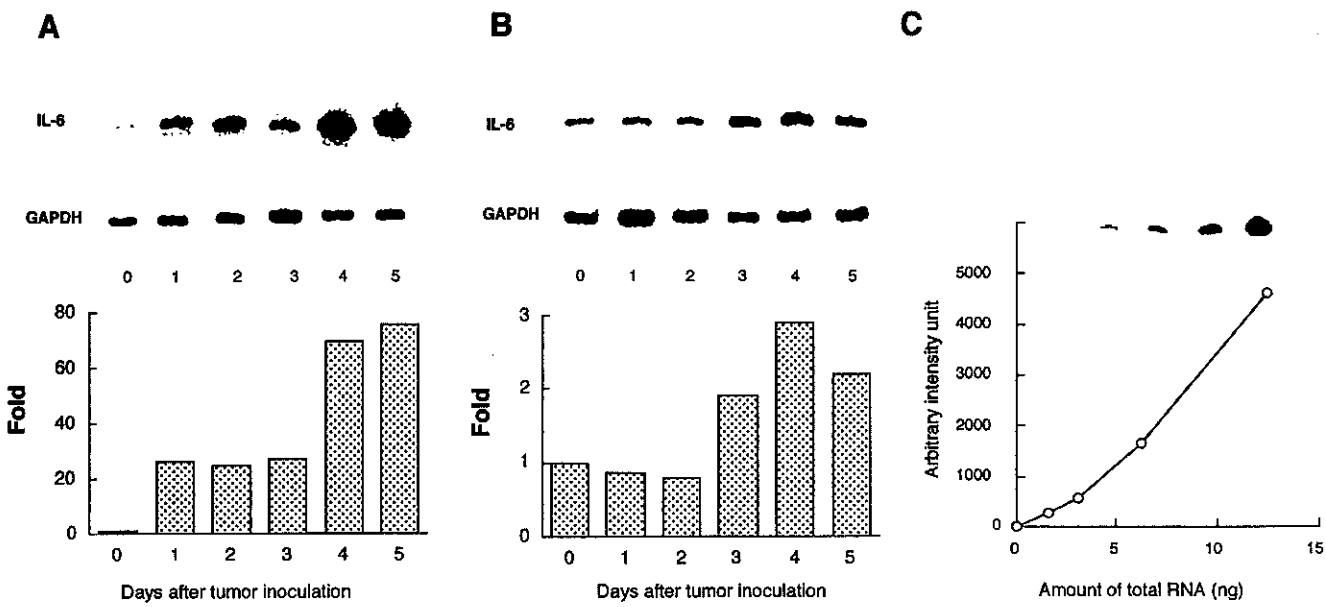

Figure 4. Time-course of IL-6 gene expression in subcutaneous tumors and lung in tumor-bearing mice. Expression of IL-6 gene in the tumor (A) and the lung (B) of tumor-bearing mice. Amount of IL-6 mRNA was calculated by standard curve (C) and expressed as -fold compared with normal skin (A) or normal lung (B).

Time course of IL-6 expression in subcutaneous tumor and lung in tumor-bearing mice. Since it is possible to think that IL-6 participated in restraint of lung colony formation as mentioned above, we examined the time-course of IL-6 gene expression in the subcutaneous tumors. Normal skin expressed only a small amount of IL-6 gene, while the tumor-bearing skin expressed a remarkably high amount of IL-6 gene even at 1 day after tumor inoculation. Fig. 4A shows the increased amount of IL-6 gene from 1 to 5 days later. The amount of IL-6 gene was ~76 fold, 5 days later, compared with that in normal skin. It is important to note that expression of IL-6 gene in the lungs started to increase on the third day in tumor-bearing mice, and the increase was roughly double that of the control (Fig. 4B).

Discussion

The results obtained in our experiments demonstrated that antitumor resistance can be achieved by inoculation of colon 26 cells into tumor-bearing mice, where the number of artificial metastatic colonies developed in the lungs of subcutaneous tumor-bearing versus control mice serves as an identification of antitumor resistance to a second challenge. For many years, this phenomenon was believed to be mediated through an immunological mechanism (1-5), and earlier reports showed immune effectors were produced from splenocytes in tumor-bearing mice. Our experimental system using tumor-bearing mice of colon 26 cells demonstrated that removal of NK cell or T cell subsets using NK^-, $CD4^-$ or/and $CD8^-$-specific monoclonal antibodies *in vivo* inhibited lung colony formation in normal mice as strongly as in non-treated tumor-bearing mice, though the immunological effector cells were not determined. Thus, we considered the importance of non-immunological systemic factors, such as nutritional deficiencies (25) and tumor by-products or cytokines, that influence tumor growth rate (26,27). We paid attention to the fact that colon 26 cells produce IL-6 spontaneously, and we found that in subcutaneous tumor-bearing mice, concentration of IL-6 in serum gradually increased, while colon 26 cells developed tumors. Concentration of IL-6 in sera was 3.2±1.2 pg/ml in mice with lung colony formation, and without subcutaneous tumors. Concentration of serum IL-6 in tumor-bearing mice varied greatly, depending on the organ inoculated with colon 26 cells, which suggests that IL-6 was produced not only in colon 26 cells, but also in parenchymal tissues, and, further-more, that inoculated tumor cells affected monocytes/macrophages, fibroblasts, keratinocytes, endothelial cells, T cells, and B cells in the mice skin in a paracrine manner. IL-6 is known as an inflammatory cytokine (28-30) that affects B cells, T cells and macrophage differentiation, stimulates keratinocyte growth, inhibits fibroblast proliferation, and stimulates or inhibits tumor cell growth (31-34). However, the specific role of IL-6 in antimetastasis has not yet been determined. We present here the first evidence of the close correlation between serum IL-6 level and IL-6 gene in the lung, along with consequent inhibition of lung colony formation (antimetastatic effect) in tumor-bearing mice. Since the infiltration of tumor cells (colon 26 cells) in the lung of tumor-bearing mice was not observed microscopically, it is assumed that IL-6 released in the sera induces IL-6 gene expression in the lung tissue, probably in alveolar macrophages, and then inhibits tumor growth in the lung after the second challenge is conducted intravenously. The elevation of IL-6 gene in the lung of tumor-bearing mice could have progressed as follows: first, subcutaneous tumor cells may have penetrated into blood stream to reach the lung, resulting in micrometastases of the tumor cells, or their reaction with endothelial cells, fibroblasts, monocytes/macrophages or T cells in the lung. Second, immune effector cells,

