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Monosodium glutamate-induced diabetic mice are susceptible to azoxymethane-induced colon tumorigenesis

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Obese people and diabetic patients are known to be high risk of colorectal cancer (CRC), suggesting need of a new preclinical animal model, by which to extensively study the diverse mechanisms, therapy and prevention. The present study aimed to determine whether experimental obese and diabetic mice produced by monosodium glutamate (MSG) treatment are susceptible to azoxymethane (AOM)-induced colon tumorigenesis using early biomarkers, aberrant crypts foci (ACF) and β-catenin-accumulated crypts (BCACs), of colorectal carcinogenesis. Male Crj:CD-1 (ICR) newborns were daily given four subcutaneous injections of MSG (2 mg/g body wt) to induce diabetes and obesity. They were then given four intraperitoneal injections of AOM (15 mg/kg body wt) or saline (0.1 ml saline/10 g body wt). Ten weeks after the last injection of AOM, the MSG-AOM mice had a significant increase in the multiplicity of BCAC (13.83 ± 7.44, $P < 0.002$), but not ACF (78.00 ± 11.20), when compare to the Saline-AOM mice (5.45 ± 1.86 of BCAC and 69.27 ± 8.06 of ACF). Serum biochemical profile of the MSG-treated mice with or without AOM showed hyperinsulinemia, hypercholesteremia and hyperglycemia. The mRNA expression of insulin-like growth factor-1 receptor (IGF-1R, $P<0.01$) was increased in the MSG-AOM mice, when compared with the mice given AOM alone. IGF-1R was immunohistochemically expressed in the BCAC, but not ACF, in the AOM-treated mice. Our findings suggest that the MSG mice are highly susceptible to AOM-induced colorectal carcinogenesis, suggesting potential utility of our MSG-AOM mice for further investigation of the possible underlying events that affect the positive association between obese/diabetes and CRC.

Introduction

Epidemiological studies have shown that obesity and diabetes mellitus may be one of the risk factors for colorectal cancer (CRC) development (1–7). At present, hyperinsulinemia (8,9), hypercholesterolemia (10,11), hyperglycemia (9,12) and hyperlipidemia (7) are considered to be the possible risk factors of CRC. In addition, insulin-like growth factor (IGF) pathway is involved in colorectal carcinogenesis (13–16) and the signaling pathway is reported to be a potential target of CRC treatment (17–19) and CRC chemoprevention (20,21). Thus, importance of the growth hormone/IGF-1 axis (22) and IGF/IGF-1 receptor (IGF-1R) axis (15,23,24) is postulated in carcinogenesis in CRC development. In fact, our experimental studies indicated that the IGF/IGF-1R axis is altered during carcinogenesis in colorectum (25,26) and other tissue (27,28) and the axis is a good target for cancer chemoprevention (25–28). However, the underlying mechanisms of how these chronic diseases promote colon carcinogenesis still remain unknown (19). On this context, new research animal models are needed to investigate the diverse aspects of the mechanisms.

We have previously reported that development of AOM-induced precancerous lesions is enhanced in C57BL/KsJ-*db/db* mice with hyperleptinemia and hyperinsulinemia (29). Such an animal model may give important implications for further exploration of the possible underlying events that affect the positive association between CRC and obesity and/or diabetes (30–32). A number of animal models for diabetes and/or obesity have been reported. One such model is produced by injection of monosodium glutamate (MSG). When MSG is applied to Crj:CD-1 (ICR) newborn mice (MSG mice), they develop diabetic condition (hyperinsulinemia, hyperglycemia and hyperplastic islets) without polyphagia (33,34).

It is believed that colorectal carcinogenesis is a representative multistep tumorigenesis with events of genetic alterations. Several small lesions, including aberrant crypt foci (ACF) (35,36), mucin-depleted foci (37) and β-catenin-accumulated crypts (BCACs) (38) are proposed as early-appearing preneoplastic lesions (37). While ACF and mucin-depleted foci are recognized on the surface of cancer-predisposed colons of rodents and human (37), BCAC are identified in colonic mucosa at the early stages of colon carcinogenesis (39). Accumulating evidence suggests that BCAC are independent small dysplastic lesions and/or microadenomas and progressed precancerous lesions (40) in colon carcinogenesis when compared with ACF and mucin-depleted foci (39). These early lesions are widely used for investigating pathobiology of colorectal carcinogenesis (37).

In the current study, new born Crj:CD-1 (ICR) mice were treated with MSG to produce diabetes and obesity and, subsequently, they received a colonic carcinogen, azoxymethane (AOM). Our results indicated that the MSG mice are highly susceptible to AOM-induced colorectal carcinogenesis by counting the number of BCAC, but not ACF, and possible involvement of the IGF/IGF-1R axis in colorectal tumorigenesis of diabetic and obese mice induced by MSG and AOM. Our main goal is to assess the involvement of obesity/diabetes-associated events, such as hyperinsulinemia, in colorectal carcinogenesis *in vivo*.

Materials and methods

Animals and chemicals

The pregnant Crj:CD-1 (ICR) mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and their newborns were used in the study. MSG was obtained from Wako Pure Chemical Industries, Ltd (Tokyo, Japan) and AOM from Sigma Chemical Co. (St Louis, MO). Mice used for the experiment were maintained in the well-controlled room with a high-efficiency particulate air filter, a 12 h lighting (7:00–19:00), 25 ± 2°C room temperature and 55 ± 15% humidity. Mice (3–6 mice/cage) were housed in polycarbonate cages measuring W225 × D338 × H140 mm (Japan CLEA, Inc., Tokyo, Japan) with the floor covered with a sheet of roll paper (Japan SLC). MF (Oriental Yeast Co., Ltd, Tokyo, Japan) was used as a basal diet throughout the study. Groundwater that was chlorine-treated and subjected to ultraviolet disinfection was used as drinking water in a bottle. We fully complied with the 'Guidelines Concerning Experimental Animals' issued by the Japanese Association for Laboratory Animal Science and exercised due consideration so as not to cause any ethical problem.

Experimental procedure

The newborns were divided into two groups according to the treatments. The birth date was the beginning of four daily subcutaneous injections of MSG (2 mg/g body wt, MSG mice) and physiological saline (Saline mice). Among these mice, males were subjected to the study. They were divided into four groups at 4 weeks of age: groups 1 (12 males) and 2 (6 males) of the MSG mice received four weekly intraperitoneal injections of AOM (15 mg/kg body wt,

Abbreviations: ACF, aberrant crypts foci; AOM, azoxymethane; BCAC, β-catenin-accumulated crypt; CRC, colorectal cancer; IGF-1R, insulin-like growth factor-1 receptor; MSG, monosodium glutamate.

the MSG-AOM mice) and physiological saline (0.1 ml/10 g body wt, the MSG-Saline mice), respectively. Similarly, two groups of the ICR-Saline mice were given AOM or saline, belonging to groups 3 (11 males, the Saline-AOM mice) and 4 (5 males, the Saline–Saline). At the termination of the experiment (10 weeks after the last injection of AOM and 17 weeks of age of mice), all animals were killed to analyze the number of colonic ACF and BCAC, clinical serum chemistry and mRNA expression of IGF1, IGF2 and IGF-1R in the colonic mucosa.

Counting the numbers of ACF and BCAC

The ACF and BCAC were determined according to the standard procedures described previously (30,31,41). ACF are defined as single or multiple crypts that have altered luminal openings, exhibit thickened epithelia and are larger than adjacent normal crypts (35). BCAC, which have high-frequency mutations in β-*catenin* gene, demonstrate histological dysplasia with a disruption of the cellular morphology (Figure 2A) and an accumulation of this protein (Figure 2B) (39). BCAC do not have a typical ACF-like appearance because the lesion is not recognized on the mucosal surface like ACF and is only identified in the histological sections of 'en face' preparations. Both of these lesions are utilized as biomarkers to evaluate a number of agents for their potential chemopreventive (42–44) and tumor promotion (45) properties. After the colons were fixed flat in 10% buffered formalin for 24 h, the mucosal surface of the colons were stained with methylene blue (0.5% in distilled water) and then the number of ACF were counted under a light microscope. Thereafter, the distal parts (5 cm from anus) of the colon were cut to count the number of BCAC. To identify BCAC intramucosal lesions, the distal part of the colon (mean area: 0.7 cm^2/colon) was embedded in paraffin and then a total of 20 serial sections (4 μm thick each) per colon were made by an en face preparation (30,31,41). For each case, two serial sections were used to analyze BCAC.

Histopathology and immunohistochemical analyses for β-catenin and IGF-1R

Three serial sections were made from paraffin-embedded tissue blocks. Two sections were subjected to hematoxylin and eosin (H and E) staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC. Immunohistochemistry for β-catenin and IGF-1R was performed using the labeled streptavidin-biotin method (LSAB kit; DAKO, Glostrup, Denmark), as described previously (30,31). Primary antibodies of anti-β-catenin antibody (1:1000 final dilution) and anti-IGF-1R antibody (1:100 final dilution) obtained from Transduction Laboratories (catalog no. 610154; San Jose, CA) and Santa Cruz Biotechnology, Inc. (sc-7907; Santa Cruz, CA), respectively, were applied on the sections. Negative control sections were immunostained without the primary antibody.

