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Inhibition by Green Tea Extract of Diethylnitrosamine-initiated but Not Choline-deficient, L-Amino Acid-defined Diet-associated Development of Putative Preneoplastic, Glutathione *S*-Transferase Placental Form-positive Lesions in Rat Liver

Kazutoshi Tamura, Dai Nakae, Kohsuke Horiguchi, Hiroyuki Akai, Yozo Kobayashi, Hiroshi Satoh, Toshifumi Tsujiuchi, Ayumi Denda and Yoichi Konishi[1]

Department of Oncological Pathology, Cancer Center, Nara Medical University, 840 Shijo-cho, Kashihara 634

The effects of green tea extract (GTE) on exogenous and endogenous models of rat liver carcinogenesis using diethylnitrosamine (DEN) and a choline-deficient, L-amino acid-defined (CDAA) diet were studied. For the exogenous carcinogenesis study, male Fischer 344 rats, 6 weeks old, were given a single intraperitoneal dose of 200 mg/kg body weight of DEN, partially hepatectomized at week 3, and administered GTE at doses of 0, 0.01 and 0.1% in the drinking water from week 2 for 10 weeks. For the endogenous carcinogenesis study, rats were fed the CDAA diet and simultaneously given GTE for 12 weeks. All rats were killed at the end of week 12. After DEN-initiation, the apparent numbers of glutathione *S*-transferase placental form-positive foci, assayed as putative preneoplastic lesions, were decreased by the administration of GTE, though their sizes were not altered. In contrast, GTE did not significantly reduce the numbers of the lesions induced by the CDAA diet or affect their sizes. While the levels of 8-hydroxyguanine, a parameter of oxidative DNA damage, were reduced by the GTE administration in both experimental models, GTE did not protect against the CDAA-diet-associated liver tissue damage in terms of either histology or plasma marker enzyme levels. We conclude that, while GTE may be a possible chemopreventive agent for nitrosamine-initiated hepatocarcinogenesis in the absence of chronic hepatocyte damage, it does not significantly inhibit lesion development in hepatocarcinogenesis associated with the CDAA diet, a cirrhosis-associated model.

Key words: Green tea extract — Diethylnitrosamine — Choline-deficient L-amino acid-defined diet — Rat liver carcinogenesis

Green tea is a very popular beverage in Japan, and is rich in polyphenols such as (−)-epigallocatechin gallate (EGCG), (−)-epicatechin and (−)-epicatechin gallate. In 1987, it was first demonstrated that topical applications of EGCG inhibited tumor promotion of mouse skin initiated with 7,12-diethylbenz[*a*]anthracene.[1] Since then, inhibitory effects in a variety of experimental carcinogenesis models of EGCG, a mixture of green tea polyphenols and green tea extract (GTE) have been reported.[2–11] Epidemiological studies have demonstrated that mortality rates from total cancers, including stomach cancer, are significantly lower in Shizuoka Prefecture, where green tea plants are extensively cultivated, than in the other prefectures in Japan.[12] Similarly, an inverse correlation between green tea consumption and lung cancer risk in Okinawa Prefecture, Japan, was reported.[13] With regard to liver carcinogenesis, however, only a few reports have appeared. Klaunig first described the reduction by GTE of bromodeoxyuridine-labeling indices in and outside of the preneoplastic foci induced in the livers of male $B_6C_3F_1$ mice initiated with diethylnitrosamine (DEN) followed by a phenobarbital-derived promotion, but did not mention whether GTE altered the development of such foci.[14] Nishida *et al.* reported that EGCG inhibits the development of spontaneous liver tumors in male C3H/HeNCrj mice.[15] Quite recently, Matsumoto *et al.* demonstrated the inhibitory effects of EGCG and other green tea polyphenols as well as black and oolong tea extracts on the development of putative preneoplastic, glutathione *S*-transferase (EC2.5.1.18) placental form (GST-P)-positive focal lesions in the livers of male Fischer 344 (F344) rats initiated with DEN and promoted by phenobarbital.[16] In the latter two studies, however, individual green tea polyphenol components were used in place of the more physiologically relevant GTE or, at least, a mixture of such polyphenols.[15,16] In the study of Matsumoto *et al.*, each green tea polyphenol was administered through dietary intake instead of in drinking water.[16] There have been no reports about the effects of GTE or green tea polyphenols on liver carcinogenesis induced by a regime other than DEN and phenobarbital.

