

Prevention of dual promoting effects of pentachlorophenol, an environmental pollutant, on diethylnitrosamine-induced hepato- and cholangiocarcinogenesis in mice by green tea infusion

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In order to explore a possibility that the custom of drinking green tea infusion is efficacious for reducing the carcinogenic risk of environmental exposure to pentachlorophenol (PCP), we examined the effects in a hepato- and cholangiocarcinogenesis model in mice exposed to diethylnitrosamine (DEN). In the first experiment, groups of 15 male mice were initially treated with DEN at a dose of 20 p.p.m. in the drinking water for the first 8 weeks followed by a 4 week recovery interval by PCP at concentrations of 0 (basal diet), 300 or 600 p.p.m. in the diet for 23 weeks. Further groups of animals were treated with DEN and PCP in the same manner and received 2% green tea infusion (GT) instead of the drinking water from week 10 until death. PCP exposure at the high dose promoted DEN-induced hepatocarcinogenesis, and also caused progression of cystic hyperplasias of the intrahepatic bile ducts to cholangiocellular tumors. Co-administration of GT was able to prevent the increases of incidences and multiplicities of DEN-induced hepatocellular tumors and also arrest the progression of cholangiocellular tumors. In the second experiment, co-treatment with GT in the drinking water from 1 week before 300 or 600 p.p.m. PCP treatment in the diet to the end of the experiment at week 3 in B6C3F1 male mice suppressed increases of serum ALT activities, 8-oxodeoxyguanosine levels in liver DNA and bromodeoxyuridine labeling indices of hepatocytes and intrahepatic biliary epithelial cells induced by PCP. These findings suggest that regular intake of green tea may reduce the carcinogenic risk posed by an environmental pollutant, PCP, presumably due to effects on oxidative stress.

Introduction

Pentachlorophenol (PCP) has been widely used as a wood preservative, herbicide and insecticide all over the world (1). This has resulted in the ubiquitous environmental pollution and the hazard risk of human exposure is now a matter of concern (2). Especially in Japan where PCP was applied as

Abbreviations: ALT, serum alanine aminotransferase; BrdU, bromodeoxyuridine; DEN, diethylnitrosamine; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; GT, green tea infusion; LI, labeling index; 8-oxodG, 8-oxodeoxyguanosine; PCP, pentachlorophenol.

a paddy herbicide in the 1960s, there is serious pollution of soil and ground water (3). As PCP can be converted to polychlorinated dibenzo-*p*-dioxins by photolysis (4), it is one of the major sources responsible for Japanese dioxin pollution (5). PCP itself has the potential to induce hepatocellular carcinomas in the mouse liver (6) and we have reported promoting effects on diethylnitrosamine (DEN)-induced hepatocarcinogenesis in mice, probably involving oxidative stress generated in redox cycling through its metabolism (7,8). Recently, it has also been found that PCP causes progression of cystic lesions of intrahepatic biliary epithelia initiated by DEN to cholangiocellular tumors (9). The overall data suggest that PCP exerts promoting actions on both hepato- and cholangiocarcinogenesis, so that it could be hazard to humans. Results obtained with an interspecies extrapolation approach also indicated PCP to pose a possible public health hazard (10).

Components of green tea, one of the most popular beverages in Japan, have been demonstrated to exert preventive effects on carcinogenesis in various organs in rodents (11–13). These include (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG) and (–)-epigallocatechin gallate (EGCG), thought to play crucial roles in its chemopreventive potential (14). In addition to antioxidant activities, various biological actions including inhibition of metabolic enzymes (15) and induction of detoxifying enzymes are considered to contribute to the antitumorigenic action of green tea (16). In fact, epidemiological data have demonstrated a positive association between green tea consumption and delayed onset of cancer (17). Thus, regular drinking of green tea might be expected to be efficacious against carcinogenic effects due to environmental contaminants.

In order to assess the possibility that daily intake of green tea reduced the carcinogenic risk of PCP, the present study was conducted using a DEN-initiated carcinogenesis model focusing on hepatocyte and intrahepatic biliary epithelium of mice. In addition, the effects of co-treatment with green tea on PCP-induced increases in serum alanine aminotransferase (ALT) activity, 8-oxodeoxyguanosine (8-oxodG) levels in liver DNA and the bromodeoxyuridine (BrdU)-labeling index (LI) for hepatocytes and intrahepatic biliary epithelial cells were assessed.

Materials and methods

Chemicals

PCP (purity 98.6%) and DEN were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alkaline phosphatase and BrdU were obtained from Sigma Chemical (St Louis, MO) and nuclease P1 was from Yamasa Shoyu Co. (Chiba, Japan).