Blood chemistry

At 17 weeks of age, blood samples (0.5–1.0 ml/mouse) were collected for determination of total cholesterol, triglyceride and glucose by a simple measurement device (DRICHEM Fujifilm Medical Co., Ltd, Tokyo). The concentration of blood insulin was measured by an LBIS insulin measuring kit for mice (Shibayagi Co., Ltd, Gunma).

RNA extraction and quantitative real-time reverse transcription–polymerase chain reaction analysis

A quantitative real-time reverse transcription–polymerase chain reaction analysis was carried out in the scraped colonic mucosa of the MSG-AOM mice and the Salin-AOM mice. Total RNA was isolated from the scraped colon mucosa of the mice using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX). The cDNA was synthesized from 0.2 μg total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The primers used for the amplification of IGF-1-, IGF-2- and IGF-1R-specific genes were as follows: IGF-1 forward, 5′-CTGGACCAGAGACCCTTTGC-3′ and reverse, 5′-GGACGGGGACTTCTGAGTCTT-3′; IGF-2 forward, 5′-GTGCTGCATCGCTGCTTAC-3′ and reverse, 5′-ACGTCCCTCTCGGACTTGG-3′; and IGF-1R forward, 5′-GTGGGGGCTCGTGTTTCTC-3′ and reverse, 5′-GATCACCGTGCAGTTTTCCA-3′. Real-time PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The expression levels of the IGF-1, IGF-2 and IGF-1R genes were normalized to the β-actin gene expression level.

Statistical analysis

Measurements are expressed as mean ± SD, and differences if present were compared by one-way analysis of analysis of variance (Tukey–Kramer's multiple comparison's test) or two-tailed unpaired *t*-test. The incidences of intestinal tumors were compared by Fisher's exact probability test. The results were considered statistically significant if the P values were <0.05.

Results

General observations

As shown in Figure 1, the mean body weight of MSG–Saline mice (group 2) was much greater than Saline–Saline mice (group 4) during the study. The average body weights of the AOM-injected groups belonging to groups 1 (MSG-AOM) and 3 (Saline-AOM) were smaller than that of saline-injected groups, groups 2 (MSG-Saline) and 4 (Saline–Saline), during the study. At the termination of the experiment, the mean body weights of groups 1 and 3 were significantly lower than groups 2 and 4, respectively ($P < 0.001$), as listed in Table I.

The numbers of ACF and BCAC

ACF and BCAC (Figure 2A and B) developed in all the mice belonging to group 1 (the MSG-AOM mice) and 3 (the Saline-AOM mice) but not in the mice of groups 2 (the MSG-Saline) and 4 (the Saline-Salin mice) that did not receive AOM. Table I summarizes the total numbers of ACF and BCAC in all groups. The number of BCAC of group 1 was significantly greater than group 3 ($P < 0.001$), whereas the numbers of ACF developed in groups 1 and 3 were comparable.

Serum levels of glucose, total cholesterol, triglyceride and insulin

The serum concentrations of glucose, total cholesterol, triglyceride and insulin at 17 weeks of age are shown in Table II. MSG treatment significantly elevated all measures regardless of the AOM exposure (group 1 versus group 3, $P < 0.001$; and group 2 versus group 4, $P < 0.001$). However, the AOM administration did not affect all the measurements (group 1 versus group 2; and group 3 versus group 4).

β-Catenin and IGF-IR immunohistochemistry

Immunohistochemical expression of β-catenin revealed the presence of BCACs, where β-catenin was accumulated in the nucleus and/or cytoplasm (Figure 2B). Immunohistochemical expression of IGF-IR in the cytoplasm of BCAC that develop in the MSG-AOM mice was intensive when compared with the surrounding crypts (Figure 2C). Inflammatory cells infiltrated into the surrounding stroma of BCAC also showed positive reaction against IGF-1R.

mRNA expression levels of IGF-1, IGF-2 and IGF-1R in the colonic mucosa

The expression levels of IGF-1, IGF-2 and IGF-1R mRNAs of the colonic mucosa from the MSG-AOM mice (group 1) and the Saline-AOM mice (group 3) were determined. As illustrated in Figure 3, the MSG + AOM treatment mice showed significantly increased mRNA levels of IGF-1 (1. 81-fold increase) and IRF-1R (2.43-fold increase), when compared with the Saline-AOM mice. The increase of IGF-1R was statistically significant ($P < 0.01$). mRNA levels of IGF-2 of the two groups were comparable.

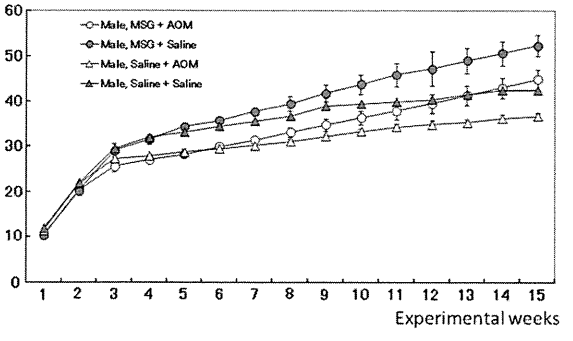

Fig. 1. Body weight gains of all groups during the study.

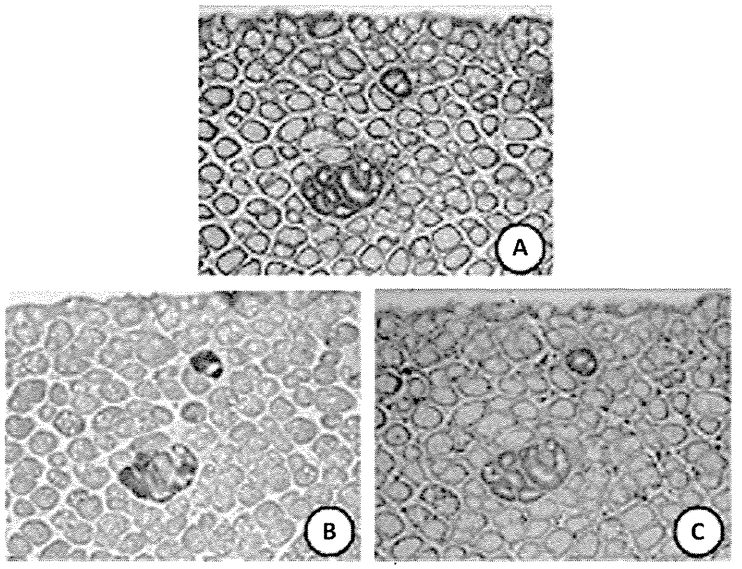

Fig. 2. Histopathology (**A**) of a BCAC developed in an MSG-AOM mouse. Immunohistochemistry of (**B**) β-catenin and (**C**) IGF-1R in the BCAC shows positive reaction of β-catenin in their cell membrane and cytoplasm and IGF-1R in their cytoplasm. Bars = 50 μm. (A) H and E stain, (B) β-catenin immunohistochemistry and (C) IGF-1R immunohistochemistry.

Table I. Body weights and numbers of ACF and BCAC per colon of mice treated with AOM and/or MSG at the end of study (17 weeks of age)

Group number	Number of mice examined	Treatments		Body weight (g)	Total number of ACFs/colon	Total number of BCACs/colon
		MSG (2 mg/kg body wt)	AOM (15 mg/kg body wt)			
1 (MSG-AOM)	12	+ (4 times daily)	+ (4 times weekly)	44.82 ± 2.22[a,b,c]	78.00 ± 11.20[b]	13.83 ± 7.44[b,c]
2 (MSG-Saline)	6	+ (4 times daily)	− (saline)	52.29 ± 2.32[d]	0	0
3 (Salin-AOM)	11	− (saline)	+ (4 times daily)	36.62 ± 4.15[e]	69.27 ± 8.06[f]	5.45 ± 1.86
4 (Saline–Saline)	5	− (saline)	− (saline)	42.42 ± 0.78	0	0

[a]Mean ± SD.
[b]Significantly different from group 2 ($P < 0.001$).
[c]Significantly different from group 3 ($P < 0.001$).
[d]Significantly different from group 4 ($P < 0.001$).
[e]Significantly different from group 4 ($P < 0.01$).
[f]Significantly different from group 4 ($P < 0.001$).

Table II. Serum levels of total cholesterol, triglycerides, glucose and insulin of mice treated with AOM and/or MSG at the end of study (17 weeks of age)

Group number	Number of mice examined	Treatments		Total cholesterol (mg/dl)	Triglycerides (mg/dl)	Glucose (mg/dl)	Insulin (ng/dl)
		MSG (2 mg/kg body wt)	AOM (15 mg/kg body wt)				
1 (MSG-AOM)	12	+ (4 times daily)	+ (4 times weekly)	167.92 ± 19.96[a,b]	96.25 ± 14.38[b]	196.67 ± 34.09[b]	10.66 ± 1.31[b]
2 (MSG-Saline)	6	+ (4 times daily)	− (saline)	177.50 ± 23.09[c]	93.17 ± 12.64[c]	202.67 ± 15.24[c]	11.18 ± 1.41[c]
3 (Salin-AOM)	11	− (saline)	+ (4 times daily)	107.64 ± 18.65	49.45 ± 13.87	110.36 ± 10.48	0.49 ± 0.14
4 (Saline–Saline)	5	− (saline)	− (saline)	104.60 ± 13.69	45.20 ± 9.98	123.60 ± 11.30	0.50 ± 0.07

[a]Mean ± SD.
[b]Significantly different from group 3 ($P < 0.001$).
[c]Significantly different from group 4 ($P < 0.001$).

