Jpn. J. Cancer Res. 87, 1034–1038, October 1996

Inhibitory Effects of Tea Catechins, Black Tea Extract and Oolong Tea Extract on Hepatocarcinogenesis in Rat

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Inhibitory effects of individual tea catechins ((−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate), black tea extract and oolong tea extract on hepatocarcinogenesis were investigated. Male F344 rats received a single dose of diethylnitrosamine (200 mg/kg, i.p.), and thereafter phenobarbital (0.05%) was administered in the drinking water for a period of 6 weeks. Tea catechins, black tea extract or oolong tea extract were given during the entire experimental period, during only the initiation period or during only the promotion period. All four tea catechins, black tea extract and oolong tea extract (0.05 or 0.1%) significantly decreased the number and area of preneoplastic glutathione *S*-transferase placental form-positive foci in the liver. These results suggest that tea catechins, black tea extract and oolong tea extract have a chemopreventive action against hepatocarcinogenesis.

Key words: Tea catechin — Black tea extract — Oolong tea extract — Hepatocarcinogenesis — GST-P

Tea catechins are responsible for the astringent taste of green tea, and our laboratory has demonstrated that they have a variety of biological activities, including antibacterial,[1–3] antioxidative,[4,5] hypocholesterolemic[6] and antitumor[7] activities. There have been many reports that tea catechin and green tea extract inhibit carcinogenesis of various organs in rodent.[8–15] Some epidemiologic studies have suggested an inverse correlation between green tea consumption and cancer incidence.[16,17] Green tea leaves contain about 10% catechins, which have the following composition: (−)-epigallocatechin gallate (EGCg) 50%, (−)-epigallocatechin (EGC) 30%, (−)-epicatechin gallate (ECg) 10% and (−)-epicatechin (EC) 10%. In most of the above-mentioned studies the effects of a crude catechin mixture or EGCg, the main component of green tea catechin, were investigated. Little is known about the individual effects of EC, EGC and ECg on tumorigenesis.

There are only a few reports on the inhibitory effect of green tea on hepatic carcinogenesis. Klaunig has reported that green tea extract prevents the induction of hepatocyte lethality by oxygen free radicals and the induction of hepatocyte DNA synthesis[18]; and Nishida *et al.* have indicated that EGCg inhibits the growth of spontaneous hepatoma in mice and that of a human hepatoma cell line.[19] In the present study we report the effects of EC, EGC, ECg and EGCg on hepatocarcinogenesis, using glutathione *S*-transferase placental form (GST-P)-positive foci as a marker for preneoplasia.[20] We also investigated the effect on hepatocarcinogenesis of the polyphenolic fraction of black tea and that of oolong tea.

MATERIALS AND METHODS

Catechins, black tea extract and oolong tea extract EC, EGC, ECg and EGCg were prepared from crude catechins (90% catechin content) as described in our previous paper.[7] Polyphenolic fraction of black tea was prepared according to the following method. Black tea hot water extract (1 kg) was adsorbed on a Sepabeans HP-20 column (318 $\phi \times 730$ mm), then eluted with 10% (30 liters), 40% (40 liters), 60% (40 liters) and 70% (70 liters) methanol at a flow rate of 1 liter/min. Peaks in the eluted fractions were detected by absorbance measurement at 340 nm, and the fraction obtained from the 70% methanol eluate was washed with chloroform, concentrated and freeze-dried. This process was repeated twice to afford the polyphenolic fraction of 22.7 g. Polyphenolic fractions of oolong tea were prepared according to the following method. Oolong tea leaves were extracted with boiling water, and the extract obtained was washed with chloroform then further extracted with butanol. The components of these extracts are shown in Table I.

Animals and diet Male F344 rats were purchased from Charles River Japan, Inc., Atsugi. They were housed in plastic cages in an air-conditioned room at 23 ± 2°C and 60 ± 10% humidity. The basal diet used was MF (Oriental Yeast Co., Tokyo).

Experiment 1: In this experiment, individual catechins were fed throughout the whole experimental period. The experimental protocol is shown in Fig. 1. Six-week-old rats were divided into 9 groups of 6 to 12 animals per group. The rats in group 1 were fed a basal diet for 12

weeks. In week 4 the animals received an intraperitoneal injection of diethylnitrosamine (DEN, 200 mg/kg), and, starting from week 6, phenobarbital (PB, 0.05%) was administered in the drinking water for a period of 6 weeks. Groups 2–9 were given a diet containing 0.05 or 0.1% catechin (EC, EGC, ECg, or EGCg) in the basal diet for 12 weeks. All animals were killed at week 12, and the livers were removed.