Preparation of green tea infusion

Medium grade green tea produced in Schizuoka, Japan, was obtained commercially and stored in a sealed plastic bag at 4°C before use. A 2% green tea infusion (GT) was prepared every 3 days by adding 750 ml boiling water to 15 g green tea leaves, followed by filtration and centrifugation at 3000 r.p.m. after

allowing the mixture to stand at room temperature for 30 min, as described previously (18). Sealed bottles of GT were given to mice as their only source of drinking water. In order to confirm the stabilities of the major catechins in GT used in the present study, we measured EGC, EGCG, EC and ECG in GT in sealed bottles 1, 4 and 7 days after placement in the animal facilities using a HPLC system with UV detection (280 nm) as described previously (18). The results are as follows (in µg/ml): 1 day, EGC 454, EGCG 847, EC 190, ECG 136; 4 days EGC 435, EGCG 847, EC 209, ECG 136; 7 days, EGC 472, EGCG 843, EC 190, ECG 150. Exchange for new GT bottles was every 3 days.

Animals

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old male B6C3F1 mice (specific pathogen-free) were purchased from Japan SLC (Shizuoka, Japan) and kept under quarantine for 1 week before starting the experiments. The animals were housed in plastic cages, 5 mice/cage, and maintained under controlled conditions of temperature (23 ± 2°C), and relative humidity (55 ± 5%), with a 12 h light/dark cycle.

Animal treatments

Experiment I. One hundred animals were randomly divided into seven groups, as shown in Figure 1. Fifteen animals each in groups 2–7 were given DEN as an initiator at 20 p.p.m. in the drinking water for 8 weeks. After a 4-week recovery period, those in groups 2 and 5, 3 and 6, and 4 and 7 received PCP at concentrations of 0, 300 and 600 p.p.m., respectively, in the diet for 23 weeks. The doses of PCP used in the present study were earlier demonstrated to exert the promoting effects on DEN-induced hepatocarcinogenesis (8). Additionally, animals in groups 5–7 were given GT in sealed bottles as their drinking water from 2 weeks before the start of the PCP treatment until the end of the experiment. Ten animals were maintained untreated during the experimental period as a control group (group 1). The actual doses of PCP in groups 3, 4, 6 and 7 were calculated to be 1.2, 2.2, 1.2 and 2.5 mg/mouse/day, respectively, and the actual calculated intakes of GT in groups 5–7 were 5.6, 4.6 and 5.3 g/mouse/day, respectively. At necropsy, mice were killed at week 35 under ethyl ether anesthesia and the livers were immediately removed and slices taken from each lobe were fixed in buffered formalin. Using 4 µm sections stained with hematoxylin and eosin (H&E), liver lesions were diagnosed as hepatocellular adenomas and carcinomas, and cystic hyperplasias, cystic atypical hyperplasias, cholangiomas and cholangiocarcinomas, as described previously (8,9).

Experiment II. Thirty animals were randomly divided into six groups, 1–3 being given PCP at doses of 0, 300 and 600 p.p.m. in the diet for 2 weeks. Groups 4–6 received PCP in the same manner but also were administered GT in the drinking water from 1 week before PCP exposure until the end of the experiment. The actual doses of PCP in groups 2, 3, 5 and 6 were calculated to be 1.3, 2.2, 1.2 and 2.6 mg/mouse/day, respectively. The actual intakes of GT in groups 4–6 were calculated to be 4.0, 3.2 and 3.7 g/mouse/day, respectively. BrdU (20 mg/kg) was given by i.p. injection twice a day for the final 2 days of the experiment and once on the day of termination 2 h before death, as described previously (19). Animals were killed under ethyl ether anesthesia, blood was collected from the orbital venous plexus and the serum obtained was frozen at –80°C until measurement of ALT. Then the livers were immediately removed and weighed, and slices taken from each lobe were fixed in buffered formalin for BrdU immunostaining. The remaining pieces for the livers were

frozen with liquid nitrogen and stored at –80°C until measurement of 8-oxodG in nuclear DNA.

Serum biochemistry

Determination of serum ALT activity was carried out with a Hitachi Automatic Analyzer 7150 (Tokyo, Japan) using commercially available kits.