Discussion

As expected, the findings described suggest that development of AOM-induced precancerous lesions, BCAC, of the colon in the MSG mice with hyperinsulinemia, hypercholesterolemia, hyperglycemia and hyperlipidemia was increased, when compared with the Salin-AOM mice. Our findings are in accordance with our previous findings that AOM-induced colon carcinogenesis is enhanced in another obese model using C57BL/KsJ-db/db mice (29–31). In the current study, the number of ACF was not different between the

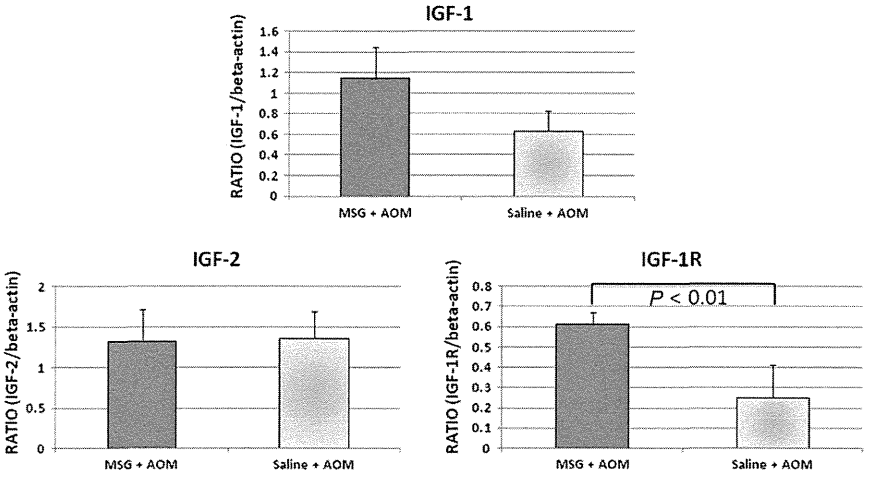

Fig. 3. mRNA expression of IGF-1, IGF-2 and IGF-1R in the colonic mucosa of the MSG-AOM mice and the Saline-AOM mice.

MSG-AOM mice and Saline-AOM mice, suggesting that BCAC rather than ACF has potential to progress to malignancies (39,42). This may be explained by the differences of pathobiological characteristics between two lesions: BCAC is dysplastic and microadenomas and ACF is consisted of hyperplastic and dysplastic lesions.

ACF have attracted attention as putative precancerous lesions in the colon in both experimental models and in humans (36). A number of molecular abnormalities, including increased expression of K-*ras* and *APC* gene mutations, are demonstrated in human ACF (46–48). BCAC, which accumulate β-catenin protein in the nucleus and cytoplasm, are also regarded as putative precursors to colorectal adenomas (37). Several rodent studies have shown that both of these lesions are useful as biomarkers to evaluate the chemopreventive properties of specific agents (49,50). In human colorectum, increased plasma IGF-1 levels are associated with the number of dysplastic ACF (51), suggesting that IGF-1 may be a promoter of the growth of dysplastic ACF and an independent risk factor of CRC. It may be possible that the number of dysplastic ACF, but not hyperplastic ACF, may be increased in the MSG-AOM mice, although we did not analyze two types of ACF.

Neonatal injections of MSG to mice or rats cause hyperthalamic damage (52,53), and as a consequence, these animals present several neuroendocrine and metabolic alterations, which lead central obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, hypertriglyceridemia and hyperlipidemia (33). These abnormalities are risks for the development of CRC (9). The pancreatic islets in the mice subcutaneously injected MSG in neonatal period are hyperplastic up to 54 weeks of age (33). Therefore, our model described here may be suitable to study the pathobiology of diabetes- and obesity-associated colorectal carcinogenesis.

In this study, blood insulin level of the MSG-Saline mice (group 2) was the highest among the groups. There is accumulating evidence suggesting that hyperinsulinemia is involved in colon carcinogenesis, obesity and diabetes (3,8,9,12,54,55). Several epidemiological studies indicate that type 2 diabetic patients with hyperinsulinemia increases risk for CRC (3,12). Additionally, continuous injections of insulin promote AOM-induced colon carcinogenesis in rats (56,57). Hence, it seems likely that hyperinsulinemia in the MSG-AOM mice enhanced the development of AOM-induced lesions in the present study. Hyperglycemia and hypercholesterolemia observed in the MSG-AOM mice also contribute the development of BCAC and ACF in the colorectum because these conditions are positively associated with CRC occurrence (9,11,12). Hyperinsulinemia, hyperglycemia and hypercholesterolemia may singly or synergistically promote the development of preneoplastic and neoplastic colonic lesions. Although insulin resistance in colorectal carcinogenesis of obese people and/or type 2 diabetic patients is reported (7,55,58,59), we did not investigate presence or absence of insulin resistance in this study, Corpet *et al.* (60) reported that diet that increase some indirect insulin resistance markers does not promote colon carcinogenesis in female rats when ACF are used as a biomarker.

Regarding the mode of action, the current consensus assumes that the IGF-1 pathway plays a role in insulin-related tumor promotion in the colon (20,61). IGF-1 binds to the IGF-1R, activates a signal cascade and triggers cell proliferation in several tissues, including colon (62). Insulin at supra-physiological levels also binds to and activates the IGF-1R because of its homology with the insulin receptor (62). Furthermore, hyperinsulinemia was shown to indirectly increase bioavailability of IGF-1 by regulating levels of IGF-binding proteins (63). In this study, IGF-1R immunohistochemical expression of IGF-1 was strongly positive in the cytoplasm of BCAC developed in the MSG-AOM mice. Indeed, overexpression of IGF-1R was also reported in human CRC (64). Accordingly, it may be possible that hyperinsulinemia in MSG-AOM mice activates the signaling cascades involving the IGF-IR, resulting in a proliferative response (19,59,61). Another interesting findings regarding IGF-1R immunohistochemistry is that inflammatory cells infiltrated around BCAC were positively reacted against the IGF-1R antibody. Significance of the findings is not known, but similar findings have been reported in human Crohn's disease (65). IGF-1 and IGF2 are potentially relevant mediators in the chronic inflammation (27) and mediate the majority of their biological action through IGF-1R (66). Thus, our findings on the IGF-1R immunohistochemical positivity in inflammatory cells suggest that inflammation in the microenvironment of precancerous lesions for CRC may contribute to the growth of the lesions (67,68).

In conclusion, our data indicate that the MSG-AOM mice with hyperinsulinemia, hypercholesterolemia, hyperglycemia and hypercholesterolemia are highly susceptible to colorectal carcinogenesis and the MSG-AOM mouse model could be useful for investigating the mechanisms of obesity/diabetes-associated events involving in colorectal carcinogenesis and the therapeutic and chemopreventive strategies of CRC in obese people and/or type 2 diabetic patients.

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Conflict of Interest Statement: None declared.

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REVIEW

Challenging the effectiveness of green tea in primary and tertiary cancer prevention

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Abstract
Purpose Drinking green tea daily is part of Japanese culture, and various studies have revealed that green tea is a cancer preventive. We here review our progress in cancer prevention with green tea on 12 main topics, from basic to clinical level.
Topics and methods Biochemical and biological studies of green tea catechins, a prospective cohort study, preclinical safety trials with tablets of green tea extract, double-blind randomized clinical phase II prevention trial for recurrence of colorectal adenomas, and synergistically enhanced inhibition by the combination of green tea catechins and anticancer drugs. All results were significant, including human studies with informed consent.
Results Drinking 10 Japanese-size cups of green tea per day delayed the cancer onset of humans 7 years for females. For tertiary cancer prevention, consuming 10 cups of green tea per day fortified by green tea tablets, 50 %, significantly prevented the recurrence of colorectal adenomas. A minimum effective amount of green tea catechins for cancer prevention was found in humans. In addition, the combination of green tea catechins and anticancer drugs engendered a new cancer therapeutic strategy.
Conclusion The consumption of 10 Japanese-size cups of green tea per day is a significant factor in primary cancer prevention for the general population, and the preventive effect on recurrence of colorectal adenomas in patients is vital evidence in tertiary cancer prevention.

Keywords Apoptosis · Delayed cancer onset · GADD153 · Green tea tablet · Phase II prevention trial · Prospective cohort study · TNF-α

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Introduction

Japanese have the longest life span in the world, 86 years for females and 79 years for males. Even so, cancer mortality increases after age 60 and is the highest for age groups around 80 years of age for both males and females (Fig. 1). The multistage carcinogenesis study of B. Vogelstein revealed that the first neoplastic change in cells in human colon development starts many years earlier, probably 20–30 years earlier than the clinical appearance of cancer in patients (Vogelstein et al. 1988). M. Sporn coined the term "Cancer chemoprevention," and defined it as "prevention of the occurrence of cancer by administration of one or several compounds (Sporn et al. 1976)." Today, it is generally accepted that it is possible to delay the clinical appearance of cancer by slowing down the development of cancer. At present, for example, 50 % of breast cancer of high-risk group can be prevented by the administration of tamoxifen, in the United States (Fisher et al. 1998).