such as granulocytes, monocytes/macrophages or B cells, may have been activated in subcutaneous tumors and then have affected the lung tissue. Third, this process may be facilitated by IL-1 and TNF-α, which are major inducers of IL-6 gene expression. Thus, the process of paracrine, endocrine, and autocrine IL-6/IL-1 loops (28,35) in lung tissue can regulate the proliferative capacity of colon 26 cells, and leave them metastatically incompetent in the lung. These considerations also suggest the possibility of acquisition of resistance to lung metastases in tumor-bearing host, due to expression of organ inhibitional factors, conceptually similar to the acquisition of organ specific metastases in cancer.

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Preventive effects of drinking green tea on cancer and cardiovascular disease: Epidemiological evidence for multiple targeting prevention

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Abstract. The significance of drinking green tea in prevention of two of the main lifestyle-related diseases, cancer and cardiovascular disease, was demonstrated in terms of a prospective cohort study on a total of 8,552 general residents in Saitama Prefecture, Japan. On the basis of the follow-up study, we revealed decreased relative risk of cancer incidence for those consuming over 10 cups a day, compared with those consuming below 3 cups: 0.54 (95% confidence interval, 0.22–1.34) for men, 0.57 (0.34–0.98) for women, and 0.59 (0.35–0.98) for both sexes. Furthermore, a significant delay in cancer onset was associated with increased consumption of green tea. Next, decreased relative risk of death from cardiovascular disease was 0.58 (0.34–0.99) for men, 0.82 (0.49–1.38) for women, and 0.72 (0.60–1.04) for members of both sexes consuming over 10 cups a day. Finally, we evaluated the life-prolonging effects of drinking green tea on cumulative survival, using the life table.

Keywords: Green tea, cancer, cardiovascular disease, prospective cohort study

1. Introduction

Green tea has recently obtained significant acceptance as a cancer preventive, on the basis of numerous studies from around the world, which have been accumulating since 1987, when Dr. H. Fujiki's group for the first time reported the cancer-preventive activity of (-)-epigallocatechin gallate (EGCG), the main constituent of green tea polyphenols [1]. Subsequent laboratory studies have revealed that EGCG or green tea extract in drinking water inhibited carcinogenesis in various organs in rodents [2–4]. In addition to the experimental evidence, our prospective cohort study previously reported the cancer-preventive effects of drinking green tea on the basis of a nine-year follow-up study (384 cancer cases) [5]. Our Phase I trial with green tea tablets found that blood examination showed no adverse effects among the participants [6].

On the other hand, tea polyphenols were reported to reduce the levels of serum lipids in animal models, implying the preventive effects of green tea on cardiovascular disease [7,8]. We previously reported

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that drinking green tea resulted in lower serum levels of total cholesterol and triglyceride, along with a decreased atherogenic index, in terms of a cross sectional analysis of the prospective cohort study [9]; a further study using follow-up data remains to be reported.

In this paper we demonstrated the significance of green tea not only in prevention of cancer and cardiovascular disease, but also in prolonging life activity, indicating that green tea is effective for multiple targeting prevention.

2. Subjects and methods

In 1986 we began a prospective cohort study in residents over 40 years of age in a town in Saitama Prefecture, Japan. We used a self-administered questionnaire covering 90 lifestyle factors, where green tea consumption was categorized as below 3, 4–9, and over 10 cups a day. The questionnaire covered a total of 8,552 individuals. The details were described elsewhere [5]. Our eleven-year follow-up study from 1986 to 1997 found a total of 488 cancer cases. The most frequent cancers among both sexes were, in order, cancers of the stomach (140), lung (69), colorectum (60), and liver (35).

In the follow-up study, we surveyed death from all causes by death certificate from 1986 to 1997. A total of 1,109 deaths from all causes (338, 222, 193, and 356 deaths from cancer, cardiovascular disease, cerebrovascular disease, and other causes, respectively) were used in the analysis of cumulative proportion surviving. In the analysis of green tea and cardiovascular disease, we added 52 cardiovascular deaths in 1998 and 1999 to those from 1986 to 1997, for a total of 274 deaths from cardiovascular disease (ICD-9: 390–429).

3. Results

3.1. Age-standardized incidence rates and relative risk of cancer incidence

The overall cancer incidence rates, specifically, truncated age-standardized cancer incidence rates, were calculated by daily consumption of green tea, using a truncated world population, age over 40 years per 32,000 per annum. The rates (± SE) among women strongly indicate that high consumption (over 10 cups a day) of green tea resulted in a significant reduction of cancer incidence by about 40%: 116.5 ± 15.6, 159.6 ± 12.7, and 81.6 ± 18.6 for those consuming below 3, 4–9, and over 10 cups a day, respectively. On the other hand, men revealed an increased cancer incidence among those consuming over 10 cups a day: 190.0 ± 23.3, 193.6 ± 17.2, 264.2 ± 32.0 for those consuming below 3, 4–9, and over 10 cups a day, respectively. In men, an increased percentage of current smokers and their cigarette consumption was observed among those consuming the most green tea, compared with other groups (data not shown). Thus, the deleterious effects of cigarette smoking seem to cancel the benefits of drinking green tea. In fact, male non-smokers (never and ex-smokers) revealed a decreased cancer incidence associated with high consumption of green tea: 185.5 ± 35.5, 194.0 ± 27.6, 154.8 ± 45.2 for those consuming below 3, 4–9, and over 10 cups a day, respectively.