Measurement of nuclear 8-oxodG

Nuclear fractions were obtained by centrifugation at 1000 g from 0.3 g samples of liver tissue after homogenization in 2.7 ml of 20 mM EDTA, 5 mM phosphate buffer (pH 7.5). DNA extraction was performed with a Nucleic Acid Purification System (Model 341, Applied Biosystem, Foster City, CA). The nuclear fractions were lysed with proteinase K and lysis buffer and DNA was extracted with phenol/water/chloroform reagent and then precipitated with isopropanol. All reagents for this process were supplied by Applied Biosystems. The DNA extraction procedures were automatically conducted in glass vessels filled with helium gas and in the dark in order to reduce auto-oxidation of dG. The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase. 8-OxodG levels (8-oxodG/10⁵ deoxyguanosine) were assessed by HPLC with an electrochemical detection system (HPLC pump 420, Kontron AG, Zurich Inst., Switzerland; Coulochem Model-5100A, ESA, Bedford, MA).

Immunohistochemical procedures

For immunohistochemical staining of BrdU, after first denaturing DNA with 4 N HCl, the sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (Becton Dickinson, CA) (1:100), biotin-labeled horse anti-mouse IgG (1:400), and avidin–biotin–peroxidase complex. The sites of peroxidase binding were demonstrated by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical). Immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

Quantification of proliferation of hepatocytes and intrahepatic biliary epithelial cells

For each animal at least 3000 hepatocytes and 1000 bile duct epithelial cells were counted. The LI was calculated as the percentage of cells positive for BrdU incorporation.

Statistics

The significance of differences in the results for relative liver weights, serum ALT activities, 8-oxodG levels and LIs was evaluated with the use of ANOVA, followed by Dunnett's multiple comparison test. Regarding the initiation–promotion bioassay, the significance of differences in the results for the multiplicity of lesions was evaluated using the Student's *t*-test and that for the incidence with the Fisher's exact probability test.

Results

Experiment I

All animals survived until the termination of the tumor-prevention bioassay at 35 weeks. The final body weights in groups 1–7 were 40.6 ± 2.2 (distilled water/basal diet), 39.1 ± 2.7 (DEN/BD), 37.6 ± 2.6 (DEN/PCP-L), 33.5 ± 4.0 (DEN/PCP-H), 34.7 ± 2.1 (DEN/GT), 38.2 ± 3.3 (DEN/PCP-L/GT) and 36.0 ± 2.0 g (DEN/PCP-H/GT), respectively. Values for mice given GT following DEN treatment were significantly decreased but reduction was not observed in the other two GT-treated groups. On the contrary, although PCP treatment was associated with a tendency for decrease, co-treatment with GT prevented the reduction.

The incidences and multiplicities of hepatocellular lesions in mice exposed to PCP with or without GT in the two-stage model are given in Table I. PCP exposure for 23 weeks in the diet following DEN initiation increased the incidences and multiplicities dose-dependently, statistical significance being attained with the highest dose. Co-treatment with GT significantly suppressed the promotion effects.

Incidences of proliferative lesions in intrahepatic bile ducts are summarized in Table II. As reported previously, PCP promoted development of cholangiocellular tumors from cystic atypical hyperplasias, the incidences of cholangiomas

No. of animals	0	8	10	12	35 weeks
10	DW				BD
15	DEN				BD
15	DEN				PCP (L)
15	DEN				PCP (H)
15	DEN		GT		GT+BD
15	DEN		GT		GT+PCP (L)
15	DEN		GT		GT+PCP (H)

Fig. 1. Protocol for Experiment I. DW, distilled water; BD, basal diet; DEN, 20 p.p.m. diethylnitrosamine in drinking water; PCP(L), 300 p.p.m. pentachlorophenol in diet; PCP(H), 600 p.p.m. pentachlorophenol in diet; GT, 2% green tea infusion in drinking water.

Table I. Preventive effects of GT on development of hepatocellular tumors in mice given PCP following DEN treatment

Treatment	Number of mice at risk	Number of mice with adenomas (%)	Number of mice with adenocarcinomas (%)	Average number of tumors per mouse
DW ^a /BD ^b	10	0 (0)	0 (0)	0
DEN ^c /BD	15	1 (6.7)	0 (0)	0.33 ± 1.29
DEN/PCP(L) ^d	15	4 (26.7)	1 (6.7)	0.47 ± 1.06
DEN/PCP(H) ^e	15	11 (73.3) ^h	3 (20.0)	2.00 ± 2.67 ^g
DEN/GT ^f -BD	15	4 (26.7)	2 (13.3)	0.53 ± 0.92
DEN/GT-PCP(L)	15	3 (20.0)	2 (13.3)	0.40 ± 0.63
DEN/GT-PCP(H)	15	5 (33.3) ⁱ	1 (6.7)	0.47 ± 0.74 ⁱ