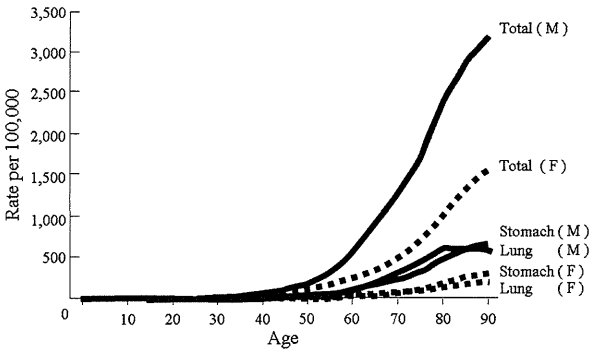


Fig. 1 Cancer mortality by age in Japan (1997). *M* male, *F* female

When research in cancer chemoprevention began in Japan in 1983, we were lucky to receive tannins or polyphenols isolated from medicinal plants and herbs from T. Okuda at Okayama University (Okuda et al. 1985). To screen for cancer preventive agents, we tried to find inhibitors of tumor promotion in two-stage carcinogenesis experiments on mouse skin, consisting of initiation and tumor promotion. The three diterpene ester tumor promoters discovered by E. Hecker were very useful for our further study (Hecker et al. 1984). From a total of 30 polyphenols, (−)-epigallocatechin gallate (EGCG) and penta-*O*-galloyl-*β*-D-glucose (5GG) showed binding to the phorbol ester receptor and inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced activation of protein kinase C (PKC). In 1987, we first reported, in the British journal Phytotherapy Research, that EGCG prevented tumor promotion of teleocidin, one of the TPA-type tumor promoters on mouse skin (Yoshizawa et al. 1987). Later, we also confirmed that repeated applications of 5 mg EGCG completely prevented tumor promotion of okadaic acid (Yoshizawa et al. 1992), a potent tumor promoter working through the inhibition of protein phosphatases 1 and 2A. The tumor-promoting activity by okadaic acid is as potent as TPA (Suganuma et al. 1988).

After we published the results in 1987, the Journal "New Scientist" soon introduced our results with EGCG in an article entitled "Green tea cuts cancerous growths" (November 12, 1987). In winter of 1987, an Australian TV scientific series entitled "Beyond 2000" visited us at the National Cancer Center Research Institute in Tokyo to make a TV film about our research. This 8-min-long TV film was shown all over the world in 1988 and 1989, and many overseas scientists took greater interest in cancer prevention with green tea than Japanese scientists. A putative carcinogenic potential for tannins or polyphenols suspected previously was eliminated by establishing the non-mutagenicity of EGCG and green tea catechins (Okuda et al. 1984), and by EGCG inhibition of TPA-induced activation of PKC (Yoshizawa et al. 1987). Moreover, EGCG and green tea extract are non-toxic for rodents and humans (Japanese). These findings encouraged us to intensively study cancer prevention by green tea (Fujiki and Suganuma 2002; Fujiki 2005).

Results and discussion

Green tea and green tea extract

Green tea is made from steamed fresh tea leaves and is a non-oxidized non-fermented product containing at least four green tea catechins—EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin (EC) and (−)-epicatechin gallate (ECG)—and caffeine on high-performance liquid chromatography (HPLC). EGCG is the main constituent (Fujiki and Okuda 1992). In addition to EGCG, we used for our experiments green tea extract, the dried green tea infusion containing all green tea catechins, a condensed form of green tea, and Japanese have been drinking green tea for 800 years. Green tea catechins are active in preventing carcinogenesis in a wide range of target organs, including the digestive tract, lung, liver, pancreas, breast, bladder, prostate and skin in rodents (Fujita et al. 1989; Yamane et al. 1991, 1995; Conney et al. 1992; Wang et al. 1992; Narisawa and Fukaura 1993; Yang and Wang 1993; Nishida et al. 1994; NCI et al. 1996; Fujiki et al. 1996; Gupta et al. 2001). Moreover, drinking 0.05 and 0.1 % EGCG solutions significantly prevented the spontaneous metastasis of B16-BL6 cells from foot pad to the lungs of male C57BL/6 mice (Taniguchi et al. 1992). It is interesting to note that green tea catechins are active in most of these organs.

Tissue distribution of ^{3}H-EGCG

To prove the systemic effects of EGCG, ^{3}H-EGCG was intubated into the stomach of mice. The microautoradiography of the lungs showed silver grains of radioactive EGCG in some cells, but not all (Suganuma et al. 1998), apparent confirmation that EGCG was incorporated from the digestive tract into the lungs, one of the target organs. Microautoradiography of human lung cancer cell line PC-9 cells treated with ^{3}H-EGCG showed silver grains in the membrane, cytosol and nuclei, and that ^{3}H-EGCG had been incorporated from culture medium into the cells (Okabe et al. 1997). Using cold spray ionization-mass spectrometry and surface plasmon resonance assay (Biacore), the direct binding of EGCG to single-strand 18 mers of DNA and RNA was demonstrated (Kuzuhara et al. 2006). The incorporation of ^{3}H-EGCG into target organs is shown in Table 1. Various amounts of the total administered radioactivity were found in the digestive tract, liver, brain, kidney, lung, pancreas and skin 24 h after intubation

Table 1 Incorporation of ^{3}H-EGCG into target organs

Organs	% of total administered radioactivity (24 h after)	Reduction in tumor incidence
Stomach	3.93	62.0 → 31.0
Duodenum	0.35	63.0 → 20.0
Small intestine	5.69	ND
Colon	4.52	77.3 → 38.1
		67.0 → 33.0[a]
Liver	0.89	83.3 → 52.2
Brain	0.32	ND
Kidney	0.28	ND
Lung	0.16	96.3 → 65.5
Pancreas	0.07	54.0 → 28.0
Skin	1.9×10^4/100 mg[b]	65.0 → 28.0[a]

ND, not determined

[a] Green tea extract

[b] dpm

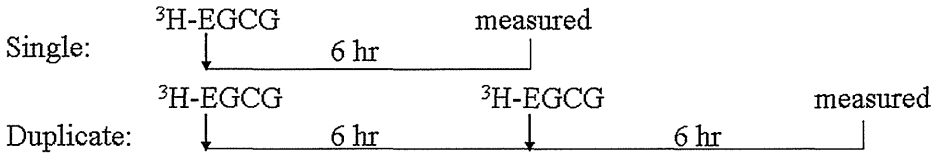
(Suganuma et al. 1998). The right column shows the reduction in tumor incidence in carcinogenesis by EGCG and green tea extract in rodents (Table 1) (Fujiki et al. 1996). Thus, radioactivity was found in the organs where EGCG and green tea extract had previously been shown to inhibit carcinogenesis.

Significance in multiple administrations of EGCG

Japanese drink green tea throughout the day. To study the effects of frequent consumption, ^{3}H-EGCG was intubated into the stomach of mice at 6-h intervals. Duplicate administrations enhanced incorporation of ^{3}H-EGCG 4- to 9-fold in most organs compared with a single administration, suggesting the accumulation of EGCG in cells. Radioactivity in blood and urine increased as well. We named this synergistic enhancement by EGCG the "Fujiki-Suganuma Effect," which is supported by frequent drinking of green tea (Table 2) (Suganuma et al. 1998). To further study the effects on enhanced incorporation of EGCG by multiple administrations, the induction of *growth arrest and DNA damage-inducible 153 (GADD153)* and *p21* gene expressions in human lung cancer cell line A549 cells was determined at 6-h intervals. *GADD153* (*CHOP*) is an apoptosis-regulating gene. The overexpression of *GADD153* gene induces apoptosis of the cells and leads to antiproliferative effects (Novoa et al. 2001), and EGCG was reported to induce apoptosis of the cells (Okabe et al. 1997; Ahmad et al. 1997; Yang et al. 1998; Okabe et al. 1999). *p21* is an inhibitor of *cyclin-dependent kinase* gene. The upregulation of *p21* gene inhibits proliferation by blocking the cell cycle (El-Deiry et al. 1994). And multiple treatments with EGCG induced enhancement of *GADD153* and *p21* gene expressions (Fig. 2) (Kuzuhara et al. 2007a).