In this context, the present study was conducted to investigate whether green tea polyphenols would be effective in different models of rat liver carcinogenesis when administered in a form extrapolatable to the human situ-

[1] To whom correspondences should be addressed.

ation (i.e., giving GTE via drinking water). We used two rat liver carcinogenesis models; one caused by the exogenous carcinogen DEN and the other due to endogenous changes in response to a choline-deficient, L-amino acid-defined (CDAA) diet. Development of GST-P-positive foci was used as the end-point marker because of its reliability and sensitivity.[17] DEN is a well-known liver carcinogen in rats, forming alkyl DNA adducts in the liver and inducing hepatocellular carcinomas without cirrhosis through the development of putative preneoplastic, enzyme-altered focal lesions.[18-21] The CDAA diet, not containing any known carcinogen, induces a high incidence of hepatocellular carcinomas with cirrhosis in rat liver when given chronically for 52 weeks,[22] again through the development of enzyme-altered focal lesions, but in this case, being accompanied by severe liver injuries.[23, 24] The formation of 8-hydroxyguanine (8-OHG), a representative form of oxidative DNA damage, in rat liver DNA has been demonstrated after DEN administration[25] and has been suggested to play an important role in the CDAA diet-associated liver carcinogenesis.[26-29]

MATERIALS AND METHODS

Animals A total of 106 male F344 rats, 5 weeks old, were purchased from Japan SLC Inc., Shizuoka. They were housed individually in stainless-steel, wire-mesh cages in an air-conditioned (10–15 air replacements/h) environment with a constant temperature (23±3°C) and relative humidity (50±20%) and a 12-h dark/light cycle. After a 1-week acclimation period on the basal diet (Oriental MF diet, Oriental Yeast Co., Ltd., Tokyo), the rats were divided into 10 groups each consisting of 10 or 11 rats for the experiments. The animals were allowed access to food and tap water *ad libitum* throughout the acclimation and experimental periods.

Chemicals and diet for the animal experiments DEN (Wako Pure Chemical Industries, Ltd., Kyoto) was diluted with 0.9% sodium chloride solution to the concentration of 200 mg/ml to give an injection volume of 1 ml/kg body weight. The CDAA diet and its control, a choline-supplemented, L-amino acid-defined (CSAA) diet, were obtained from Dyets Inc., Bethlehem, PA. GTE was kindly provided by Dr. Douglas A. Balentine, U.S. Tea Association, New York, NY, and dissolved in distilled water at concentrations of 0.01 and 0.1%. It was composed of EGCG (85%), (−)-epicatechin (10%) and (−)-epicatechin gallate (5%).

Animal treatments In the exogenous carcinogenesis study, the protocol was a slight modification of the medium-term liver bioassay model developed by Ito *et al.*[17] Groups 1, 2 and 3 received DEN at a single intraperitoneal dose of 200 mg/kg body weight, underwent two-thirds partial hepatectomy (PH) according to Higgins and Anderson[30] in week 3 and were given GTE in drinking water at concentrations of 0, 0.01 and 0.1%, respectively, from week 2 for 10 weeks. Groups 4 and 5 received the vehicle (0.9% sodium chloride solution, the diluent of DEN) in place of DEN, underwent PH on week 3 and were given GTE in drinking water at concentrations of 0 and 0.1%, respectively, from week 2 for 10 weeks. In the endogenous carcinogenesis study, groups 6, 7 and 8 received the CDAA diet and GTE simultaneously in drinking water at concentrations of 0, 0.01 and 0.1%, respectively, for 12 weeks. Groups 9 and 10 received the CSAA diet and simultaneously GTE in drinking water at concentrations of 0 and 0.1%, respectively, for 12 weeks.

