

## Review

### Green tea and cancer prevention

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**Abstract:** We found that (-)-epigallocatechin gallate (EGCG), the main constituent of green tea and green tea extract, the lyophilized form of green tea infusion, inhibited both activation of protein kinase C and tumor promotion on mouse skin: The results led us to suggest green tea as a cancer preventive for humans. Inhibition of chemical carcinogenesis in rodents, inhibition of cell growth and induction of apoptosis on human cancer cell lines, organ distribution of <sup>3</sup>H-EGCG in mice, multifunctional actions, and the synergistic cancer preventive activity of green tea with sulindac were studied. All our results show that green tea is a promising beverage for the purpose of cancer prevention in humans.

**Key words:** EGCG; tumor promotion; tea polyphenols; synergistic effects.

**Introduction.** Our study with green tea began within the program of cancer chemoprevention in Japan in 1983. The term “cancer chemoprevention”, defined as “prevention of the occurrence of cancer by administration of one or more compounds” was coined by Michael B. Sporn in 1976.<sup>1)</sup> When looking for Japanese cancer preventive agents, we received tannins or polyphenols derived from medicinal plants and drugs from Takuo Okuda at Okayama University. Among these was (-)-epigallocatechin gallate (EGCG), which is the main constituent of green tea polyphenols. Our experiment in 1983 had indicated that EGCG bound to phorbol ester receptor, and EGCG dose-dependently inhibited activation of protein kinase C. Based on evidence that tumor promotion on mouse skin was induced by — in addition to phorbol ester — teleocidin, aplysiatoxin, palytoxin, and okadaic acid class compounds,<sup>2)-5)</sup> we thought that cancer prevention was somehow related to reversing the process of tumor promotion. In the study of EGCG as cancer preventive, topical applications of EGCG inhibited tumor promotion on mouse skin induced by both teleocidin and okadaic

acid.<sup>6),7)</sup> These results encouraged us to search a possibility that green tea would be a practical cancer preventive.<sup>8)</sup> This review article includes our research activities along with some results of other scientists.

**Inhibition of chemical carcinogenesis.** In 1987, in a two-stage carcinogenesis experiment, we reported that repeated topical applications of EGCG to mouse skin treated with 7,12-dimethylbenz(a)anthracene (DMBA) as an initiator inhibited tumor promotion by teleocidin, one of the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters.<sup>6)</sup> Since then, many scientists have joined the study of green tea as a cancer preventive. EGCG and green tea extract, the lyophilized form of green tea infusion, have a wide range of target organs: EGCG and green tea extract in drinking water inhibited carcinogenesis of various organs, including esophagus, stomach, duodenum, colon, lung, liver, pancreas, skin, breast, bladder, and prostate in rodents (Table I).<sup>7)-11)</sup> Also significant was the fact that EGCG and green tea extract were not toxic for rodents. For example, a solution of 0.05% EGCG p.o. for 15 weeks to male Wistar/KOB rats did not show any significant difference in body weight between the groups treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) alone and with MNNG plus EGCG.<sup>11)</sup> Furthermore, EGCG in drinking water inhibited metastasis of mouse melanoma cells into lung.<sup>12)</sup> Recently Mukhtar's group used the autochthonous transgenic adenocarcinoma of a mouse prostate model for the study of green tea infusion:

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Table I. Anticarcinogenic effects of EGCG and green tea extract in various organs

Organs	Species	Carcinogens/ Tumor promoters	Tumor incidence (%)	
			Non-treated	Treated <sup>a)</sup>
Skin	Mouse CD-1	DMBA/Teleocidin	53.0	13.0
		DMBA/Okadaic acid	73.3	0
Glandular stomach	Rat Wistar	MNNG	62.0	31.0
Duodenum	Mouse C57BL/6	ENNG	63.0	20.0
Colon	Rat	AOM	77.3	38.1
	Rat	MNU	67.0	33.0*
Liver	Mouse C3H/HeN	Spontaneous	83.3	52.2
Lung	Mouse A/J	NNK	96.3	65.5*
Pancreas	Hamster	BOP	54.0	33.0*

<sup>a)</sup>Treated with EGCG or green tea extract (\*); DMBA: 7,12-dimethylbenz(a)anthracene; MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ENNG: *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; AOM: azoxymethane; MNU: methylnitrosourea; NNK: 4-(methylnitrosoamine)-1-(3-pyridyl)-1-butanone; and BOP: *N*-nitrosobis-(2-oxopropyl)amine.