We then evaluated cancer risk by considering differences in lifestyle. Relative risk of cancer incidence by consumption of green tea was estimated in Table 1 by the Cox proportional hazard model, adjusting for the potent confounding factors. Women who consumed over 10 cups a day revealed a significantly decreased risk of 0.57, taking the risk of those consuming below 3 cups a day as reference. Men also showed a reduction in relative risk with increased consumption of green tea. Combining data for men

Table 1
Relative risk[a] of cancer incidence by daily consumption of green tea

	Consumption of green tea (cups/day)		
	⩽ 3	4–9	⩾ 10
Men	1.0	1.00 (0.50–2.04)[b]	0.54 (0.22–1.34)
Women	1.0	0.92 (0.64–1.31)	0.57 (0.34–0.98)
Both sexes	1.0	0.81 (0.52–1.27)	0.59 (0.35–0.98)

[a]Adjusted for cigarette smoking, alcohol consumption, intake of green and yellow vegetables, and intake of rice, using age as the fundamental time variable.
[b]In parentheses, 95% confidence interval.

Table 2
Mean age (± SE, years) at cancer onset by daily consumption of green tea among 488 cancer patients found in the follow-up study

	Consumption of green tea (cups/day)			
	⩽ 3	4–9	⩾ 10	All categories
Men	65.7 ± 0.6 (69)[a]	68.0 ± 0.9 (130)	68.9 ± 1.0 (86)	67.7 ± 0.6 (285)
Never smokers[b]	70.2 ± 3.7 (14)	71.4 ± 2.2 (19)	74.1 ± 2.5 (6)	71.4 ± 1.7 (39)
Current smokers	62.6 ± 1.6 (40)	67.3 ± 1.1 (76)	68.6 ± 1.1 (70)	66.8 ± 0.7 (186)
Women	68.3 ± 1.6 (58)	67.5 ± 1.1 (117)	74.5 ± 2.0 (28)	68.7 ± 0.9 (203)
Both sexes	66.9 ± 1.0 (127)	67.7 ± 0.7 (247)	70.2 ± 0.9 (114)	68.1 ± 0.5 (488)

[a]In parenthesis, number of patients.
[b]Excluding ex-smokers.

and women, both sexes revealed a significantly decreased risk of 0.59 associated with high consumption of green tea.

We next analyzed the cancer-preventive effects of green tea by organs. Green tea most significantly exerted its preventive effects on lung cancer in both sexes, with a relative risk of 0.33 (95% CI, 0.11–0.94) among those consuming over 10 cups a day, adjusting for sex and lifestyle factors. High consumption of green tea showed decreased relative risk of 0.56 (0.22–1.40), 0.53 (0.17–1.57) and 0.69 (0.23–1.88) for cancers of the colorectum, liver, and stomach, respectively. This epidemiological observation showed close correlation with the target organs of EGCG or green tea extract in rodent carcinogenesis experiments [2–4].

3.2. *Age at cancer onset*

Analysis of mean age at cancer onset among the 488 cancer patients found in the follow-up study revealed that increased consumption of green tea was associated with delay of cancer onset (Table 2). Mean age at cancer onset among female patients consuming over 10 cups a day was 6.2 years higher than that among those consuming below 3 cups a day ($P < 0.01$). Among male patients, the difference in mean age at cancer onset by consumption of green tea was 3.2 years, possibly being influenced by cigarette smoking.

We found that cigarette smoking was associated with earlier onset of cancer ($P < 0.01$): mean age ± SE at cancer onset was 66.8 ± 0.7, 68.2 ± 1.3, and 71.4 ± 1.7 years in 186 male current smoker patients, 60 ex-smoker patients, and 39 never-smoker patients, respectively. Thus, earlier onset of cancer among smokers included in the highest consumption group of green tea cancelled the effects of green tea on age at cancer onset. In fact, delay of cancer onset was found in both male never and current smoker patients

Table 3
Serum concentration (Mean ± SE, nmol/ml) of lipid peroxides (thiobarbituric assay) among male smokers by consumption of green tea

	Consumption of green tea (cups/day)		
	⩽ 3	4–9	⩾ 10
Current smokers	9.38 ± 0.23	8.87 ± 0.18	8.23 ± 0.20[a]
Smokers consuming > 20 cigarettes a day[c]	10.30 ± 0.55	9.79 ± 0.43	7.79 ± 0.40[b]

[a] $P < 0.01$, compared with those in ⩽ 3 cups; $P = 0.1$ after adjustment for age and cigarette consumption.
[b] $P < 0.01$, compared with those in ⩽ 3 cups; $P < 0.05$ after adjustment for age and cigarette consumption.
[c] The upper quartile in distribution of cigarettes consumed a day (177 smokers).

in association with increased consumption of green tea ($P < 0.05$ for current smokers, Table 2). Our theoretical model predicted that delay of cancer onset by 5, 10, and 15 years generates a reduction of 25, 50, and 70% in cancer incidence, respectively.

3.3. *Serum risk markers and relative risk of cardiovascular death*

We previously reported an inverse association between consumption of green tea and serum risk markers for cardiovascular disease, in terms of a cross sectional analysis of 3,625 cohort members, 1,371 men, who gave peripheral blood samples at baseline [9]. A more detailed analysis including female subjects revealed: Decreased serum levels of total cholesterol were found among men with increased consumption of green tea, although this lowering effect was not observed among postmenopausal women; Decreased serum triglyceride and atherogenic index were consistently observed among men and women with increased consumption of green tea. Drinking green tea influenced not only serum levels of lipids and lipoproteins but also the oxidative status of serum lipids. Serum levels of lipid peroxides among 766 current smokers (mean ± SE, 8.83 ± 0.12 nmol/ml) were higher than those (8.37 ± 0.21) among 197 never smokers; smokers consuming over 10 cups a day revealed significantly decreased levels of serum lipid peroxides, compared with those consuming lower amounts (Table 3). The reduction of serum lipid peroxides was more remarked among smokers who consumed > 20 cigarettes a day.