All rats were weighed and killed under light ether anesthesia at the end of week 12, when the livers were excised and plasma samples were prepared from blood collected from the abdominal aorta. The livers were weighed, and 5-mm-thick slices were immediately taken from the three major lobes (cranial and caudal parts of right lateral lobe and caudate lobe in the exogenous study; left lateral, median and right lateral lobes in the endogenous study), fixed in ice-cold acetone and embedded in paraffin. Two serial 40-μm-thick sections were prepared from each fixed liver slice for histological examination after routine hematoxylin and eosin staining, and for the immunohistochemical demonstration of putative preneoplastic lesions. The remaining liver portions were quickly frozen under liquid nitrogen and stored at −80°C until use for the measurement of the 8-OHG levels. Body weight and food consumption were monitored weekly, and water intake was monitored twice a week throughout the experimental period.

Quantitative analysis of the development of the putative preneoplastic liver lesions The development of putative preneoplastic, enzyme-altered focal lesions in the livers was quantitatively analyzed utilizing their GST-P-positive phenotype. Anti-GST-P binding was demonstrated by the avidin-biotin-peroxidase complex method as previously described[31] using rabbit anti-rat-GST-P polyclonal antibody (Medical and Biological Laboratories Co., Aichi) and a Vectastain *Elite* ABC kit (rabbit IgG; Vector Laboratories, Inc., Burlingame, CA). The numbers and sizes of GST-P-positive foci were determined using an image analyzing system as described elsewhere[26] featuring the three-dimension correction procedure of Campbell *et al.*[32] Only the foci consisting of more than 10 altered hepatocytes were considered positive, since such foci are considered to result from the clonal expansion of the initiated cells and thus to have more preneoplastic potency and less reversibility than single-positive cells or foci of a few positive cells.

Measurement of the 8-OHG levels in liver DNA The levels of 8-OHG in genomic/nuclear DNA of viable cells

in the livers were individually measured as described elsewhere.[33] Briefly, portions of the livers weighing approximately 100 mg from groups 1–5, 9 and 10 and 200 mg for groups 6–8 were pulverized under liquid nitrogen. DNA was extracted using a Sepagene kit (Sanko Junyaku Co., Ltd., Tokyo)[33] and digested completely to nucleosides by combined treatment with nuclease P1 (EC 3.1.30.1, Yamasa Shoyu Co., Ltd., Chiba) and alkaline phosphatase (EC 3.1.3.1, Sigma Chemical Co., St. Louis, MO).[34] The levels of 8-OHG in the resultant samples were then quantitated using a high-performance liquid chromatography technique with electrochemical detection by an adaptation of the method of Kasai *et al.*,[35] as previously described.[29,36] The levels of 8-OHG were determined as numbers of 8-hydroxydeoxyguanosines (8-OHdGs) per 10^5 deoxyguanosines (dGs), by calibration against curves from runs of standard samples containing known amounts of authentic 8-OHdG (Wako) and dG (Sigma).

Measurement of plasma alanine aminotransferase (EC 2.6.1.2, ALT) activity The ALT activity in plasma samples was determined by means of an ultraviolet-assay method[37] using an automatic analyzer, Monarch Chemistry System (Instrumentation Laboratory Inc., Lexington, MA).

Statistical analysis To assess the statistical significance of inter-group differences in quantitative data, the Student-Newman-Keuls multiple comparisons test was performed after a one-way analysis of variance to determine the variations among the group means, followed by Barlett's test to determine the homology of variance.