The results clearly showed that green tea inhibited development, and metastasis of prostate cancer.<sup>13)</sup> Considering all of these results, this simple beverage of every-day life now has a significant potential as the most promising cancer preventive for humans.

**Growth inhibition and apoptosis.** Green tea, the non-oxidized/non-fermented product of tea leaves, contains at least four tea polyphenols: EGCG, (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epicatechin (EC) (Fig. 1). In *in vitro* experiments, tea polyphenols inhibited growth of human lung cancer cell line PC-9 dose-dependently, with the concentrations for 50% growth inhibition (IC<sub>50</sub> values) being 78  $\mu$ M for ECG, 140  $\mu$ M for EGCG and 275  $\mu$ M for EGC. EC, which does not contain a galloyl moiety, did not significantly inhibit cell growth.<sup>14)</sup> Growth inhibition was associated with G<sub>2</sub>/M arrest in PC-9 cells.<sup>14)</sup> When growth inhibitory activity of EGCG was compared with that of an anti-cancer agent adriamycin, EGCG was approximately 200 times less effective than adriamycin, the IC<sub>50</sub> values being 41.5  $\mu$ M for EGCG and 0.17  $\mu$ M for adriamycin in PC-9 cells.<sup>15)</sup> Growth inhibition by EGCG and green tea extract was similarly observed in various human cancer cell lines, including lung cancer cell line PC-14, and mammary cancer cell lines MCF-7 and BT-20.<sup>15)</sup>

Although the potencies of tea polyphenols for inhibition of cell growth correlated well with their induction of apoptosis, we also discovered that EC, an apparently inactive tea polyphenol, showed synergistic induction of apoptosis with other tea polyphenols, such as EGCG, ECG or EGC, in PC-9 cells. For example, 200  $\mu$ M EC with 75  $\mu$ M EGCG synergistically enhanced 20 times the

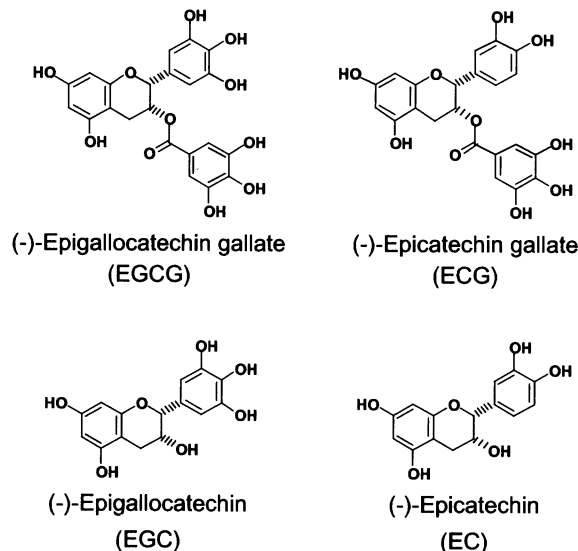


Fig. 1. Structures of green tea polyphenols.

DNA fragmentation induced by 75  $\mu$ M EGCG alone.<sup>16)</sup> As for the mechanisms of action of EC, we found that EC increased enhancement of <sup>3</sup>H-EGCG incorporation into PC-9 cells.<sup>16)</sup> The results indicated that EC, which has no galloyl moiety, enhances incorporation of EGCG and other tea polyphenols that do have galloyl moiety.<sup>16)</sup>

**Organ distribution of EGCG.** Since animal experiments indicated that EGCG and green tea extract had systemic effects on inhibition of carcinogenesis, we assumed that orally administered EGCG was easily distributed from the digestive tract to various organs, where they showed their anticarcinogenic effects. To study the bioavailability of tea polyphenols,