We then estimated the relative risk of death from cardiovascular disease by consumption of green tea (Table 4), on the basis of our follow-up study. Men consuming over 10 cups a day revealed a significantly decreased risk of 0.58, adjusted for lifestyle factors; high consumption of green tea prevents cardiovascular death even among smokers with a significantly low relative risk of 0.51. Ten cups (150 ml per cup) of green tea contain 360–540 mg of EGCG or about 1 g of tea polyphenols, which is thought to be the daily required amount for prevention of cancer and cardiovascular disease.

3.4. *Cumulative proportion surviving*

Deaths from cancer and cardiovascular disease account for about half of all deaths in Japan. Since high consumption of green tea prevents these two major lifestyle-related diseases, we compared mean age at death from all causes by consumption levels of green tea (Table 5): We found that mean age at all death among both men and women became higher with increased consumption of green tea ($P < 0.01$), producing a difference of 3.8 and 6.0 years between the highest and lowest consumption groups, respectively. Combining men and women, the difference in mean age at all death in both sexes was 4.4 years ($P < 0.001$), a result which implies that high consumption of green tea may generate a

Table 4
Relative risk[a] of cardiovascular death by daily consumption of green tea

	Consumption of green tea (cups/day)		
	⩽ 3	4–9	⩾ 10
Men	1.0	1.09 (0.71-1.65)[b]	0.58 (0.34-0.99)
Never smokers	1.0	1.03 (0.41-2.58)	0.49 (0.12-1.97)
Current smokers	1.0	0.96 (0.55-1.67)	0.51 (0.26-0.99)
Women	1.0	0.90 (0.60-1.37)	0.82 (0.49-1.38)
Both sexes	1.0	1.02 (0.76-1.36)	0.72 (0.50-1.04)

[a]Adjusted for cigarette smoking, alcohol consumption, intake of meat, and relative body weight, using age as the fundamental time variable.
[b]In parenthesis, 95% confidence interval.

Table 5
Mean age (± SE, years) at death from all causes in the follow-up study by consumption of green tea

	Consumption of green tea (cups/day)			
	⩽ 3	4–9	⩾ 10	All categories
Men	71.2 ± 1.1 (157)[a]	73.4 ± 0.7 (288)	75.0 ± 0.8 (164)	73.3 ± 0.8 (609)
Women	74.9 ± 1.1 (129)	77.5 ± 0.7 (265)	80.9 ± 0.9 (106)	77.6 ± 0.5 (500)
Both sexes	72.9 ± 0.8 (286)	75.4 ± 0.5 (553)	77.3 ± 0.6 (270)	75.2 ± 0.4 (1109)

[a]In parenthesis, number of deaths.

prolonged lifetime. We, then, studied the life-prolonging effects of drinking large amounts of green tea, in terms of cumulative survival.

We first evaluated the influence of cigarette smoking on cumulative survival by way of example, since cigarette smoking is the most potent well-known risk factor for cancer and cardiovascular disease, providing a scale to compare with the effects of green tea. Percent survivors (± SE) among male never and current smokers were 95 ± 1 and 87 ± 1% for age-period of 40–69 years, 75 ± 3 and 66 ± 2% for 40–79 years, and 50 ± 5 and 46 ± 3% for 40–84 years, respectively. Specifically, cigarette smoking reduced percent survivors at age 84 from 50% among never smokers to 46% among current smokers.

Next, we studied cumulative survival by consumption of green tea (Table 6). In both men and women, we found a significant increase of percent survivors among those consuming over 10 cups a day for each age-period, compared with those consuming smaller amounts. The differences in percent survivors by consumption of green tea increased with age, resulting in 53 and 41% among men, and 69 and 59% among women surviving at age of 84 years. This excess of 12 and 10% in percent survivors at age 84 was greater than the difference of 4% by cigarette smoking.

4. Discussion

Green tea is an acknowledged cancer preventive, specifically in prevention targeting the delay of carcinogenic processes. We demonstrated epidemiological evidence in this report on the basis of our prospective cohort study. Furthermore, various functions of tea polyphenols may generate further benefits to human health other than cancer prevention. Along this line, we examined the association between consumption of green tea and cardiovascular disease and indicated remarkable preventive effects of green tea on the disease.

Table 6
Cumulative proportion surviving (± SE, %) by daily consumption of treen tea, estimated by the life table obtained from the follow-up study

Age-periods (years)	Consumption of green tea (cups/day)		
	⩽ 3	4–9	⩾ 10
Men			
40–69	86 ± 2	90 ± 1	89 ± 1
40–79	66 ± 3	66 ± 2	71 ± 3
40–84	41 ± 4	43 ± 3	53 ± 4
Women			
40–69	94 ± 1	96 ± 0	97 ± 1
40–79	77 ± 3	83 ± 1	84 ± 2
40–84	59 ± 4	66 ± 2	69 ± 4

These results encouraged us to further investigate how much green tea contributes to the prolongation of life in a Japanese population who habitually drink large amounts of green tea. The observed excess in percent survivors associated with increased consumption of green tea became larger with age, implying that high consumption of green tea prolongs life activity and thus contributes to long and healthy lives. Green tea can be used as a multiple targeting preventive without toxicity both in the general population where target diseases of prevention are various and sometimes uncertain, and also in high-risk populations with green tea alone or in combination with other disease-specific preventives.

