher than that of aged SAMP10 mice for every effector cells/target cells (E/T) ratio (Fig. 2), suggesting that GT-catechin intake suppressed the attenuation of immune surveillance potential with age.

Suppression of Tumor Cell Accumulation in Lung by GT-Catechin Intake We next confirmed the early accumulation of lung-metastatic K1735M2 cells after intravenous in-

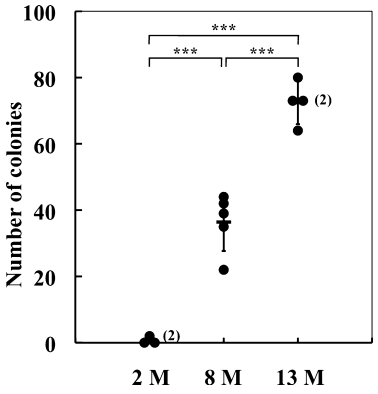

Fig. 1. Age-Dependent Increase in Experimental Tumor Metastasis in SAMP10 Mice

K1735M2 cells (5×10^4 cells/mouse) were intravenously injected into 2-month-old ($n=4$), 8-month-old ($n=5$), and 13-month-old SAMP10 mice ($n=4$), and the number of metastatic colonies in the lungs was counted on day 21 after the tumor cell implantation. The circles in the graph show the number of colonies in individual mice and black bars show the average number of colonies in each mouse. Asterisks indicate the significant differences (*** $p<0.001$). Similar results were obtained in a separate experiment.

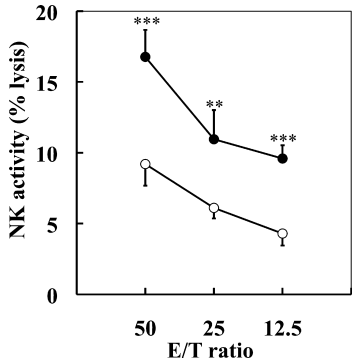

Fig. 2. Recovery of NK Activity by GT-Catechin Intake in Aged SAMP10 Mice

Cytotoxicity of NK cells from the spleen (E: effector cells) of aged (○) and aged/catechin (●) SAMP10 mice against ^{51}Cr-labeled YAC-1 target cells (T: target cells) was examined as described in Materials and Methods. The radioactivity detected in supernatant medium was measured by a gamma counter, and the NK activity was determined. Significant differences from aged control mice are indicated (** $p<0.01$; *** $p<0.001$). Similar results were obtained in a separate experiment.

jection in aged and aged/catechin SAMP10 mice, since GT-catechin intake suppressed the reduction in immune surveillance potential with age. As shown in Fig. 3A, the accumulation of [^{125}I]IUdR-labeled K1735M2 cells in the lungs of aged/catechin mice was lower than that in aged SAMP10 mice at 6 h after the intravenous injection of the cells. Furthermore, this difference in the accumulation was maintained up to 24 h (Fig. 3B). These results supported the idea that GT-catechin intake suppresses the attenuation of immune surveillance potential, and therefore the clearance of metastatic tumor cells from the target organ is not impaired.

Preventive Effect of GT-Catechin Intake on Experimental Lung Metastasis in Aged SAMP10 Mice To investigate the preventive effect of GT-catechin intake on experimental tumor metastasis in old age, lung-metastatic K1735M2 tumor cells (5×10^5 cells/mouse) were injected into the bloodstream of 8-month-old aged, or aged/catechin SAMP10 mice, and then the number of lung-metastatic colonies was determined on day 21 after injection. The number of metastatic colonies in the lung of aged/catechin SAMP10 mice was far less than that in aged SAMP10 mice given normal water (Fig. 4).