Experiment 2: In this experiment, tea catechins were administered during the initiation or promotion stage in order to investigate at which stage of carcinogenesis tea catechins are effective. The experimental protocol is shown in Fig. 2. Eight-week-old rats were divided into 5 groups. Group 1 rats were maintained on a basal diet for 9 weeks. Groups 2 and 4 were fed a 0.1% ECg or 0.1% EGCg diet, respectively, for 2 weeks (initiation stage), and the rats in groups 3 and 5 were given the experimental diet from week 3 to the last week of the experiment (promotion stage). In week 1, all rats were treated once with DEN (200 mg/kg), and commencing from 2 weeks after this time, water containing PB (0.05%) was administered for a period of 6 weeks.

Experiment 3: The experimental protocol is shown in Fig. 2. Black tea extract (0.3%) or oolong tea extract (0.2%) was mixed in the feed so the catechin concentration was 0.1%.

Analytical method The excised livers were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Paraffin sections were immunohistochemically stained for GST-P by the method of Ito *et al.*[21] The numbers and areas of GST-P-positive foci in the livers were measured with an image analyzer, LUZEX-FS (NIRECO Co., Tokyo).

Statistical analysis was performed using Student's *t* test.

Table I. Theaflavin and Catechins Contents in Black Tea and Oolong Tea Extracts

	Black tea extract (%)	Oolong tea extract (%)
Theaflavin	15.7	—
EGCg	0.2	21.4
EGC	—	8.3
ECg	0.7	4.0
EC	—	3.2
GCg	0.1	2.0
Cg	0.7	—
Total catechins	1.7	38.9

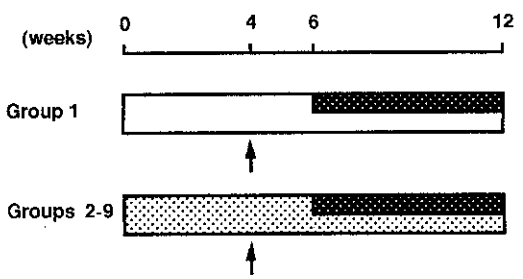

Fig. 1. Experimental protocol. □: basal diet, ▨: 0.05 or 0.1% catechin (EC, EGC, ECg or EGCg), ■: 0.05% phenobarbital in drinking water, ↑: diethylnitrosamine, i.p., 200 mg/kg.

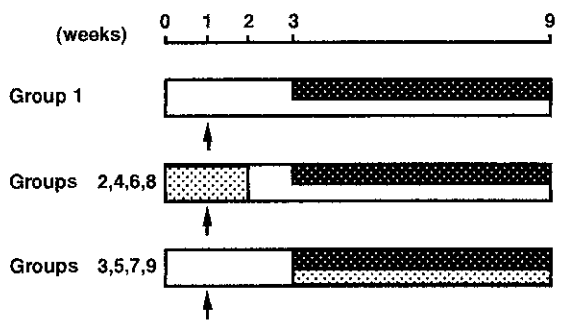

Fig. 2. Experimental protocol. □: basal diet, ▨: 0.1% catechins (ECg or EGCg), 0.3% black tea extract or 0.2% oolong tea extract, ■: 0.05% phenobarbital in drinking water, ↑: diethylnitrosamine, i.p., 200 mg/kg.

RESULTS

Experiment 1: In the 0.1% EC, 0.05% EGC and 0.05% ECg groups, food intake was significantly less than the control, but body weight was not significantly different from the control group (Table II). Tea catechins had no apparent toxic effect on the other tissues (stomach, small

Table II. Body Weight and Food Intake (Exp. 1)

Group	Treatment		No. of rats	BW (g)	Food intake (g/day)
1	basal diet		12	326.3±16.7	14.2±0.3
2	EC	0.05%	5	309.5±24.0	14.0±1.0
3	EC	0.1%	6	312.6±20.5	13.4±0.1***
4	EGC	0.05%	6	310.3±19.5	13.9±0.1*
5	EGC	0.1%	6	326.3±11.1	14.1±0.2
6	ECg	0.05%	5	305.6±17.9	13.5±0.5**
7	ECg	0.1%	6	316.6±0.8	14.1±0.2
8	EGCg	0.05%	6	327.4±8.8	13.9±0.4
9	EGCg	0.1%	9	319.4±10.7	14.1±0.7