RESULTS

General findings Data for initial and effective numbers of rats, final body and relative liver weights, food consumption, water intake and GTE intake are summarized in Table I. In the exogenous carcinogenesis study, 2 rats each from groups 1 and 2 and 1 rat from group 5 died due to a failure of PH. All other rats survived in good condition throughout the experimental period. There were no significant inter-group differences in final body weights, relative liver weights, food intake or water intake. Daily as well as total GTE intake increased in a dose-dependent manner. In the endogenous carcinogenesis study, all rats survived in good condition until the end of the experiment. Final body and relative liver weights of group 6 were significantly lighter and heavier than those of group 9, respectively. These values were not significantly different among groups 6–8 or between groups 9 and 10. There were no significant inter-group differences in food consumption or water intake. Daily as well as total GTE intake again increased in a dose-dependent manner.

Effects of GTE on the induction of GST-P-positive foci Data for the numbers and sizes of GST-P-positive foci are summarized in Table II. In the exogenous carcinogenesis study, foci were significantly induced in groups 1–3, but not in group 4 or group 5. The numbers of foci of groups 2 and 3 were significantly less than that of group 1. There were no inter-group differences among groups 1–3 in terms of the foci sizes. In the endogenous carcinogenesis study, foci were significantly induced in groups 6–8, but not in group 9 or group 10. There were

Table I. Experimental Details of Rats Given DEN or the CDAA Diet with or without GTE

Group	Treatment(s)	No. of rats		Final body weight (g)	Relative liver weight (g/100 g body weight)	Food consumption (g/rat/day)	Water intake (ml/day/rat)	GTE intake	
		Initial	Effective					Daily (mg/kg body weight)	Total (g/rat)
1	DEN	11	9	335±33[a]	2.41±0.12	12.5±5.3	16.7±8.6	0	0
2	DEN+GTE (0.01%)	11	9	323±28	2.40±0.14	13.3±3.8	17.7±5.9	7.1±0.1	0.10±0.03
3	DEN+GTE (0.1%)	11	11	318±26	2.48±0.15	13.9±3.5	21.6±6.5	83.0±15.5[c]	1.30±0.38[c]
4	vehicle	11	11	333±18	2.40±0.08	14.8±3.5	19.8±5.7	0	0
5	vehicle+GTE (0.1%)	11	10	319±26	2.36±0.11	14.8±2.2	22.7±4.1	77.0±10.0[d]	1.32±0.24[d]
6	CDAA	10	10	289±16[b]	4.78±0.18[b]	13.1±0.6	15.4±1.0	0	0
7	CDAA+GTE (0.01%)	10	10	296±19	4.75±0.16	13.5±0.7	15.3±2.1	5.8±1.5	0.13±0.02
8	CDAA+GTE (0.1%)	10	10	305±17	4.78±0.21	14.0±0.9	17.1±0.7	65.0±0.9[e]	1.44±0.06[e]
9	CSAA	10	10	334±30	2.26±0.11	13.5±1.1	15.0±2.4	0	0
10	CSAA+GTE (0.1%)	10	10	357±27	2.37±0.17	14.6±0.8	15.7±0.8	60.8±12.6[f]	1.32±0.11[f]

a) Numerical data are presented as the means±the standard deviations.
b) Significantly different from the group 9 value ($P<0.01$).
c) Significantly different from the values of groups 1 and 2 ($P<0.001$).
d) Significantly different from the group 4 value ($P<0.001$).
e) Significantly different from the values of groups 6 and 7 ($P<0.001$).
f) Significantly different from the group 9 value ($P<0.001$).