Table II. Increased incorporation of radioactivity in organs after a duplicate administration of  $^3\text{H}$ -EGCG compared with that after a single administration

	Administration				Fold
	Single	Duplicate		Total radioactivity $\times 10^{-4}$ dpm	
Blood (ml)	25.13 $\pm$	3.96*	149.40 $\pm$	9.60*	$\times 5.9$
Brain	3.00 $\pm$	2.04	20.29 $\pm$	1.47	$\times 6.8$
Lung	3.47 $\pm$	0.56	16.14 $\pm$	4.05	$\times 4.7$
Heart	2.08 $\pm$	0.11	5.13 $\pm$	1.03	$\times 2.5$
Liver	18.94 $\pm$	8.18	87.06 $\pm$	30.17	$\times 4.6$
Kidney	4.68 $\pm$	0.39	14.68 $\pm$	4.75	$\times 3.1$
Spleen	1.33 $\pm$	0.34	2.03 $\pm$	0.14	$\times 1.5$
Pancreas	0.77 $\pm$	0.49	3.29 $\pm$	0.16	$\times 4.3$
Uterus & ovary	2.04 $\pm$	0.62	7.23 $\pm$	2.44	$\times 3.5$
Bladder	0.14 $\pm$	0.07	0.74 $\pm$	0.31	$\times 5.3$
Mammary gland (100 mg)	0.29 $\pm$	0.07	0.72 $\pm$	0.01	$\times 2.5$
Bone (100 mg)	0.96 $\pm$	0.01	4.50 $\pm$	0.44	$\times 4.7$
Skin (100 mg)	3.41 $\pm$	1.73	4.91 $\pm$	2.09	$\times 1.4$
Urine	35.63 $\pm$	11.18	215.77 $\pm$	35.67	$\times 6.1$
Feces	585.67 $\pm$	388.30	217.19 $\pm$	5.94	$\times 0.4$

\*Each value represents the mean  $\pm$  variation of 2 female mice.

we obtained  $^3\text{H}$ -EGCG with a specific activity of 48.1 GBq/mmol as a very stable compound: We first found by microautoradiography that  $^3\text{H}$ -EGCG was incorporated into PC-9 cells in culture, and that silver grains of  $^3\text{H}$ -EGCG appeared in the membrane, cytosol and nuclei.<sup>14)</sup>

When an EGCG solution containing 3.7 MBq  $^3\text{H}$ -EGCG was given directly to mice by gastric tube, we found that 2% of total administered radioactivity were incorporated into blood in mouse 6 h after intubation, and within 24 h, 6.6% of total administered radioactivity was excreted in urine, 37.7% in feces.<sup>17)</sup> Table II shows incorporation of  $^3\text{H}$ -EGCG into various target organs 6 h after intubation, suggesting that radioactivity was found in the organs where EGCG or green tea extract had previously been shown to inhibit carcinogenesis. We also confirmed that silver grains derived from  $^3\text{H}$ -EGCG were present in the lungs and colon.<sup>17)</sup>

Yang's group intensively studied plasma and tissue levels of tea polyphenols in rats and mice given a 0.6% green tea preparation, and found that the plasma concentrations of EGC and EC were much higher than those of EGCG.<sup>18)</sup> They also found catechin esterase activity in human saliva, which converts EGCG to EGC.<sup>19)</sup>

Since Japanese drink many cups of green tea throughout the day, we wanted to determine whether frequent drinking of green tea could be recommended. Table II compares the results of a single administration of 3.7 MBq  $^3\text{H}$ -EGCG determined 6 h after, with those of

duplicate administrations (3.7 MBq  $\times$  2) determined 6 h after the second administration: Duplicate administrations of  $^3\text{H}$ -EGCG enhanced incorporation of  $^3\text{H}$ -EGCG 4 to 6 times in most organs.<sup>17)</sup> Judging from this experiment, it seems apparent that the more green tea we drink, the higher concentration of EGCG we get in the target organs. The specific mechanisms of enhancement in the organs are not known.