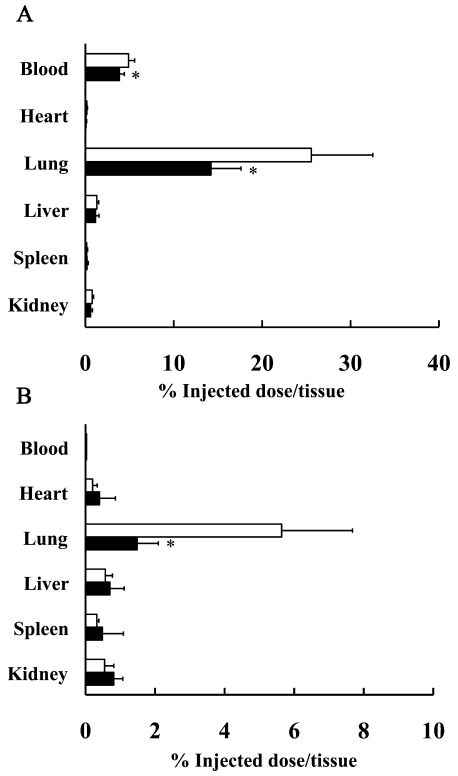

Fig. 3. Distribution of [^{125}I]IUdR-Labeled K1735M2 Melanoma Cells in SAMP10

[^{125}I]IUdR-labeled K1735M2 melanoma cells (1×10^4 cells/mouse) were injected into aged (open column) and aged/catechin (closed column) SAMP10 mice. The columns indicate the percent accumulation of [^{125}I]IUdR-labeled K1735M2 melanoma cells 6 (A) and 24 (B) h after injection. Significant differences from aged mice are indicated (* $p<0.05$). Distribution experiments were performed in two more separate experiments, and similar results were obtained.

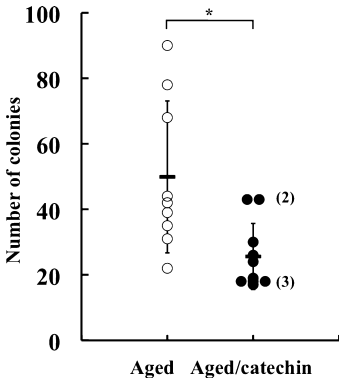

Fig. 4. Preventive Effect of GT-Catechin Intake of Experimental Tumor Metastasis in Aged SAMP10 Mice

K1735M2 cells (5×10^4 cells/mouse) were intravenously injected into aged ($n=9$) and aged/catechin SAMP10 mice ($n=10$) and the metastatic colonies in the lungs were counted on day 21 after tumor implantation. The circles in the graph show the number of colonies in individual mice and black bars show the average number of colonies in each mouse. Asterisks indicate significant differences (* $p<0.05$). Similar results were obtained in a separate experiment.

DISCUSSION

Senescence is accompanied by various changes in the body, and the internal environment in aged people is different from that in young people. Young people are able to maintain

body homeostasis, and their capacity to defend against foreign substances such as exogenous antigens is superior to that of aged people. Although the mechanism of aging is quite complicated, the accumulation of reactive oxygen species (ROS) in the body is considered to be an important factor in aging.[17] While the relationship between ROS production and cancer development has already been demonstrated, cancer usually starts to develop in middle age or later, suggesting that the aging process is involved in cancer development.[18,19] Our previous study demonstrated that the aging process caused the enhancement of susceptibility to experimental tumor metastasis in senescence-accelerated mice and the reason why it occurs was suggested to be due to a reduction in immune surveillance potential against metastatic tumor cells with age.[9] These findings suggest that the maintenance of a healthy internal environment in aged animals would improve the age-related weakening of the protective effect against experimental metastasis.

To maintain body homeostasis in a favorable condition, a number of functional components have been developed, of which GT-catechin is a well-known example.[20] Many researchers have studied GT-catechin functions and the multifunctional effects of GT-catechins against cancer have been demonstrated.[11] Young animals have been widely used in these basic studies on cancer prevention since it takes a long time to prepare aged animals. It is uncertain, however, whether this situation reflects aged humans, who have high cancer rates, and if these functional components are truly effective at preventing cancer. In the present study, we used SAMP mice as a model to investigate the preventive effect of GT-catechin intake against tumor metastasis in old age.

We first prepared SAMP10 mice of different ages and examined the difference in blood–borne metastasis after intravenous injection of K1735 melanoma cells with age. As shown in Fig. 1, the number of metastatic colonies increased with age. This result confirmed those of our previous study and demonstrated that tumor metastasis was accelerated in an age-dependent manner.[9] In an attempt to inhibit tumor metastasis in old age, we exposed aged SAMP10 mice to GT-catechin water (0.02% Polyphenon70S) for an extended period of time. When the daily intake volume of GT-catechin water was measured, the volume was about 10 ml/d/mouse and was almost the same as for the group given normal water.