Table II. Numbers and Sizes of GST-P-positive Foci and 8-OHG Levels in the Livers and Plasma ALT Activities in Rats Given DEN or the CDAA Diet with or without GTE

Group	Treatment(s)	Effective no. of rats	GST-P-positive foci		8-OHG (8-OHdG/10^5 dG)	ALT (U/liter)
			No./cm^3	Mean volume (mm^3)		
1	DEN	9	1544±378[a, b]	0.23±0.09	5.96±0.56[d]	48.8±6.7
2	DEN+GTE (0.01%)	9	1083±473	0.30±0.13	5.07±0.35	47.2±3.2
3	DEN+GTE (0.1%)	11	1044±128	0.28±0.11	5.32±0.33	47.0±6.8
4	vehicle	11	0	—	2.72±0.31	57.8±12.8
5	vehicle+GTE (0.1%)	10	0	—	2.43±0.31	48.6±5.7
6	CDAA	10	421±125[c]	6.37±2.25	9.28±1.77[e]	204.9±33.5[c]
7	CDAA+GTE (0.01%)	10	371±105	4.71±4.41	8.58±0.71[f]	232.8±17.9
8	CDAA+GTE (0.1%)	10	359±159	5.88±3.89	7.83±0.36	231.6±52.1
9	CSAA	10	0	—	2.64±0.40	36.6±3.5
10	CSAA+GTE (0.1%)	10	0	—	2.48±0.56	38.4±2.9

a) Numerical data are presented as the means±the standard deviations.
b) Significantly different from the values of groups 2, 3 and 4 ($P<0.001$).
c) Significantly different from the group 9 value ($P<0.001$).
d) Significantly different from the values of groups 2, 3 ($P<0.05$) and 4 ($P<0.001$).
e) Significantly different from the values of groups 7 ($P<0.05$), 8 and 9 ($P<0.001$).
f) Significantly different from the group 8 value ($P<0.05$).

no significant inter-group differences among groups 6–8 in terms of the foci numbers or sizes.

Effects of GTE on the levels of 8-OHG in liver DNA Estimated levels of 8-OHG in liver DNA are summarized in Table II. In the exogenous study, the 8-OHG level was significantly elevated in group 1. The group 2 and 3 values were significantly lower than the group 1 value. In the endogenous study, the 8-OHG level was significantly elevated in group 6. The group 7 and 8 values were significantly lower than the group 6 value, while the group 8 value was significantly lower than the group 7 value.

Effects of GTE on pathological changes and plasma ALT activity In the exogenous carcinogenesis study, no particular changes were found either macroscopically or microscopically, with the exception of the development of altered hepatocyte focal lesions positive for anti-GST-P binding in groups 1–3. There were no inter-group differences among groups 1–5 in terms of plasma ALT activity (Table II). In the endogenous carcinogenesis model, the liver was enlarged, with white-yellowish, multi-nodular surfaces, in groups 6–8. Histologically, hepatocellular fat accumulation, hepatocyte single cell necrosis and connective tissue deposition were apparent in groups 6–8. Since these findings were in accordance with our earlier reports,[23, 24] the data are not shown here. There were no obvious qualitative differences among groups 6–8 in terms of the above-mentioned macroscopic or microscopic changes. No particular pathological changes were found in group 9 or group 10. Plasma ALT activity was extensively elevated in groups 6–8, and those in groups 7 and 8 were not significantly different from the group 6 value (Table II).

DISCUSSION

The present results clearly indicate that GTE can exert inhibitory effects on exogenous rat liver carcinogenesis caused by DEN, but without dose-dependency. Though the reason for this lack of dose-dependency is uncertain, the lower dose (0.01%) might have already exerted the maximum effect under the present conditions, making the higher dose (0.1%) unable to exert a greater effect. Although the present results seem to be in accordance with the recent report by Matsumoto *et al.*, they claimed that the polyphenols reduced not only the numbers, but also the sizes of the GST-P-positive foci.[16] In contrast, GTE reduced the numbers of the foci, but not their sizes in the present study. There are obvious differences between their and our experimental conditions such as, respectively, the administration of the individual green tea polyphenol in diet and of GTE in drinking water, and the presence and absence of the phenobarbital-derived promotion regimen. The last factor may be particularly important since the inhibitory effects of GTE or the green tea polyphenol(s) on chemically induced promotion are suggested to be mainly due to interference with the actions of the promoters.[38] Furthermore, Matsumoto *et al.* used the ratio of the combined area of the GST-P-positive foci to the area of the liver specimen as a parameter for the lesion size instead of the average area of the foci.[16] Such a ratio, however, does not directly

reflect the sizes of the foci when the numbers of the foci are altered. Their data, therefore, may not be sufficient to permit the conclusion that the green tea polyphenols reduced the sizes of the foci.