^aDW: distilled water.^bBD: basal diet.^cDEN: 20 p.p.m. in drinking water.^dPCP(L): 300 p.p.m. in diet.^ePCP(H): 600 p.p.m. in diet.^fGT: 2% green tea in drinking water.^g*P* < 0.05 versus DEN/BD.^h*P* < 0.01 versus DEN/BD.ⁱ*P* < 0.05 versus DEN/PCP(H).**Table II.** Preventive effects of GT on development of proliferative lesions in the intrahepatic bile ducts of mice given PCP following DEN treatment

Treatment	Number of mice at risk	Number of mice with			
		Cystic hyperplasias	Cystic atypical hyperplasias	Cholangiomas	Cholangiocarcinomas
DW ^a /BD ^b	10	0 (0) ^g	0 (0)	0 (0)	0 (0)
DEN ^c /BD	15	9 (60)	4 (27)	0 (0)	0 (0)
DEN/PCP(L) ^d	15	7 (47)	9 (60)	1 (7)	0 (0)
DEN/PCP(H) ^e	15	8 (53)	11 (73) ^h	9 (60) ⁱ	8 (53) ^j
DEN/ GT ^f -BD	15	7 (47)	2 (13)	0 (0)	0 (0)
DEN/GT-PCP(L)	15	9 (60)	8 (53)	0 (0)	0 (0)
DEN/GT-PCP(H)	15	5 (33)	10 (67)	3 (20) ^j	0 (0) ^k

^aDW: distilled water.^bBD: basal diet.^cDEN: 20 p.p.m. in drinking water.^dPCP(L): 300 p.p.m. in diet.^ePCP(H): 600 p.p.m. in diet.^fGT: 2% green tea in drinking water.^gIncidence (%): number of mice with the lesions per number of mice at risk.^h*P* < 0.05 versus DEN/BD.ⁱ*P* < 0.01 versus DEN/BD.^j*P* < 0.05 versus DEN/PCP(H).^k*P* < 0.01 versus DEN/PCP(H).

and cholangiocarcinomas in DEN-initiated mice exposed to the highest dose of PCP were significantly higher than those in mice treated with DEN alone. Although GT treatment failed to prevent the progression from cystic hyperplasia to atypical hyperplasia due to PCP exposure, cholangiocarcinomas did not occur in mice exposed to PCP along with GT treatment and the incidence of cholangiomas was significantly reduced by the co-treatment.

Experiment II

Changes in relative liver weights, serum ALT activity, 8-oxodG levels in liver nuclear DNA, and BrdU LIs for hepatocytes and intrahepatic biliary epithelial cells of mice exposed to PCP in the diet for 2 weeks together with GT administration in the drinking water are shown in Table III. All of the parameters examined in the present study were increased by PCP exposure in a dose-dependent manner, in line with our previous data (7). GT treatment effectively inhibited their rise except for the relative liver weights. In particular, 8-oxodG

levels, and BrdU LIs in hepatocytes and bile duct epithelial cells following PCP exposure at either dose were reduced by co-treatment with GT with statistical significance.

Discussion

While PCP has not shown mutagenicity in a number of *in vitro* assays (20), the major metabolite of PCP, tetrachlorohydroquinone (TCHQ) does cause micronuclei and mutations in V79 hamster cells (21,22). With reference to metabolism of PCP in mammalian livers it has been proposed that cytochrome P450 monooxygenase dechlorinates PCP to produce TCHQ and tetrachlorocatechol, which are subsequently oxidized to tetrachlorobenzoquinones via the respective semiquinone (23). In part, these benzoquinones might be reduced to hydroquinones by quinone reductase (24). Thus, redox cycling could give rise to reactive oxygen radicals, which could be a trigger for chains of events associated with oxidative stress (25–27). In fact, we have documented increases of 8-oxodG levels in mouse liver

Table III. Effects of GT on liver weights, serum ALT activity, 8-oxodG levels in liver DNA and LIs for epithelial liver cells of mice given PCP