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TETRAHEDRON LETTERS

Syntheses of isocitric acid derivatives and biological evaluation

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Abstract

Isocitric acid methyl esters and their derivatives have been synthesized and their biological activities, superoxide release inhibition and tumor necrosis factor-alpha (TNFα)'s release inhibition, have been studied. Linoleic and linolenic acid derivatives showed strong activity against TNFα's release inhibition comparable to sarcophytol A and cryptoporic acid E.

Keywords: biologically active compounds; carboxylic acids and derivatives; esters; terpenes and terpenoids.

We have previously reported the isolation of cryptoporic acids A–F from the fungus *Cryptoporus volvatus* and established their structures based on high resolution 2D NMR techniques, X-ray analysis, and chemical degradations.[1–5] It is very interesting to note that cryptoporic acids exhibit strong superoxide release inhibition and anti-tumor promotion activities.[5] The structures are unusual; namely, they are drimane-type sesquiterpenes linked to isocitric acid moiety with an ether bond.[6–10] We were interested in their biological activities and planned to prepare synthetic substitutes to the natural products.[11] We first intended to prepare isocitric acid with the same stereochemistry as natural products. Then fatty acids were attached to the isocitric acid and biological activities of these esters were evaluated. We now report our preliminary results.

Since Seebach et al. reported the alkylation of malic acid and synthesis of isocitric acid,[12] we have modified their methodology to prepare monocarboxylic acid derivatives[13] (Scheme 1). The allylated compound **2** of (*R*)-(+)- or racemic malic acid was protected with the MOM group and subjected to ozonolysis. The Jones' oxidation of aldehyde **4** afforded acid **5**, which was converted to trichloroethyl ester **6** using DCC. The MOM group was deprotected by TMSBr and fatty acids were used for the preparation of esters, whose trichloroethyl group was removed under Zn–AcOH–MeOH conditions to afford **8**, **9** and **10**.[14] Trimethyl isocitrate (**11**)[14] was synthesized by methylation (CH_2N_2) of acid **5** and deprotection of the MOM group in 65% yield from **5**.

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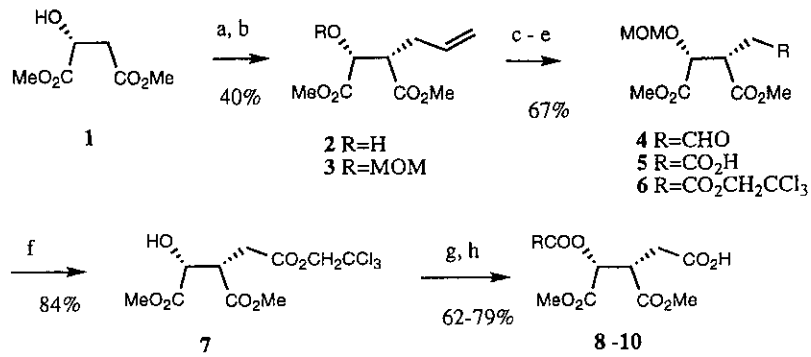
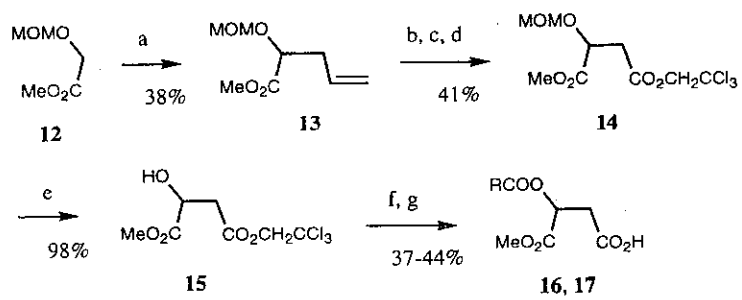


Scheme 1. *Reagents and conditions*: (a) LDA, $BrCH_2CH{=}CH_2$; (b) MOM–Cl, EtN^iPr_2; (c) O_3, CH_2Cl_2–MeOH, then SMe_2; (d) Jones; (e) DCC, $HOCH_2CCl_3$, DMAP; (f) $BrSiMe_3$, CH_2Cl_2; (g) RCOOH, DCC, DMAP, CH_2Cl_2; (h) Zn, AcOH, MeOH

Malic acid derivatives were similarly prepared (Scheme 2). The MOM-protected methyl glycolate was allylated, and similarly converted to methyl trichloroethyl ester of the malic acid **15**. The esterification with fatty acids using DCC and deprotection afforded **16** and **17**.[14]



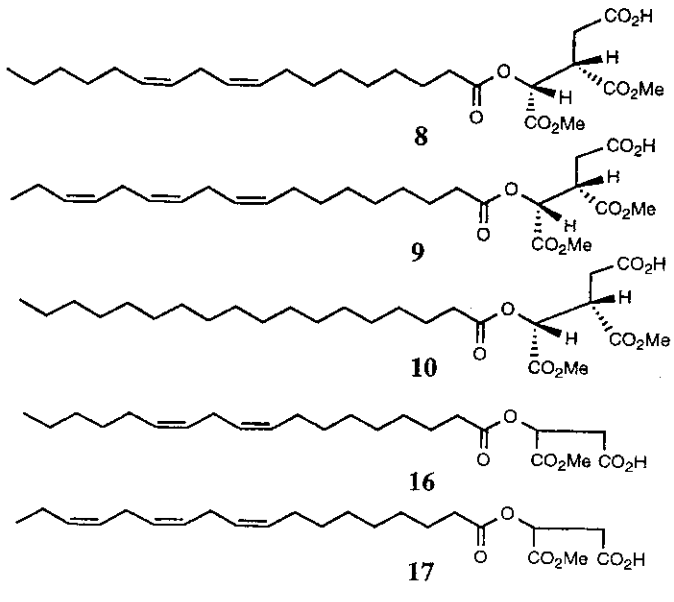
Scheme 2. *Reagents and conditions*: (a) LDA, $BrCH_2CH{=}CH_2$; (b) O_3, CH_2Cl_2–MeOH, then SMe_2; (c) Jones; (d) DCC, $HOCH_2CCl_3$, DMAP; (e) $BrSiMe_3$, CH_2Cl_2; (f) RCOOH, DCC, DMAP, CH_2Cl_2; (g) Zn, AcOH, MeOH

Because the natural products exhibit strong superoxide release inhibition activities,[4,5] these compounds were subjected to the same tests.[4,5] The results are listed in Table 1.[15] Among those tested, compound **16** was the most effective (IC_{50} 0.39 μM). The malic acid derivatives were 10 times more effective than isocitric acid derivatives. The linoleic acid derivatives were more than twice as strong as linolenic acid derivatives.