Significantly different from control; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table III. Effect of Tea Catechins on GST-P-positive Foci Development in the Liver (Exp. 1)

Group	Treatment	No. of rats	No. (No./mm²)	Area (μm²/mm²)
1	basal diet	12	0.38±0.06	2622.2±422.4
2	EC 0.05%	5	0.29±0.07***	1739.0±410.7**
3	EC 0.1%	6	0.29±0.04***	1994.1±305.0**
4	EGC 0.05%	6	0.28±0.03***	1825.9±226.3***
5	EGC 0.1%	6	0.32±0.05***	1916.1±321.9**
6	ECg 0.05%	5	0.24±0.06***	1642.8±433.9***
7	ECg 0.1%	6	0.28±0.04***	1601.4±254.6***
8	EGCg 0.05%	6	0.32±0.04***	2459.7±337.2*
9	EGCg 0.1%	9	0.26±0.05***	2036.3±387.4**

Significantly different from control; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table IV. Body Weight and Food Intake (Exp. 2, 3)

Group	Treatment	No. of rats	BW (g)	Food intake (g/day)
1	basal diet	6	336.5±14.1	15.2±0.6
2	EGCg→basal diet	6	325.2±12.8	14.8±0.4
3	basal diet→EGCg	6	337.6±16.6	14.8±0.4
4	ECg→basal diet	6	326.5±15.8	15.2±0.1
5	basal diet→ECg	6	327.1±15.4	15.5±0.2
6	BT→basal diet	6	325.2±12.8	15.2±0.6
7	basal diet→BT	6	337.6±16.6	15.3±0.3
8	OT→basal diet	6	326.5±15.8	15.3±0.5
9	basal diet→OT	5	327.1±15.4	14.8±1.3

intestine, cecum, lung, spleen and kidney) as observed histologically.

The number of GST-P-positive foci in groups 2–9 showed a significant decrease as compared with the control group. Except for the 0.05% EGCg group, the area of foci/mm² in groups 2–9 was significantly smaller than that of the control group (Table III). ECg was the most effective at inhibiting both the number and area of foci.

Experiments 2 and 3: There were no significant differences in body weight or food intake between the control and the experimental groups administered ECg, EGCg, black tea or oolong tea extract (Table IV). The number and areas of GST-P-positive foci were decreased significantly in both the initiation and promotion stages with the addition of EGCg, ECg, and black tea and oolong tea extracts (Table V). ECg was more effective than EGCg, and the effects of black tea and oolong tea extracts were comparable.

DISCUSSION

In the present study we showed that tea catechins have inhibitory effects on hepatocarcinogenesis. Most studies

Table V. Effects of Tea Catechins, Black Tea (BT) Extract and Oolong Tea (OT) Extract on GST-P-positive Foci Development in the Liver (Exp. 2, 3)

Group	Treatment	No. of rats	No. (No./mm²)	Area (μm²/mm²)
1	basal diet	6	0.46±0.06	1844.6±240.8
2	EGCg→basal diet	6	0.37±0.07*	1360.2±241.7**
3	basal diet→EGCg	5	0.38±0.04*	1308.2±126.3***
4	ECg→basal diet	6	0.30±0.06***	1143.2±234.8***
5	basal diet→ECg	6	0.33±0.06**	1051.5±194.2***
6	BT→basal diet	6	0.33±0.02**	1179.6±86.2***
7	basal diet→BT	6	0.35±0.05**	1347.5±197.3**
8	OT→basal diet	5	0.35±0.07*	1246.3±247.2**
9	basal diet→OT	5	0.35±0.06*	1058.5±173.6***

Significantly different from control; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

investigating the effect of tea on carcinogenesis have used EGCg[12–14, 19] since it is the major component, accounting for 50% of the tea catechin fraction. The results of these studies indicate that the effect of tea catechins on tumorigenesis may be predominantly due to EGCg. However EC, EGC and ECg combined account for the remaining 50% of tea catechin fraction, and while it has been reported that these components have antioxidative[4] or enzyme-inhibiting activity,[22, 23] little is known about their effects on tumorigenesis. In the present report, not only EGCg but also EC, EGC and ECg significantly decreased the number and area of GST-P-positive foci in the liver. These results suggest that EC, EGC and ECg contribute significantly to the inhibitory effect of tea catechin fraction on carcinogenesis.