The mechanisms by which GTE reduced the numbers of GST-P-positive foci in the livers of rats initiated with DEN remain obscure. The antioxidative activities of GTE or the green tea polyphenols are well-known.[39,40] We ourselves verified a sufficient antioxidative activity of the GTE used here against rat liver microsomal auto-oxidation by hydrogen peroxide in the presence of ferric chloride and sodium ascorbate (data not shown). In fact, GTE reduced the 8-OHG levels in liver DNA of rats initiated with DEN. This is in line with earlier reports on mouse lung carcinogenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone[41] and rat colon carcinogenesis by 1,2-dimethylhydrazine.[42] Nevertheless, it is doubtful whether the antioxidative action can account for the inhibition by GTE, since GTE was administered from 2 weeks after DEN administration and thus could not affect the DEN-induced formation of 8-OHG. One might still argue that GTE enhanced the repair of 8-OHG or related mutations and exerted inhibitory effects in the post-initiation phase, but no evidence is available concerning the effects of GTE or the green tea polyphenols on the repair system(s) for 8-OHG, though tea catechins are known to induce antioxidant and phase II detoxifying enzymes.[43] Furthermore, since no particular oxidative stimuli were introduced after the DEN administration, it is also unlikely that GTE inhibited oxidative subcellular damage other than the 8-OHG formation. Since GTE and EGCG were shown to inhibit DNA synthesis and proliferation of hepatoma and erythroleukemia cells,[44] GTE may inhibit the PH-accelerated proliferation of the DEN-initiated cell populations. This is, however, still insufficient to explain the inhibitory effects of GTE in view of the failure to reduce the sizes of the GST-P-positive foci. The possible involvement of other mechanisms, such as the sealing of membranes to interrupt the interaction of various growth factors, hormones and/or cytokines with their receptors in membranes,[38] should be considered.

In contrast, GTE did not significantly inhibit the development of the GST-P-positive foci induced by chronic feeding of the CDAA diet. In this case, oxidative stress existed as long as the CDAA diet was fed, resulting in the progressive accumulation of 8-OHG, which has been suggested to play important roles in the CDAA diet-associated development of GST-P-positive foci.[23,24,26–29] The levels of 8-OHG were reduced by the GTE administration, and thus GTE had been expected to inhibit the foci development. The one clear difference between the exogenous and endogenous rat liver carcinogenesis models in the present study was the absence and presence of severe liver injury, resulting in cirrhosis,[22–24] respectively. It is thus conceivable that this severe injury in the livers of rats chronically fed the CDAA diet may interfere with the anti-carcinogenic effects of GTE, even though some antioxidative effects were exerted. In the other words, GTE may not be able to exert sufficient anti-carcinogenic effects in the presence of severe tissue injury.

In the present study, the intake of GTE was 5.8–7.1 and 65–83 mg/kg body weight/day/rat, when GTE was administered at 0.01 and 0.1% in drinking water, corresponding to 0.340–0.426 and 3.9–5.0 g/day for humans weighing 60 kg, respectively. Chemopreventive effects of EGCG was reported to require at least 1 g/day for a human,[45] an amount corresponding to about 10 cups of green tea per day.[38] Among patients with greater consumption of green tea, 10 or more cups of green tea per day was reported to decrease the risk of gastric cancer.[46] The presently used doses of GTE were thus meaningful for extrapolation to the human situation. The present results indicate that GTE may be a possible chemopreventive agent for hepatocarcinogenesis in the absence of chronic hepatocyte damage, but that it might have little potential to inhibit tumor development in cirrhotic liver. Epidemiological studies of representative human cases should clarify whether the present results can indeed be extrapolated to man.

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