Compounds **8** and **9** were further subjected to inhibition tests of TNFα's release with okadaic acid by anti-tumor promoters on BALB/3T3 cells.[5,16] As shown in Fig. 1, both compounds are as strong as sarcophytol A (**18**) and cryptoporic acid E (**19**). Therefore, it is very interesting to note that the relative positions of the hydrophilic and lipophilic parts are very important, and that the lipophilic part can be substituted by fatty acids to show anti-tumor promoter activity.

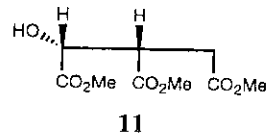
Thus, we have prepared fatty acid derivatives with isocitric and malic acid moiety and evaluated their biological activities, and two of them (**8** and **9**) were as effective as sarcophytol A (**18**) and cryptoporic acid E (**19**) in inhibition of TNFα's release.

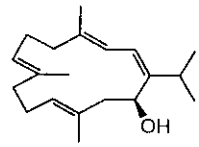
Table 1
Superoxide release inhibition activity against guinea pig macrophage



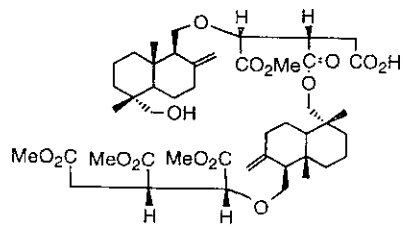
compounds	IC_{50} (μM)
8	3.1
9	8.0
10*	enhanced
11	1000
16*	0.39
17*	0.88

*racemic





Sarcophytol A (**18**)



Cryptoporic acid E (**19**)

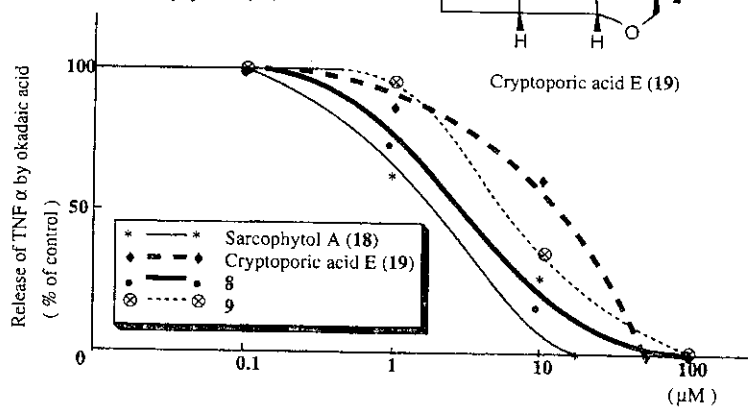


Fig. 1. Inhibition of TNFα's release with okadaic acid by anti-tumor promoters on BALB/3T3 cells

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RAPID COMMUNICATION

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Combination cancer chemoprevention with green tea extract and sulindac shown in intestinal tumor formation in Min mice

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Abstract Green tea is the most effective beverage for cancer prevention in humans. Looking at the concept of combination cancer chemoprevention, we previously reported the synergistic effects of (−)-epigallocatechin gallate (EGCG) with sulindac, and the additive effects of EGCG with tamoxifen, on cancer-preventive activity in human lung cancer cell line PC-9. This paper reports confirmation of the synergistic effects of EGCG with sulindac on the inhibition of intestinal tumors in multiple intestinal neoplasia (Min) mice. Treatment with both green tea extract and sulindac significantly reduced the number of tumors from 72.3 ± 28.3 to 32.0 ± 18.7 tumors per mouse, a decrease of 44.3%, whereas treatment with green tea extract alone or with sulindac alone reduced it to 56.7 ± 3.5 and 49.0 ± 12.7, respectively. The results also indicated that green tea extract inhibited tumor growth in Min mice almost as potently as sulindac itself did. The three treated groups did not show any adenocarcinomas, whereas 10.8% of the control group did. Since cancer-preventive agents like sulindac and tamoxifen are associated with adverse effects, we discuss the possibility of non-toxic, combination cancer chemoprevention with green tea, looking at the goal of truly effective cancer prevention.

Key words *Apc* gene · EGCG · Tea polyphenol

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Abbreviations *EGCG* (−)-epigallocatechin gallate · *FAP* familial adenomatous polyposis

Introduction

The term "Combination Cancer Chemoprevention" was introduced by M.B. Sporn in the journal *Nature* in 1980, based on evidence that the combined use of several drugs with different mechanisms of action exerted marked synergistic preventive effects (Sporn 1980). We realized the need to pay special attention to the activity of other cancer-preventive agents in combination with green tea, since we found that drinking green tea daily has cancer-preventive activity in humans (Fujiki 1999; NCI, DCPC Chemoprevention Branch and Agent Development Committee 1996). This was demonstrated in the results of our previous experiments in which cotreatment with (−)-epigallocatechin gallate (EGCG) and other cancer-preventive agents, such as sulindac and tamoxifen, synergistically and additively enhanced induction of apoptosis of human lung cancer cell line PC-9 (Suganuma et al. 1999).