In the cases of EC and EGC, the level of 0.05% was more effective than that of 0.1% in Exp. 1. The reason why dose dependency was not observed is thought to be that 0.05% tea catechins is a sufficiently high level to decrease the number and area of GST-P-positive foci. In fact, there are reports that a low catechin dose ($\leq$ 0.05%) was as effective against carcinogenesis as 0.1% catechin,[13, 19] and other reports have shown that levels lower than 0.05% were even more effective.[11]

In Exp. 2 EGCg and ECg were administered during either the initiation or the promotion stage. With both ECg and EGCg, the number and area of GST-P-positive foci decreased in both the initiation and promotion stages. There are many reports that EGCg inihibits each stage in the process of carcinogenesis,[13, 14, 24] and the present results are in accordance with that conclusion. In Exp. 2, EC and EGC were not examined, but they can be expected to show similar effects.

The mechanism by which tea catechins inhibit hepatocarcinogenesis has not been clarified. However, there are several possible mechanisms. Firstly, it has been reported

that there is a correlation between formation of 8-hydroxyguanine, which is a major species of oxidative DNA damage, and GST-P-positive foci in rat liver.[25] Tea catechins have a potent antioxidative action[4,5] and it has been reported that green tea inhibited the formation of 8-hydroxyguanine.[12,26] Moreover, inhibitory effects of some antioxidants on hepatocarcinogenesis have been reported.[27,28] Thus, the present effect may be related to the antioxidative activity of tea catechins. Secondly, Hirose *et al.* have reported that green tea catechins reduced 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1)-induced development of GST-P-positive foci in the rat liver but did not influence dimethylnitrosamine-induced development of foci. They hypothesized that the inhibitory effect may involve the modification of phase I and II enzymes and suggested that tea catechin did not directly inhibit the growth of GST-P-positive foci.[27] Tea catechins have been reported to inhibit the catalytic activities of cytochrome P-450,[8,29] and to induce phase II detoxifying enzymes.[30] Taking into account these reports, the present finding that tea catechins inhibited the growth of a DEN- and PB-induced hepatic preneoplastic lesion may be related to the modification of metabolizing enzymes by tea catechins. The influence of tea catechin on hepatocarcinogenesis could be dependent on not one factor, but a combination of actions.

In order to understand further the effect of tea catechin on carcinogenesis, it is important to examine the metabolism of tea catechin. Recently we reported that EC, EGC, ECg and EGCg were detected in the portal vein after oral administration of each catechin.[31,32] This observation raises the possibility that each tea catechin acts on the liver to inhibit hepatocarcinogenesis.

In this study, we did not investigate the possibility of carcinogenesis by tea catechin. However, Hirose *et al.* have reported that treatment with green tea catechin after DEN initiation did not increase the number or area of GST-P-positive foci,[25] and thus it is thought that tea catechin has no promotional activity on rat hepatocarcinogenesis.

Black tea extract and oolong tea extract also decreased the number and area of GST-P-positive foci in both the initiation and promotion periods. Oolong tea extract contained 38.9% catechin, so the effect of oolong tea may be due to catechin. Black tea extract contained 1.8% catechin and 15.7% theaflavin. Theaflavin is one of the oxidized products of catechin, and is produced during the manufacture of black tea in the so-called 'fermentation' process. Recently, there have been several reports on the preventive action of black tea against cancer or mutagenesis.[8,9,33] However, few studies have investigated the effect of the polyphenolic fraction on carcinogenesis. The present results suggest that the polyphenolic fraction of black tea has a potent inhibitory effect on hepatocarcinogenesis, and theaflavin may also be an effective component.

The dose of catechin administered in this study corresponds to 2 to 3 cups of tea in humans. Since the present results suggest that tea catechin, black tea extract and oolong tea extract have a chemopreventive action, tea-drinking may be protective against human hepatocarcinogenesis.

(Received April 2, 1996/Accepted July 2, 1996)

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