At first, the cytotoxicity of splenocytes derived from aged and aged/catechin mice against NK-sensitive YAC-1 cells was measured as an indicator of immune surveillance potential, since the spleen possesses a lot of immune-related cells such as NK cells and plays a critical role in the systemic immune system. GT-catechin intake enhanced the NK activity of aged SAMP10 mice (Fig. 2). Since aging is thought to involve the accumulation of oxidative stress,[21,22] it is possible that an age-dependent burden of oxidative stress causes degradation of the immune defense system against the tumor cells. In actual fact, the cytotoxicity of NK cells is known to be dependent on oxidative stress and the activity is often reduced by ROS.[23] Ferrández *et al.* previously demonstrated that an age-dependent reduction in NK activity was improved by the existence of antioxidant agents such as ascorbic acid and α-tocopherol.[24] This result suggests that the antioxidant agents up-regulated NK activity through their protection against ROS. On the other hand, GT-catechins, especially EGCG, are known to work as potent radical scavengers and show antioxidative effects against various types of oxidative stress.[22,25] Unno *et al.* demonstrated that long-term intake of EGCG water up-regulated the level of antioxidative activity in the serum of aged SAMP10 mice.[26] Thus, we speculated that GT-catechin intake provided protection from the accumulation of oxidative stress and improved the reduced NK activity with age. We also reported that consumption of GT-catechin prevented the decline of glutathione peroxidase activity and provided protection from oxidative damage of protein in mouse brain of aged SAMP10.[27] This result also supports our present speculation that GT-catechin intake up-regulates the age-induced reduction in NK activity and has the effect in aged animals.

We next evaluated the effect of GT-catechin intake on blood–borne metastasis induced by intravenous injection of tumor cells. We previously demonstrated that defense against metastatic tumor cells by the immune surveillance system was observed in the early stage of tumor metastasis by use of positron emission tomography (PET).[4,5] In the study, we examined the relationship among the real-time trafficking of lung-metastatic B16BL6 cells, metastatic potential, and the number of the cells injected. When 1×10^4 cells were injected, the accumulation of the cells in the lung was less than one-tenth of that obtained with a 1×10^5 cell-injection. Metastasis was observed when 1×10^5 cells were injected, but not when 1×10^4 cells were injected. To clarify the roles of the immune defense system at the initial phase of metastasis, mice were treated with 2-chloroadenosine prior to the tumor cell challenge. As a result, this treatment suppressed not only metastasis but also the early accumulation of the cells in lungs. These results suggest that the immune surveillance, whose action was obvious at the low dose of challenged tumor cells, functions strongly at the initial phase but not at the advanced stages of the metastatic process.[5] Therefore, in the present study, we examined the biodistribution of [^{125}I]-labeled K1735M2 cells after intravenous injection and found that early accumulation of tumor cells in their metastatic site was reduced in aged/catechin SAMP10 mice, and this status was also observed at 24 h after injection (Fig. 3).

NK cells are one type of immune cell related to innate immunity, especially immune surveillance, and together with macrophages form the first lines of defense against exogenous agents and tumor cells. Therefore, this result suggested that suppressing the decrease in NK activity with age by GT-catechin intake suppressed the age-induced accumulation of tumor cells in the target organ at the early stage. Experimental tumor metastasis in aged SAMP10 mice was also suppressed by GT-catechin intake (Fig. 4). Shimizu *et al.* reported that daily intake of green tea extract protected against metachronous colorectal adenomas after surgical removal of primary tumors in a pilot study in humans.[28] Our results in the present study may partly explain their finding.

In conclusion, the results of the present study indicate that GT-catechin intake improved the reduction in immune surveillance potential, such as NK activity with age, and suppressed the age-related increase in the susceptibility to experimental tumor metastasis in SAMP10 mice. Thus, daily intake of green tea may provide protection against the weakening of immune surveillance activity with age and thus con-

tribute to human health and longevity. In the present study, SAMP mice were used as an aging model because the internal environment of the body of aged animals and their responses to chemopreventive agents are different from those of young animals. The usage of aged animals to investigate the therapeutic and preventive efficacy of chemopreventive agents may produce more predictable results for actual human cancers.

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