Table 2 Enhanced incorporation of ^{3}H-EGCG by duplicate administrations: the "Fujiki-suganuma effect"

	Total radioactivity ($\times 10^4$ dpm)		Fold increase
	Single	Duplicate	
Blood (/ml)	25.13	149.40	×5.9
Brain	3.00	20.29	×6.8
Lung	3.47	16.14	×4.7
Liver	18.94	87.06	×4.6
Kidney	4.68	14.68	×3.1
Spleen	1.33	2.03	×1.5
Pancreas	0.77	3.29	×4.3
Uterus and ovary	2.04	7.23	×3.5
Bladder	0.14	0.74	×5.3
Mammary gland (100 mg)	0.29	0.72	×2.5
Bone (100 mg)	0.96	4.50	×4.7
Skin (100 mg)	3.41	4.91	×1.4



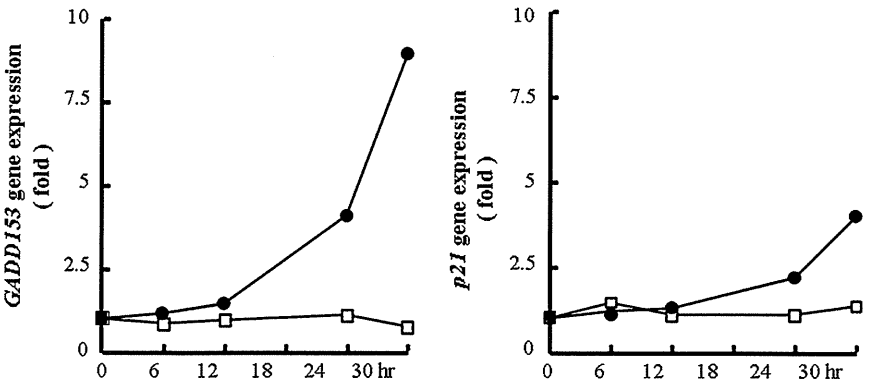


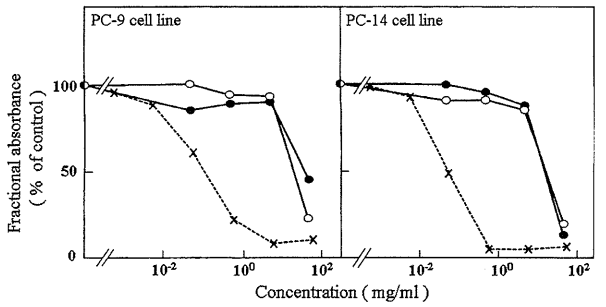
Fig. 2 The induction of *GADD153(L)* and *p21(R)* gene expressions in A549. Multiple treatments with EGCG (*filled circle*) at 6-h intervals and single treatment with EGCG (*open circle*)

We think that multiple treatments with EGCG induce synergistic effects on apoptosis in cells and that frequent drinking of green tea plays an important role in cancer prevention.

Inhibitions of in vitro cell growth and TNF-α release, and induction of apoptosis by green tea catechins

Various green tea catechins inhibited the growth of PC-9 cells, dose-dependently, with the order of potency being ECG, EGCG and EGC; EC was not effective (Okabe et al. 1997). Since the activity of EGCG was relatively weak, we compared the IC_{50} value of EGCG with that of the anticancer drug adriamycin for growth inhibition of two human lung cancer cell lines, PC-9 and PC-14. The IC_{50} value for adriamycin was 0.17 μM and that for EGCG was 41.5 μM (Fig. 3) (Komori et al. 1993a), which means that EGCG is approximately 250-fold less effective than adriamycin.

Although EGCG and green tea extract have very weak anticancer activity, they are unique in having multifunctions: inhibition of cell growth and TNF-α release from the cells induced by okadaic acid, a tumor promoter and



PC-9	IC_{50} (μM)	
EGCG	41.50	(244×)
Adriamycin	0.17	(1)

Fig. 3 Growth inhibition of two human lung cancer cell lines, PC-9 and PC-14 by EGCG (*open circle*), green tea extract (*filled circle*) and adriamycin (*times symbol*)

induction of apoptosis with green tea catechins (Table 3) (Suganuma et al. 1999a). EGCG, ECG and EGC were active in all three tests, while EC was inactive. However, cotreatment of EC with EGCG, ECG or EGC synergistically enhanced inhibition of TNF-α release from the cells (Table 4) (Suganuma et al. 1999a). Since we consider TNF-α to be an endogenous tumor promoter, EGCG inhibition of TNF-α release is a key mechanism in cancer prevention (Suganuma et al. 1999b; Fujiki and Suganuma 2011). Synergistic effects were also observed in the inhibition of cell growth and induction of apoptosis. EC obviously increased bioavailability of the catechins, since EC enhanced the incorporation of ^{3}H-EGCG into the cells (Suganuma et al. 1999a). In light of our evidence, we think that usual green tea infusion from the green tea leaves of sencha with warm water is the most appropriate and

Table 3 Three biochemical and biological effects with green tea catechins

Catechins	Inhibition of cell growth (% of control)	Inhibition of TNF-α release IC_{50} (μM)	Induction of apoptosis (A_{415} nm)
EC (100[a], 200[b] μM)	97.8[b]	>500[a]	0.01 ± 0.03[b]
EGCG (100 μM)	73.3	60	0.52 ± 0.22
ECG (50 μM)	62.2	30	0.27 ± 0.13
EGC (200 μM)	100.0	ND	0.14 ± 0.03

ND not determined

[a] 100 μM

[b] 200 μM

Table 4 Synergistic effects by cotreatment of EC with other green tea catechins on inhibition of TNT-α release

	Inhibition of TNT-α release IC_{50} (μM)	
	Without EC	With EC (100 μM)
		>500
EGCG (100 μM)	60	15
ECG (50 μM)	30	7

practical cancer preventive. Precisely how green tea catechins induce multifunctions favorable for cancer prevention is a significant question, and we have presented evidence showing that green tea catechins have potential to act as chemical chaperones (Kuzuhara et al. 2008).

Sealing effects of EGCG and green tea extract

It was exciting for us to find that repeated applications of EGCG inhibited the tumor promotion pathways of both TPA and okadaic acid, although their mechanisms of action are different: TPA and teleocidin bind to the phorbol ester receptor, whereas okadaic acid binds to protein phosphatases 1 and 2A. These two different receptors are present in the membrane and cytosol fractions of mouse skin. When mouse skin was treated with a single application of 5 mg EGCG, both the specific binding of ^{3}H-TPA and that of ^{3}H-okadaic acid decreased immediately, within 5–10 min; the levels of the specific binding gradually returned to normal. In the experiments with tumor promotion on mouse skin, EGCG was usually applied 15 min before each application of tumor promoter. Based on the evidence, we think that EGCG treatment inhibited the interaction of tumor promoters with their receptors and that EGCG can interrupt the interaction of ligand with its receptor on cell membrane: This was called the "Sealing Effects of EGCG" (Yoshizawa et al. 1992). Experimentally, EGCG caused reduction in detergent-insoluble membrane domain, that is, a decrease in lipid raft (Fujiki 2005; Adachi et al. 2007), and EGCG also inhibited the activation of specific receptor tyrosine kinases, such as epidermal growth factor receptor, insulin-like growth factor-1 receptor and vascular endothelial growth factor receptor 2 (Shimizu et al. 2011). The results indicate that treatment of cells with EGCG and green tea extract reduced kinase activity, one of the multifunctions of EGCG.

International response to cancer prevention with green tea

In 1991, the International Symposium on Physiological and Pharmacological Effects of Camellia Sinensis (Tea) was held at the American Health Foundation in New York. At a press conference, a US scientist said that since animal studies often do not have identical results in humans, more studies were needed before he could recommend green tea for its health effects on humans. However, one of our groups told the press that Japanese drink green tea every day and that green tea could be one of the most practical methods of cancer prevention available. The next day (August 27, 1991), his comments were on the first page of USA Today. We are convinced that his comments in 1991 are now more valid and widely accepted than ever. Furthermore, in 1994, the New York Times reported the cancer preventive effects of green tea in a story entitled "Green tea: more than just a soothing brew." This article was based on a report from Shangai that green tea may reduce the incidence of cancer of the esophagus in humans (June 15, 1994). And in 1995, one of us had the happy opportunity to give a 1-h lecture about our research under the title of "Inhaltsstoffe des grünen Tees, Ein Beispiel für Krebsprevention beim Menschen," within the framework of Teleakademie von Südwestdeutschen Fernsehen, Baden–Baden in Germany.

Green tea is a popular beverage in Asian countries, and cancer prevention with green tea is now a topic in Western nations. The cover of Cancer Research for September 1998 shows the leaves of the green tea plant and its small white flowers, and Molecular Carcinogenesis (February, 1999) introduced the results of T. Bowden, in which EGCG in a tea bag inhibited the activation of p38 mitogen-activated protein (MAP) kinase, resulting in the prevention of skin cancer (Fig. 4) (Chen et al. 1999). And in 2011, the 4th World Congress on Tea & Health was held at the Max Delbrück Center for Molecular Medicine in Berlin Buch, introducing new challenging applications of EGCG for neurodegenerative diseases.