**Mechanisms of action of green tea polyphenols.** After extensive study on tumor promotion with various tumor promoters, we came to the conclusion that tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is the essential cytokine in tumor promotion, acting as an endogenous tumor promoter.<sup>20)</sup> Based on these results, we thought that EGCG and green tea polyphenols might inhibit tumor promotion and chemical carcinogenesis mediated through inhibition of TNF- $\alpha$  gene expression, resulting in reduction of TNF- $\alpha$ : It is well established that TNF- $\alpha$ , induced by chemical tumor promoters, activates nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) in cancer cells, and that inhibitors of NF- $\kappa\text{B}$  activation are assumed to be cancer preventive agents. We demonstrated that treatment of EGCG and other tea polyphenols dose-dependently inhibited TNF- $\alpha$  release from human stomach cancer cell line KATO III treated with 50 nM okadaic acid, and, further, that EGCG clearly inhibited NF- $\kappa\text{B}$  activation in BALB/3T3 cells treated with 0.2  $\mu\text{M}$  okadaic acid.<sup>21)</sup> These results suggest that tea polyphenols reduce the levels of TNF- $\alpha$  and similar cytokines in tumors.

We reported that it is quantitatively easier to measure inhibition of TNF- $\alpha$  release from cells treated with a tumor promoter – e. g., okadaic acid – than to measure inhibition of both TNF- $\alpha$  gene expression and NF- $\kappa$ B activation.<sup>21)</sup> The IC<sub>50</sub> values for 50% inhibition of TNF- $\alpha$  release from KATO III cells were 26  $\mu$ M for ECG, 48  $\mu$ M for EGCG, 115  $\mu$ M for a mixture of teaflavin derived from black tea, 210  $\mu$ M for EGC and >500  $\mu$ M for EC.<sup>21)</sup> These results correlated well with those for cell growth inhibition reported earlier.<sup>14)</sup>

Since we initially found that EGCG resulted in inhibition of tumor promotion, we were interested in the mechanisms of action of tea polyphenols from the standpoints of antipromotion. We proposed as a possible mechanism the sealing effect of EGCG, based on our evidence that a membrane fraction of mouse skin treated with a single topical application of EGCG showed immediate reduction of specific binding of both <sup>3</sup>H-TPA and <sup>3</sup>H-okadaic acid to their receptors.<sup>7)</sup> This is well supported by evidence that EGCG inhibited activation of protein kinase C by TPA in the lipid bilayer membrane.<sup>22)</sup>

Many scientists have also reported that EGCG and other tea polyphenols inhibited mutagenicity of 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole (N-OH-Trp-P-2)<sup>23)</sup> and microbial growth,<sup>24)</sup> and that they up- or down-regulated expression of inflammatory cytokine genes related to carcinogenesis.<sup>8),9)</sup> Although tea polyphenols are antioxidants, we now believe that they are multifunctional and thus are completely different from enzyme inhibitors, which have one specific function.

In addition to tea polyphenols, tea contains another active compound, caffeine. Conney's group demonstrated that decaffeinated teas were inactive or less effective inhibitors of tumor formation,<sup>25)</sup> and they recently reported that administration of caffeine alone also had a strong inhibitory effect on tumorigenesis in high-risk mice.<sup>26)</sup>

**Synergistic effects of EGCG with sulindac.** We reported that EGCG with the cancer preventive agents, sulindac and tamoxifen, synergistically and additively enhanced apoptosis of PC-9 cells, quantitatively measuring apoptosis by DNA fragmentation.<sup>16)</sup> Specifically, 75  $\mu$ M EGCG with 10  $\mu$ M sulindac induced apoptosis 17 times higher than EGCG or sulindac alone did.<sup>16)</sup> We assume that the synergistic effects of EGCG with sulindac are not directly related to inhibition of cyclooxygenase-2, because an inactive derivative of sulindac did also show the synergistic effects with EGCG.