Sulindac, a non-steroidal anti-inflammatory drug, suppresses colorectal tumorigenesis in familial adenomatous polyposis (FAP) patients, whose condition is caused by germline mutations of the *Apc* gene (Kelloff et al. 1996). Although sulindac is a cancer-preventive agent for colon cancer in FAP patients, its chronic treatment is restricted because it also causes bleeding and peptic ulceration of the gastrointestinal tract. Therefore, what is urgently needed is some way to reduce the adverse effects of sulindac and increase its efficacy in cancer prevention. In the light of this, we first wanted to examine the synergistic effects of green tea extract with sulindac using multiple intestinal neoplasia (Min) mice, which have germline mutation of the murine *Apc* gene and which develop intestinal tumors similar to those of FAP patients (Moser et al. 1990; Su et al. 1992).

This paper is the first report indicating that a combination of green tea extract and sulindac significantly

suppressed the number and size of tumors more than sulindac did alone. As for the mechanisms of action of combined green tea extract and sulindac, we previously reported that green tea extract and sulindac commonly inhibit tumor necrosis factor-α (TNF-α) release from cells (Suganuma et al. 1999, 2000). These results clearly demonstrate a practical way to reduce the adverse effects of sulindac, resulting in more successful cancer prevention in humans.

Materials and methods

Treatment of Min mice with green tea extract and sulindac

Male C57BL/6J-Min/+ (Min) mice, a strain containing a dominant mutation in the *Apc* gene, were obtained from the Jackson Laboratory (Bar Harbor, Me., USA). From 6 weeks of age, Min mice were fed either a powdered CE-2 diet (Clea Japan, Tokyo, Japan) or CE-2 containing 0.03% sulindac (Sigma Chemical, St. Louis, Mo., USA). Drinking water was given with or without 0.1% green tea extract contained 11.2% EGCG, 10.3% (−)-epigallocatechin (EGC), 2.5% (−)-epicatechin (EC), 2.3% (−)-epicatechin gallate (ECG), and 6.7% caffeine. Fresh diet and drinking water were provided every 3–4 days. The numbers of mice in the non-treated control group and in the three groups treated with green tea extract, with sulindac, and with green tea extract plus sulindac were 4, 3, 4, and 4, respectively. During the course of the experiment, no significant differences in body weight or consumption of food and drinking water were noted. At 16 weeks of age, all mice were killed, and their intestinal tracts were removed, fixed with 10% formaldehyde, and stained with 0.1% methylene blue (Otori et al. 1998). Macroscopically visible tumors of more than 0.4 mm in diameter were counted by two observers separately.

Histological examination

From each group, the intestine of one mouse bearing the average number of tumors for that group (by macroscopic examination) was thoroughly examined; the middle portions of intestine of the other mice in each group were examined histologically. Tumors were classified as hyperplasia, adenoma, and adenocarcinoma. Pathohistologically, we categorized hyperplasia as enlarged crypt without nuclear atypia and irregular architectural features, adenoma as enlarged crypt with nuclear atypia, and adenocarcinoma as enlarged crypt with nuclear atypia and irregular architectural features, which was typically, a "glands within glands" structure (Schlemper et al. 1998).

Results and discussion

To identify the effects of combination cancer chemoprevention with green tea extract and sulindac, Min mice were given both drinking water of 0.1% green tea extract and diet containing 0.03% sulindac for 10 weeks. The doses administered were based on results previously reported in anticarcinogenesis experiments, i.e., inhibition of azoxymethane- and *N*-methyl-*N*-nitrosourea-induced colon carcinogenesis of F344 rats with green tea extract (Yamane et al. 1991; Narisawa and Fukaura 1993), and inhibition of tumor formation in the colon of Min mice with sulindac (Beazer-Barclay et al. 1996). Thus, the intake of green tea extract and sulindac in this experiment did not vary much in any of the groups: all mice in the groups ingested about 10 mg of green tea extract and 5 mg sulindac per day. The combination group (treated with both green tea extract and sulindac) showed an increase of body weight, as the other three groups did, indicating that the treatment of green tea extract with sulindac did not produce any toxic effects. At 16 weeks of age, the average number of tumors in the non-treated control Min mice group was 72.3 ± 28.3 tumors per mouse; tumors were distributed throughout the intestinal tract from duodenum to rectum, but 90% of the tumors were located in the jejunum and ileum. Treatment with both green tea extract and sulindac significantly reduced the number of tumors from 72.3 ± 28.3 to 32.0 ± 18.7 tumors per mouse, a decrease of 44.3%, whereas treatment with green tea extract alone or with sulindac alone reduced the number of tumors to 56.7 ± 3.5 and 49.0 ± 12.7, respectively (Table 1). When we compared the distribution of tumors by diameter (<1, 1–2, 2–3, and >3 mm), the inhibitory effects by the combination of green tea extract with sulindac were even more pronounced. Figure 1 shows the number of the intestinal tumors per mouse, classified by diameter. Treatment with the combination resulted in significantly smaller tumor size than in any of the other groups. Specifically, tumors of 1–2 mm were reduced from 26.0 ± 3.7 with green tea extract alone, and 26.3 ± 7.7 with sulindac alone, to 12.5 ± 7.8 with the combination. Green tea extract alone and sulindac alone reduced the number of tumors larger than 1 mm from 59.5 ± 13.1 to 43.3 ± 4.3 and 34.5 ± 5.7, respectively (Table 1), indicating that green tea extract inhibited formation and growth of tumors in Min mice almost potently as sulindac as did.