Group	Number of animals	Relative liver weight (%)	Serum ALT activity (IU/ml)	8-oxodG level in liver DNA (/10 ⁵ dG)	Labeling index (%)	
					Hepatocytes	Intrahepatic biliary epithelial cells
BD ^a	5	4.61 ± 0.12	10.99 ± 1.53	0.40 ± 0.06	0.34 ± 0.04	1.98 ± 0.44
PCP(L) ^b	5	5.89 ± 0.53 ^c	18.93 ± 2.46 ^c	1.15 ± 0.07 ^c	1.45 ± 0.14 ^c	4.26 ± 0.43 ^c
PCP(H) ^c	5	5.89 ± 0.18 ^c	30.91 ± 5.66 ^c	1.55 ± 0.21 ^c	2.10 ± 0.27 ^c	6.15 ± 0.73 ^c
BD/GT ^d	5	4.66 ± 0.32	9.48 ± 2.90	0.57 ± 0.17	0.43 ± 0.04	1.57 ± 0.47
PCP(L)/GT	5	6.09 ± 0.29	19.00 ± 3.39	0.91 ± 0.08 ^e	0.69 ± 0.13 ^e	3.10 ± 0.83 ^f
PCP(H)/GT	5	6.48 ± 0.34 ^h	19.69 ± 1.40 ^h	1.02 ± 0.10 ^h	0.81 ± 0.23 ^h	3.39 ± 0.83 ^h

^aBD: basal diet.

^bPCP(L): PCP 300 p.p.m. in diet.

^cPCP(H): PCP 600 p.p.m. in diet.

^dGT: 2% green tea in drinking water.

^eP < 0.01 versus BD.

^fP < 0.05 versus PCP(L).

^gP < 0.01 versus PCP(L).

^hP < 0.01 versus PCP(H).

DNA caused by a carcinogenic dose of PCP (7,8), reconfirmed in the present study. Accumulation of data on the biological significance of 8-oxodG, including the existence of the specific repair system supports the participation of this adduct, reflecting oxidative stress, in PCP carcinogenesis (28). Likewise, we have demonstrated promoting potential of PCP for hepatocellular and cholangiocellular lesions initiated by DEN (9). Increases of cell proliferation following PCP exposure to mice were always accompanied with increases of 8-oxodG levels in liver DNA (7–9), so that both could play roles in promoting activity.

In the present study, administration of GT was able to prevent increases of 8-oxodG levels in liver DNA and BrdU LIs for hepatocytes and intrahepatic biliary epithelial cells of mice exposed to PCP, and exert effective inhibitory effects on PCP promotion of lesions development in both sites. It is well known that green tea is rich in polyphenols such as EC, ECG, EG and EGCG (14), considered to be active constituents for chemoprevention (29). In fact, frequent administration of green tea maintains a high level of EGCG in the mouse liver (30). Considering the recent report that green tea catechins act as electron donors to reactive oxygen species-induced radical sites on DNA (31), we can hypothesize that the prevention observed in the present study partly resulted from their radical scavenging. However, green tea catechins can also inhibit monooxygenase activity (15) as well as induce phase II detoxifying enzymes (16) and might therefore reduce the supply of redox cycle generating radicals from PCP metabolism and/or accelerate elimination of quinones.

PCP shows hepatotoxicity, causing cell necrosis, at carcinogenic doses (2,7), with dose-dependent increases of serum ALT activity being apparent in the present study. Because regenerative cell proliferation occurs following cell death, GT may have inhibited increases of BrdU LIs as a result of preventing hepatotoxicity. However, in spite of the fact that GT did not prevent elevation of serum ALT activity in mice exposed to PCP at the low dose, GT was able to reduce increases of the LIs for hepatocytes due to the PCP exposure. Taking account of the present data revealing effective prevention of 8-oxodG formation by GT, the proposal that there might be other pathways by which oxidative stress might directly increase cell proliferation (32,33) could be a clue for

the explanation. PCP induces hepatomegaly (2,7) and in the present study, GT did not block increases of relative liver weights in mice treated with PCP, which might suggest that the biological significance underlying the morphological alteration has no relation to its carcinogenesis.

In this study, the daily intake of EGCG, a major component, by the mice was calculated to be 102 mg/kg/day. According to a previous report (18), the average daily intake of EGCG by Japanese people is 5.3 mg/kg/day because they commonly drink four or five cups of green tea a day, which is customarily ~2% infusion and consequently contains ~400 µg EGCG/ml. It has been estimated that the average daily intake of PCP by the general population of the US is 16–19 µg/day (2,10). As average concentrations of PCP in human adipose tissue have been reported to be 17.4 µg/kg in the US (34) and 107.0 µg/kg in Japan (35), average daily intake of PCP by Japanese is presumably very much higher. However, compared with the 2.5 mg/day, average daily intake of PCP by the mice at high dose in the present study, it would be much lower. Although it is impossible to make a direct extrapolation from the present data to the human case, overall our findings suggest that normal intake of green tea might reduce the carcinogenic risk with environmental exposure to PCP in Japan.

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