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

Groups	Average no. of tumors/Mouse	
Non-treated	72.3 $\pm$ 28.3	(100.0%)
Green tea extract	56.7 $\pm$ 3.5	(78.4%)
Sulindac	49.0 $\pm$ 12.7	(67.7%)
Green tea extract + sulindac	32.0 $\pm$ 18.7	(44.3%)*

\*Statistically significant ( $P < 0.05$ ).

To confirm the synergistic effects of EGCG with sulindac, we examined inhibition of tumor formation in multiple intestinal neoplasia (Min) mice. Male C57BL/6J-Min/<sup>+</sup> (Min) mice, which have a germline mutation of the murine *adenomatous polyposis coli* (*Apc*) gene, develop intestinal tumors similar to those of familial adenomatous polyposis (FAP) patients. Min mice from 6 weeks of age were fed either a powdered CE-2 diet or CE-2 containing 0.03% sulindac, and the mice were more also given drinking water with or without 0.1% green tea extract. At 16 weeks of age, the average number of tumors in the non-treated control Min mice group was 72.3 tumors per mouse (Table III).<sup>27)</sup> Treatment with both green tea extract and sulindac significantly reduced the number of tumors to 32.0 tumors per mouse, a decrease of 55.7%,<sup>27)</sup> and treatment with the combination also resulted in significantly smaller tumors than in any of the other groups. Although the reduction in the average number of tumors in this animal experiment was not synergistic, but additive, optimal doses of green tea extract and sulindac will show synergistic effects in animal experiments, as has been shown in PC-9 cells.<sup>16)</sup>

Since cancer preventive agents like sulindac and tamoxifen are known to have adverse effects on humans, we assume that drinking green tea reduces the adverse effects. Thus, we must examine the possibility of non-toxic, combination cancer chemoprevention with green tea, a combination that will lead to truly effective cancer prevention.<sup>28)</sup>

**Molecular effects of EGCG on gene expression.** To investigate modulation of gene expression by EGCG, we used CLONTECH's Atlas<sup>TM</sup> cDNA Expression Array, which can deal with 588 genes. The levels of gene expression were studied in one human lung cancer cell line, PC-9 cells. The cells were processed in one of four ways: some PC-9 cells were treated with 200  $\mu$ M EGCG alone for 7 h; some were treated with 0.1  $\mu$ M of the tumor promoter okadaic acid alone for 6 h; others were pretreated with 200  $\mu$ M

Table IV. Effects of cotreatment with EGCG and sulindac on gene expression

Genes	Relative expression level compared with control (fold)		
	EGCG	Sulindac	EGCG +sulindac
Up-regulated			
<i>GADD153</i>	0.87	0.78	11.61
<i>WAF 1</i>	1.02	1.43	2.97
Down-regulated			
<i>T-plasminogen activator</i>	1.87	0.64	0.05
<i>TIMP3</i>	0.77	0.74	0.29
<i>IL-1<math>\beta</math></i>	0.78	0.95	0.30
<i>Integrin <math>\beta 4</math></i>	0.87	0.89	0.34

EGCG for 1 h followed by further treatment with 0.1  $\mu$ M okadaic acid for 6 h; and non-treated cells were used for control.<sup>29)</sup> Looking at the expression pattern of the genes in the four groups, we identified the genes commonly affected by EGCG: EGCG up-regulated expression of one gene, *retinoic acid receptor  $\alpha 1$*  (*RAR  $\alpha 1$* ) and down-regulated expression of four other genes, *NF- $\kappa$ B inducing kinase* (*NIK*), *death-associated protein kinase* (*DAPK 1*), *rho B* and *tyrosine protein kinase* (*SKY*).<sup>29)</sup> Since it is well known that *NIK* activates the *I $\kappa$ B* kinase  $\alpha$  (*IKK $\alpha$* ) - *IKK $\beta$*  complex, which then leads to activation of *NF- $\kappa$ B*, down-regulated expression of the *NIK* gene seems to be involved in growth inhibition of PC-9 cells. These results supported our previous evidence that treatment with EGCG inhibited both *TNF- $\alpha$*  gene expression regulated by *NF- $\kappa$ B* activation in the cells and *TNF- $\alpha$*  release from the cells.<sup>30)</sup>