Most tumors in the intestine of Min mice were adenomas in all groups. However, only the non-treated control group produced adenocarcinomas; eight (10.8%) among 74 tumors examined. The adenocarcinomas were

Table 1 Synergistic effects on inhibition of tumor formation in Min mice by combination of green tea extract with sulindac

Group	Average no. of tumors/mouse	Average no. of tumors classified by diameter (mm)	
		<1	≥1
Non-treated	72.3 ± 28.3 (100%)	12.8 ± 1.3 (100%)	59.5 ± 13.1 (100%)
Green tea extract	56.7 ± 3.5 (78.4%)	13.3 ± 4.5 (103.9%)	43.3 ± 4.3 (72.8%)
Sulindac	49.0 ± 12.7 (67.7%)	15.0 ± 4.9 (117.2%)	34.5 ± 5.7 (58.0%)
Green tea extract + sulindac	32.0 ± 18.7 (44.3%)*	14.5 ± 3.5 (113.3%)	17.0 ± 5.1 (28.6%)*

* Statistically significant ($P < 0.05$)

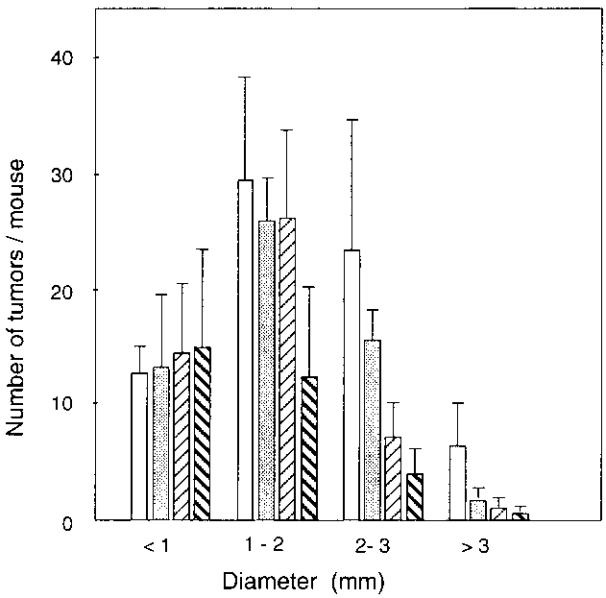

Fig. 1 Inhibitory effects of combination with green tea extract and sulindac on the number of tumors, classified by diameter. Non-treated control group (□), and groups treated with green tea extract alone (▤), with sulindac alone (▨), and with both green tea extract and sulindac (▩)

not associated with invasion, but showed histologically irregular architectural features with nuclear atypia (Schlemper et al. 1998). The three treated groups did not show any adenocarcinomas. We found that treatments with green tea extract alone, with sulindac alone, and with the combination, were more effective for prevention of adenocarcinoma than for adenoma. The two groups treated with sulindac alone and with green tea extract plus sulindac showed 25.6% and 25.8% of hyperplasia among 39 and 31 tumors examined, respectively, indicating that the groups treated with sulindac and the combination suppressed tumor progression. All our results indicated that the preventive activity of sulindac was enhanced by green tea extract in Min mice.

Several investigators had already reported synergistic activity with drug combinations; specifically retinoids (9-*cis*-retinoic acid and fenretinide) with selective estrogen receptor modulators (tamoxifen, raloxifen, and LY-353381), which were studied in rat mammary glands (Anzano et al. 1996; Chemoprevention Working Group 1999). Similar synergistic effects with these compounds were also found in carcinogenesis of rat prostate and rat colon (Chemoprevention Working Group 1999). In addition to our presented results, a combination of green tea extract and tamoxifen has been reported to synergistically reduce spontaneous mammary carcinogenesis in C3H/Ouj mice (Sakata et al. 1995) and carcinogen-induced mammary tumors in rodents (M. Sporn, personal communication). Thus, it is important to establish the compounds with which green tea is able to show synergistic or additive effects.

Our recent investigation revealed that the cancer-preventive benefits of green tea can be achieved by drinking 10 Japanese-size cups per day (about 1.5 to 2.0 g green tea extract per day) (Nakachi et al. 2000). In a practical sense, we recommend drinking as much green tea as you want and making up any lack with green tea tablets (prepared in collaboration with the Tea Experiment Station of Saitama Prefecture) (Fujiki 1999). Continuing our study, we have started clinical trials on the possible preventive effects of green tea on colon polyps, and prostate and breast cancers. One breast cancer patient, who had had surgical resection and was taking tamoxifen, complained of some adverse effects with tamoxifen. She has now started to reduce the tamoxifen dose and drink more green tea every day. This single example illustrates what we think is the key point: that green tea is a practical, non-toxic way to enhance the beneficial effects of cancer-preventive drugs, a way that any person – cancer patient or healthy – can choose with a physician's guidance.

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1.2

Green Tea as a Cancer Preventive

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1 Abstract

Green tea is now an acknowledged cancer preventive in Japan and will possibly soon be recognized as such in other countries. Initially, we found that (−)-epigallocatechin gallate (EGCG), the main constituent of green tea inhibited tumor promotion on mouse skin in a two-stage carcinogenesis experiment. Numerous additional studies revealed the anticarcinogenic effects of EGCG and green tea on various organs in rodent experiments. This paper reviews the unique role of green tea in cancer chemoprevention, its anticarcinogenic effects and other preventive activities, bioavailability of tea polyphenols and epidemiological studies with green tea. Of particular interest are studies which showed that daily consumption of green tea delayed clinical onset of various cancers and led to more hopeful prognoses for breast cancer patients in Stage I and II following treatment. Based on these results, I propose two stages of cancer prevention with green tea: prevention before cancer onset, and following cancer treatment. Since green tea is a common beverage, the knowledge that it inhibits cancer will be a great comfort to, especially, aging folk concerned with cancer prevention and any high risk population.

2 Introduction

Green tea is a beverage commonly ingested in Japan every day. The tea plant, Camellia sinensis, was brought from China to Japan by a Japanese Zen priest in the twelfth century for use as a medicine. From that time, we Japanese have simply believed in the benefit of drinking green tea for many years, while not necessarily being aware of its therapeutic effects.

When we started to study cancer chemoprevention in 1983, we had our first