Prospective cohort study in Saitama Prefecture

The cancer preventive effect of green tea was first confirmed through a prospective cohort study with humans in Saitama Prefecture. In 1986, we first surveyed 8,552 individuals aged over 40 on their living habits, including their daily consumption of green tea. During the 10 years after 1986, a total of 419 cancer patients, 244 males and 175 females, were detected (Nakachi et al. 2000). Female and male cancer patients were divided into three groups based on consumption of green tea per day: under 3 cups, 4–9

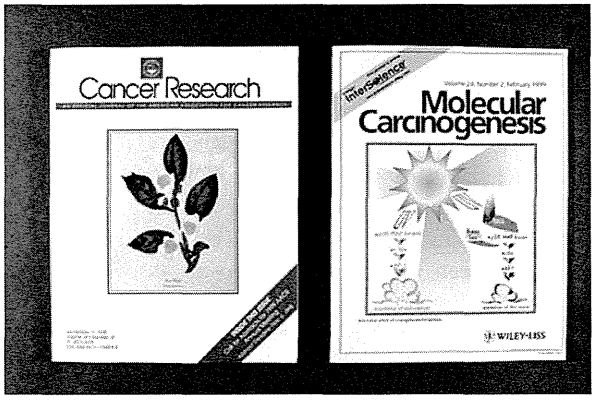


Fig. 4 The covers of Cancer Research (*L*) and Molecular Carcinogenesis (*R*)

Table 5 Average age at cancer onset and daily green tea consumption

Gender	Average age at cancer onset and consumption of green tea per day (cups)		
	≤3	4–9	≥10
Female (175)	67.0 ± 1.7 (28.0 %)	66.4 ± 1.3 (58.3 %)	74.3 ± 2.2 (13.7 %)
Male (244)	65.0 ± 1.5 (24.2 %)	67.2 ± 1.0 (46.7 %)	68.2 ± 1.1 (29.1 %)

cups and over 10 cups (Table 5). Next, the average age at cancer onset of all these cancer patients was obtained from clinical documents at hospitals. The average age at cancer onset and daily green tea consumption per patient were studied.

The cancer onset in female patients who had consumed over 10 Japanese-size cups (120 ml/cup) of green tea per day was 7.3 years later than that of female patients who had consumed less than three cups per day. The cancer onset in male patients who had consumed over 10 cups of green tea per day was 3.2 years later than that of patients who had consumed less than three cups per day (Table 5) (Nakachi et al. 2000). The difference between females and males is partly due to higher tobacco consumption by males. It is important to note that the delay of cancer onset is significant evidence of primary cancer prevention in humans. Key factors in primary cancer prevention include: tamoxifen for breast cancer (Fisher et al. 1998), cessation of smoking, diet modifications and physical activity. In addition, the cohort study first demonstrated that consumption of 10 cups of green tea per day is an additional significant factor in primary cancer prevention for the general population. Since the consumers of over 10 cups of green tea per day were only 13.7 % for female patients and 29.1 % for male patients, it would be possible to increase consumption to over 10 cups per day with green tea tablets, resulting in effective primary cancer prevention (Fujiki et al. 2002).

We next studied the reduced relative risk in human organs by high consumption of green tea in Saitama Prefecture. Green tea most significantly prevented lung cancer—a relative risk of 0.33 with over 10 cups of green tea per day. High consumption of green tea also prevented cancers of the colorectum, liver and stomach, in that order (Fig. 5) (Nakachi et al. 2000). It is important to note that consumption of less than ten cups is not effective as a preventive tool, suggesting that there is a minimum effective amount of green tea catechins for cancer prevention. In addition to the cancer preventive effects, the prospective cohort study provided other significant results. Increased green tea consumption was associated with decreased serum total cholesterol, decreased triglyceride levels and decreased atherogenic index. Moreover, the prevalence rates of cardiovascular disease and diabetes mellitus were significantly lower among the population consuming over 10 cups of green tea per day (Imai et al. 1997). Since green tea apparently prevents many major lifestyle-related diseases, including cancer, cardiovascular disease and diabetes mellitus, we reported that among women, the mean age at death for those consuming over 10 cups per day was 6 years later than that for those consuming less than 3 cups, suggesting that green tea reduces the risk of lifestyle-related diseases, resulting in a longer life span (Imai et al. 1997; Nakachi et al. 2000; Sueoka et al. 2001). All the results of this study indicate that the more green tea we drink, the higher cancer preventive activity we get in the target organs. Of course, cancer prevention for general population before cancer onset is a matter of individual responsibility, not the duty of clinicians. We call this the first stage of cancer prevention with green tea (Fig. 6).

Our cohort study was conducted in Saitama Prefecture, one of the main tea-producing areas in Japan. Other investigators have reported non-preventive effects of green tea against human cancers, such as no association between the consumption of green tea and the risk of gastric cancer

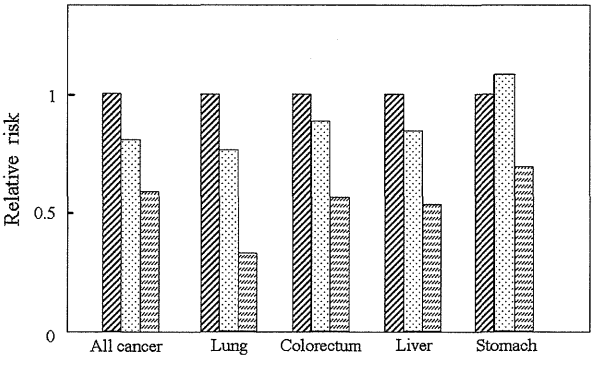


Fig. 5 Reduced relative risk of cancer in human organs with a high consumption of green tea. ▨ ≤3, ⊡ 4–9, ▤ ≥10

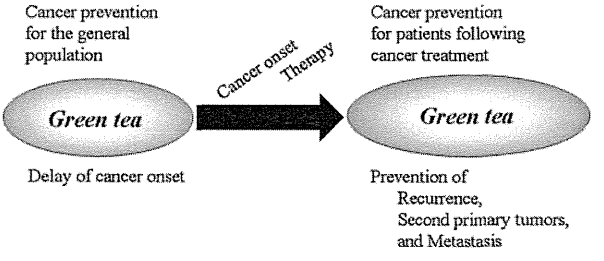


Fig. 6 Two stages of cancer prevention with green tea

in Miyagi Prefecture (Tsubono et al. 2001), and no association between green tea drinking and the risk of breast cancer in Japan (Iwasaki et al. 2010). It is important to note that various kinds of green tea are produced in Japan, such as sencha, hojicha and oolong tea. Sencha contains 13.6 % green tea catechins, hojicha made from roasted lowest grade of tea leaf contains only 2.9 %, and oolong tea 7.6 %. This clearly shows that the results of epidemiological study are dependent on the kinds of green tea consumed and the quality of green tea catechins. Thus, an ingested amount of green tea catechins is a limiting factor for demonstrating the preventive or non-preventive effects in a cohort study.

In addition, an interesting study reported that no association between green tea consumption and breast cancer incidence was found in the cohort studies of the United States, but that increased green tea consumption (more than three cups a day) was inversely associated with breast cancer recurrence (Pooled RR = 0.73, 95 % CI: 0.56-0.96) (Ogunleye et al. 2010). In 1998, we previously reported the decreased recurrence of human breast cancer with increased consumption of green tea, on the basis of results obtained from 472 cancer patients. Stages I and II cancer patients consuming over five cups of green tea per day (average 8 cups) showed a lower recurrence rate, 16.7 %, and a longer disease-free period, 3.6 years, than those consuming less than four cups (average 2 cups) per day, 24.3 % and 2.8 years (Nakachi et al. 1998). The results showed that drinking an average 8 cups of green tea per day prior to cancer onset results in more hopeful prognosis for breast cancer patients.

Preclinical safety trials for 10 cups of green tea

Next, we will think about cancer prevention for patients following cancer treatment, since surviving cancer patients are seriously looking for preventives. Our epidemiological results showed that the effective cancer preventive amount is also 10 Japanese-size cups of green tea per day, corresponding to 2.5 g green tea extract (Nakachi et al. 2000). To facilitate consumption, tablets of green tea extract are produced by the Tea Institute of Saitama Prefecture. A tablet dissolved in warm water becomes usual Saitama tea (Fujiki et al. 2001). 10 cups of green tea per day may seem to be a large amount, but the results of Phase I clinical trials with green tea extract conducted in USA reported that the maximum tolerated dose corresponds to 21–24 Japanese-size cups per day (Pisters et al. 2001), or even higher amounts (Laurie et al. 2005). Therefore, we think 10 cups of green tea is tolerable for most people.

Before going on to a clinical trial, we first asked some 102 healthy and voluntary citizens to consume this amount for 3 months, with informed consent. Group A of 51

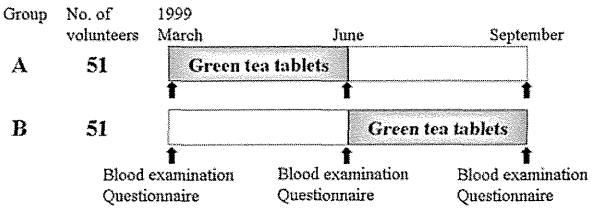


Fig. 7 Preclinical safety trials among 102 healthy and voluntary citizens of Saitama Prefecture for 6 months. 10 Cups of daily green tea beverage fortified by green tea tablets for 3 months

volunteers took 10 cups of daily green tea beverage supplemented with green tea tablets for the first 3 months, and group B took the same amount for the next 3 months. During the 6-month trial, all of the 102 volunteers answered questionnaires and their blood samples were biochemically examined three times (Fig. 7) (Fujiki et al. 2001). The blood examination did not show any serious side effects, so important answers to just two of the questions are presented. To the question, "Did you experience any discomfort from taking green tea tablets?", 93 % of the participants said that they were able to continue drinking green tea and taking green tea tablets. For another question, "Did you observe any changes in living habits or meals?", Over 90 % of the participants found their living habits or appetite unaffected by 10 cups of green tea daily.