Since EGCG significantly modulates the expression pattern of genes in PC-9 cells – and knowing that a combination of green tea extract with sulindac is effective for prevention of intestinal tumors – we paid special attention to the finding of a new modulation of gene expression in PC-9 cells after cotreatment with 200  $\mu$ M EGCG and 10  $\mu$ M sulindac.<sup>31)</sup> Comparing the expression patterns of the genes in PC-9 cells treated with EGCG alone, with sulindac alone, and with EGCG plus sulindac — and using non-treated cells as a control — we found that cotreatment newly induced up-regulated expression of *GADD153* and *WAF1* genes dramatically, about 12 times and 3 times, whereas those genes were not affected by treatment with either EGCG or sulindac alone.<sup>31)</sup> These results were well supported by evidences that overexpression of *GADD153* gene induces apoptosis of the cells and leads to antiproliferative effects,<sup>32)</sup> and that administration of green tea to mice enhanced UV-induced increase in the number of

p21(*WAF1/CIP1*)-positive cells and apoptotic sunburn cells in the epidermis.<sup>33)</sup> Since p21(*WAF1/CIP1*) is a potent inhibitor of cyclin-dependent kinase, the up-regulation of p21 seems to inhibit proliferation by blocking the cell cycle.<sup>34)</sup> Cotreatment also induced down-regulated expression of *T plasminogen activator*, *TIMP3*, *IL-1 $\beta$*  and *integrin  $\beta 4$*  genes, all less than 0.3 times (Table IV).<sup>31)</sup> This is the first evidence that cotreatment with EGCG plus sulindac induces new expression patterns in genes, patterns not observed with EGCG or sulindac alone. This suggests that the combination of green tea and a cancer preventive agent is beneficial for cancer prevention.

**Discussion.** Beverage and drug: Green tea is the most popular beverage in Japan. But it was once reported that tannin was a carcinogen.<sup>35)</sup> With the goal of developing green tea as a beverage associated with cancer preventive activity, we therefore studied animal experiments, pharmacokinetics, molecular mechanisms of action, and human epidemiology. Based on two reports on the preventive or nonpreventive effects of green tea against human cancers in the prospective cohort studies in Saitama Prefecture and northern Japan,<sup>36),37)</sup> we found the presence of the effective daily amount of green tea polyphenols for cancer prevention in humans. The amounts are assumed to be 10 Japanese-size cups of green tea per day, about 2.5 g green tea extract. Green tea has two facets as beverage and drug.

Lung cancer prevention: Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) was discovered as an early biomarker of human lung cancer by Tockman's group in 1988.<sup>38)</sup> We then found that our specific antibody for hnRNP B1, which has an additional 12 amino acids in *N*-terminal domain and hnRNP A2 lacks, increased the specificity for detection of early lung cancer.<sup>39)</sup> Furthermore, we recently demonstrated that

treatment with EGCG and ECG significantly inhibited the expression of hnRNP B1 protein dose-dependently in human lung cancer cell line A549.<sup>40)</sup> These results suggest that the lung is a reasonable and efficient target organ for human cancer prevention with green tea using monitoring by this new biomarker, hnRNP B1.

Long life with prevention of life-style related diseases: As we reported previously, TNF- $\alpha$  is induced by chemical and endogenous tumor promoters, and it subsequently induces the activation of NF- $\kappa$ B, which was recently reported to be directly associated with life-style related diseases.<sup>41)</sup> If this is indeed so, green tea would have preventive effects on both chronic inflammatory diseases and life-style related diseases.

**Conclusion.** That EGCG and green tea are now acknowledged cancer preventives in Japan makes it possible for us to establish the concept of cancer preventive beverage. The original definition of cancer chemoprevention by Sporn<sup>1)</sup> was adjusted to cancer prevention with green tea beverage. Our definition is: The administration of cancer preventives to delay the carcinogenic processes in humans, no matter when the carcinogenesis starts, thereby blocking the appearance of clinical symptoms.<sup>8)</sup>

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