However, about 50 % of the volunteers experienced very mild temporary disorders due to the caffeine in green tea extract. It is reported that the maximum tolerable dose of caffeine is about 1.0 g/day, and even though 10 cups do not contain total 1.0 g caffeine, the Tea Institute developed a new method to reduce the caffeine by washing the green tea leaves with warm water. They reduced the caffeine content from 5 % to less than 3 %, without using an organic solution. It is important to note that the tablets are a dried green tea infusion, not a powder of green tea leaves. The tablets are not controlled by the Pharmaceutical Affairs Act in Japan because they belong to the category of green tea beverage, not a drug.

Significant factors for studying cancer prevention with humans

Before going on to human studies, we had to clarify the following factors. (1) It is necessary to have evidence that green tea is non-toxic for humans and that there is bioavailability in rodents (Suganuma et al. 1998). (2) We obtained the minimum effective amounts of green tea per day for humans from the results of human epidemiological study (Nakachi et al. 2000). (3) Collaboration of healthy volunteers with informed consent was established (Fujiki et al. 2001). (4) Clinicians developed an established clinical biomarker for target organ (Shimizu et al. 2008).

(5) Green tea tablets should be quality controlled, and the absence of pesticides and herbicides confirmed (Fujiki et al. 2001). (6) We were fortunate to be able to collaborate with the Tea Institute of Saitama Prefecture, which provided green tea extract and tablets. (7) Results should be published in reviewed international journals (Nakachi et al. 2000; Shimizu et al. 2008).

Double-blind randomized clinical phase II prevention trial for recurrence of colorectal adenomas

Table 6 shows the protocol for phase II prevention trial of colorectal adenoma recurrence with green tea extract, with informed consent, which was conducted at Gifu University, Department of Medicine and its related hospitals (Shimizu et al. 2008). One green tea tablet (GTE, 500 mg) contains 52.5 mg EGCG, 34.6 mg EGC, 11.1 mg ECG, 12.3 mg EC and 15.7 mg caffeine, approximately equivalent to two Japanese-size cups of green tea. Colorectal adenomas were removed by endoscopic polypectomy at the first colonoscopy, and 12 months later, the absence of polyps was confirmed by second colonoscopy. The patients were then double-blind randomized into two groups: one group maintained their usual daily consumption of green tea beverage without a placebo, and the other group (GTE group) took 10 cups of green tea, supplemented with six green tea tablets, daily for 12 months. The incidence of recurrent adenomas was determined by end-point colonoscopy 12 months later. The recurrence rate of the control group was 31 % and that of the GTE group was 15 % (Shimizu et al. 2008). The chi-square test, p was <0.05 (Table 6). In addition, the size of recurrent adenomas was smaller in the GTE group than in the control group. Thus, drinking 10 Japanese-size cups of green tea, supplemented with green tea tablets, 50 %, significantly prevented recurrence of colorectal adenomas in patients (Shimizu et al. 2008).

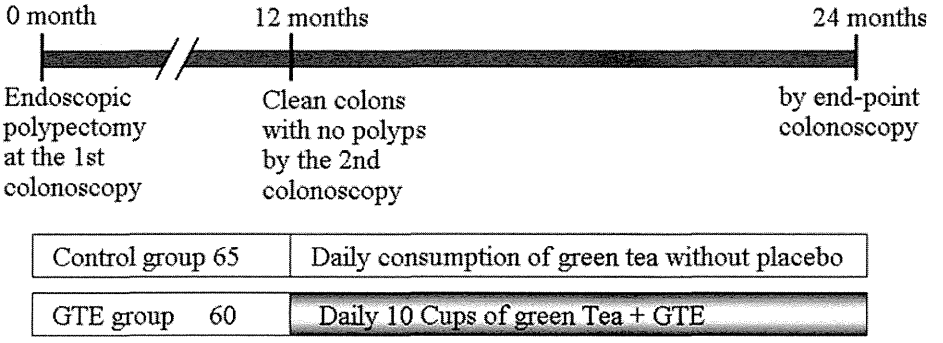
It is important to note that we did not use placebo in our phase II prevention trial because green tea is a daily beverage of Japanese, so, we aimed to raise the consumption to a target level, with supplemental tablets of green tea extract. The patients in the trial were told to take a minimum effective amount of green tea catechins. A randomized, placebo-controlled multicentre trial was recently begun to investigate the effects of diet supplementation with green tea extract containing 300 mg EGCG on the recurrence of colon adenomas for patients who have undergone polypectomy for colon polyps in Germany (Stingl et al. 2011).

Moreover, the prevention of prostate cancer development in patients with high-grade prostate intraepithelial neoplasia (PIN) using capsules of green tea catechins was confirmed in Italy. In the group using green tea capsules, only one tumor was diagnosed among 30 patients, whereas nine cancers were found among 30 placebo-controlled patients. Green tea catechins reduced incidence of prostate cancer from 30 to 3 % (Bettuzzi et al. 2006). In the United States, a phase I trial of green tea extract was first conducted at two Institutions (Pisters et al. 2001), and a phase II randomized, placebo-controlled trial of green tea extract in patients with high-risk oral premalignant lesions revealed that the two higher doses (750 and 1,000 mg/m^2) of green tea extract produced a trend toward a greater clinical response in association with a high baseline level and downregulation of angiogenic stromal vascular endothelial growth factor (Tsao et al. 2009).

Necessity of cancer prevention in our daily lives

Tumor promotion in human cancer is now understood as a disease affected by TNF-α upregulation and NF-κB

Table 6 Phase II prevention trial of colorectal adenoma recurrence with green tea extract in patients, with no polyps after polypectomy



Groups (cases)	Recurrence rate (%)	Average no. of polyps/patient
Control (20/65)	31.0	0.43
GTE (9/60)	15.0 ($p < 0.05$)	0.20

activation (Komori et al. 1993b; Suganuma et al. 1999b; Ben-Neriah and Karin 2011), induced by pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Suganuma et al. 2002). Pro-inflammatory cytokines are easily induced in humans by bacterial focal infections (Fujiki et al. 2004; Kuzuhara et al. 2007b); Cytokines and chemokines are numerous in aged people; and so, humans are always at risk of tumor promotion. With these physiological conditions, we need to establish our own cancer prevention strategy, based on reduction in TNF-α and inactivation of NF-κB. For this purpose, we believe cancer prevention with green tea is the most appropriate preventive, since it is effective on a wide range of target organs and has no toxic effects. Green tea can be ingested by everybody in the world.

Due to advancements in diagnosis and treatment, there are many healthy surviving cancer patients in Japan. They are seriously looking for drugs and preventives that will prevent recurrence, second primary tumors and metastasis. For these patients, green tea supplemented with green tea tablets is vital following cancer treatment. We call this the two stages of cancer prevention with green tea (Fig. 6) (Fujiki 1999; Fujiki et al. 2002). The left part of Fig. 6, the first stage, deals with primary cancer prevention, and the right part of Fig. 6, the second stage, shows tertiary cancer prevention.

New cancer treatment strategy using the combination of green tea catechins and anticancer drugs

Considering the cancer prevention for patients following cancer treatment (Fig. 6), anticancer drugs may be administered to Japanese cancer patients who consume green tea every day. So we raised the following questions: Is it advantageous for Japanese cancer patients to take cancer preventive green tea catechins and anticancer drugs together? Does the combination have the potential to enhance efficacy and decrease adverse effects of anticancer drugs? What kinds of anticancer drugs and compounds can work with green tea catechins? The names of the compounds are presented: gefitinib, 5-fluorouracil, taxol, doxorubicin, erlotinib, antiestrogen, COX inhibitors, sulindac and celecoxib, retinoids and curcumin.

We had two significant results. The combination of green tea extract with sulindac enhanced the inhibition of tumor development in multiple intestinal neoplasia (Min) mice (Suganuma et al. 2001). Min mice have a germline mutation of *APC* gene and develop intestinal tumors similar to those of familiar adenomatous polyposis, FAP patients. Intestinal tumors are seen as blue spots on the organ by staining with methylen blue. The combination reduced the number of tumors per mouse from 72.3 to 32.0, a decrease of 55.7 %, $p < 0.05$ (Suganuma et al. 2001). The second result was as follows: the combination of green tea extract with celecoxib enhanced the inhibition of tumor development in the lungs of A/J mice induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a carcinogen. The combination reduced the number of tumor-bearing mice from 100 to 73.3 %, a decrease of 26.7 %, $p < 0.05$ (Suganuma et al. 2011).

To understand the molecular mechanisms involved in synergistic enhancement by the combination of EGCG with sulindac, we first studied the upregulation of two gene expressions, *GADD153* and *p21* genes, and as previously reported, their inductions of gene expression were enhanced to be about 12-fold and 3-fold, respectively, (Okabe et al. 2001; Fujiki and Suganuma 2002). These gene expressions were not affected by treatment with either EGCG alone or sulindac alone. The combination of EGCG with sulindac induced apoptosis in PC-9 cells 10.5-fold stronger than EGCG alone or sulindac alone, and the combination of EGCG with celecoxib induced apoptosis in PC-9 cells 14.9-fold stronger than EGCG alone or celecoxib alone (Table 7) (Suganuma et al. 2006). The results show that the combination additionally induces new gene expressions that are not induced by EGCG alone or COX inhibitors alone. A schematic illustration of the combination is shown in Fig. 8. The combinations indicate the presence of a new therapeutic activity that will greatly expand cancer treatment and also bring successful tertiary cancer prevention for patients with good prognosis (Suganuma et al. 2011).

Our definition of cancer prevention with green tea

The administration of cancer preventives delays the carcinogenic processes in humans, no matter when the carcinogenesis starts, thereby blocking the appearance of clinical symptoms (Fujiki et al. 2002).

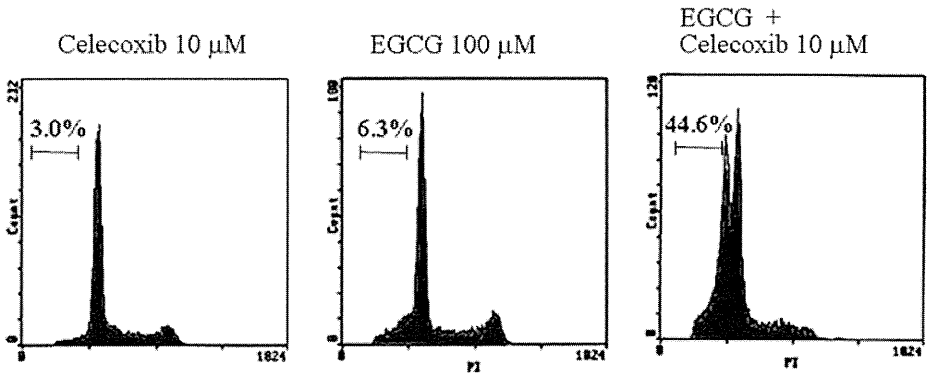
Afterword

Strong support by Professor James Watson

In May 2002, the International Conference on Green Tea and Cancer, A Critical Review, was held at the Banbury Center, Cold Spring Harbor Laboratory in New York. This Conference was strongly supported by the Director, J. Watson. After the Conference at the Banbury Center, Dr. Watson listened closely while one of the organizers noted the success of the Conference. The organizer told him that green tea clearly prevented cancer in rodents, whereas human epidemiological studies had proved inconclusive. Dr. Watson immediately answered that since green tea prevented carcinogenesis in rodents, it should prevent it in humans as well, since humans and mice have

Table 7 Synergistic induction of apoptosis by combination of EGCG with COX inhibitors

	Induction of apoptosis (% of apoptotic cells)	
	Without	With EGCG
Human lung cancer cell line PC-9		
Control	2.0	6.3
Sulindac	4.1	42.9 (×10.5)
Celecoxib	3.0	44.6 (×14.9)



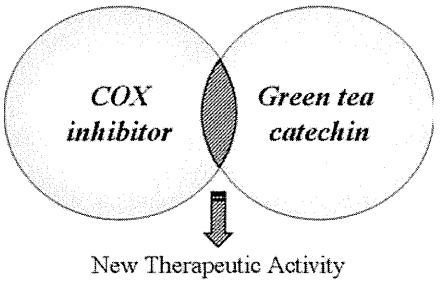


Fig. 8 Combination of green tea with COX inhibitor

similar genomes. It was wonderful encouragement for green tea scientists like us, because we got the impression that a world-famous molecular biologist now believes in the cancer preventive effects of green tea for humans. Seven years later, we successfully confirmed the prevention of colon adenomas in humans by green tea supplemented with green tea tablets.

The history of green tea in Japan

Drinking green tea is really part of Japanese culture. The Japanese Zen priest Eisai returned to Japan after 4 years training as Zen priest in China, carrying some seeds of green tea, as a medicine. When the third Shogun became ill, Eisai wrote a book entitled "Kitusa yohjohki" (Maintaining Health by Drinking Green Tea), which was presented to the Shogun together with green tea in the year 1211. The Shogun's illness, probably a disorder of the digestive tract, was cured soon, and this is the first case of therapeutic effects with green tea in Japan.

Numerous illnesses of course existed 800 years ago, but cancer and aging-associated diseases did not, because the average life span was only 40 years at that time. Thus, our study for the first time demonstrated that green tea prevents a lifestyle-related disease. Eisai concluded his book this way: I hope an excellent doctor will elucidate the mechanism of green tea action in the near future. We believe our research project with green tea is a present from Eisai.

Acknowledgments To Prof. Erich Hecker, we express gratitude for his fruitful discussion. We thank Prof. Takuo Okuda for providing numerous polyphenols including (−)-epigallocatechin gallate (EGCG) and penta-*O*-galloyl-*β*-D-glucose (5GG), Prof. Takashi Sugimura for his encouragement during the course of this work, and our Japanese colleagues cited in our references for their research. This work was supported by Japan Society for the Promotion of Science: Scientific Research on Priority Areas for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan; Comprehensive Research on Aging and Health; and Cancer Research from the Ministry of Health, Labor, and Welfare, Japan; Selective Applied and Developed Research, and Green Tea Extracts Research Development for Cancer Prevention by the Department of Agriculture and Forests and the Department of Health and Human Services of Saitama Prefecture, Japan; and the Smoking Research Fund.

Conflict of interest We declare that we have no conflict of interest.

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Cancer Prevention Research

Research Article

Possible Role of Visfatin in Hepatoma Progression and the Effects of Branched-Chain Amino Acids on Visfatin-Induced Proliferation in Human Hepatoma Cells

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Abstract

Obesity and related metabolic abnormalities, including adipocytokine dysbalance, are risk factors for hepatocellular carcinoma (HCC). Visfatin, an adipocytokine that is highly expressed in visceral fat, is suggested to play a role in the progression of human malignancies. Branched-chain amino acids (BCAA) reduce the incidence of HCC in obese patients with liver cirrhosis and prevent obesity-related liver carcinogenesis in mice. In this study, we investigated the possible role of visfatin on HCC progression and the effects of BCAA on visfatin-induced proliferation of HCC cells. In patients with HCCs, serum visfatin levels were significantly correlated with stage progression and tumor enlargement. Visfatin preferentially stimulated the proliferation of HepG2, Hep3B, and HuH7 human HCC cells compared with Hc normal hepatocytes. Visfatin phosphorylated extracellular signal–regulated kinase (ERK), Akt, and GSK-3β proteins in HepG2 cells. LY294002 [a phosphoinositide-3-kinase (PI3K) inhibitor], PD98059 [a MAP/ERK 1 kinase (MEK1) inhibitor], CHIR99021 (a GSK-3β inhibitor), and BCAA significantly inhibited visfatin-induced proliferation in HepG2 cells. BCAA also inhibited phosphorylation of GSK-3β, increased cellular levels of $p21^{CIP1}$, caused cell-cycle arrest in G_0/G_1 phase, and induced apoptosis in HCC cells in the presence of visfatin. These findings suggest that visfatin plays a critical role in the proliferation of HCC cells and may be associated with the progression of this malignancy. In addition, BCAA might inhibit obesity-related liver carcinogenesis by targeting and, possibly, by overcoming the stimulatory effects of visfatin. *Cancer Prev Res; 4(12); 2092–100. ©2011 AACR.*

Introduction

In addition to established risk factors such as hepatitis and alcohol consumption, obesity and its related metabolic abnormalities raise the risk of hepatocellular carcinoma (HCC; refs. 1–4). Several pathophysiologic mechanisms linking obesity and liver carcinogenesis have been shown, including the emergence of insulin resistance and the subsequent inflammatory cascade (5). In obese individuals, increased adipose tissue leads to the expression of a variety of adipocytokines. Recently, the role of obesity-associated dysfunctional adipose tissue and subsequent adipocytokine dysbalance in carcinogenesis has attracted attention (6). Clinical trials have shown that adipocytokine disorders, including increased levels of leptin and decreased levels of adiponectin in the serum, are implicated in hepatocarcinogenesis (7, 8). Leptin induces proliferation and inhibits apoptosis in human HCC cells (9). These findings suggest that adipocytokine dysbalance may play an important role in the development and progression of HCCs.

Visfatin/pre-B-cell–enhancing factor, which was originally isolated from peripheral lymphocytes, has been described as a secreted growth factor for early B-cell proliferation (10). More recently, visfatin has also been characterized as an adipocytokine that is highly expressed in the visceral fat of humans and rodents. Increased levels of visfatin, which are positively correlated with the size of visceral fat deposits, are observed in various clinical conditions such as obesity and diabetes mellitus (11, 12). Abnormalities in serum levels of visfatin have also been reported in nonalcoholic fatty liver disease, which is a hepatic manifestation of metabolic syndrome (13). These results are somewhat conflicting, however, as both increased and decreased serum levels of this adipocytokine have been found in patients with nonalcoholic fatty liver disease (14, 15).

Furthermore, previous studies have shown that visfatin may play a role in the development and progression of certain types of human malignancies (16). For instance,

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