Prevention of the down-regulation of gap junctional intercellular communication by green tea in the liver of mice fed pentachlorophenol

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Introduction

Gap junctions are channels connecting adjacent cells, composed of connexons, which are hexamers of connexins (Cx), in the plasma membrane. They play a role in cell–cell communication by exchanging ions and small molecules (<~1 kDa), including sugars, nucleotides, amino acids and second messengers such as Ca2+, cAMP and inositol triphosphate. Gap junctional intercellular communication (GJIC) is known to be important in maintaining tissue homeostasis, normal cell growth, differentiation and development (1). Amelioration of GJIC by toxicants is known to cause abnormal function and cause acute or chronic diseases in tissues and to affect tumor promotion, i.e. the clonal expansion of initiated cells, by inhibiting the suppressing effect of surrounding, normal gap junction coupled cells via epigenetic mechanisms (2–5). Since the discovery of GJIC inhibition by a tumor-promoting agent (6,7) and observation of the up-regulating effects of an anti-promoting agent (8), much evidence in vitro (2–4) and in vivo (10) has been reported supporting the hypothesis that GJIC inhibition is the cellular mechanism underlying tumor promotion and that prevention of the down-regulation of GJIC is critical for preventing tumor promotion. A recent investigation in mice deficient in Cx32, a major Cx molecule expressed in the liver, revealed evidence of their high susceptibility for hepatic tumorigenesis (9), emphasizing the validity of the hypothesis.

Recent research on the chemopreventive potential of green tea has demonstrated in a variety of carcinogenesis models that green tea and/or its components, such as (-)-epigallocatechin gallate (EGCG), are effective in preventing tumor development at each step of carcinogenesis, e.g. the initiation, promotion and progression phases (11). Multiple biological actions of green tea, which are assumed to contribute to its anti-tumorigenic action, have been suggested (12), such as anti-mutagenic activity (13) and epigenetic actions, including inhibition of enzymatic activities for ornithine decarboxylase (14) or metabolic enzymes (15), inhibition of cytokine release (16), induction of apoptosis (17) or induction of detoxifying enzymes (18). In vitro studies, as with cell culture, revealed the protective effects of green tea or its components on GJIC inhibition caused by oxidative stress-inducing toxicants and tumor promoters (19–21), suggesting that they have a significant action in maintaining GJIC. However, in vivo evidence is lacking for the protection afforded by drinking green tea against GJIC inhibition under conditions in which a tumor is developing.

Pentachlorophenol (PCP), which has been used as a wood preservative, is non-mutagenic in the Ames test but is known to induce hepatic tumors in mice (22). It has been speculated that oxidative stress produced during the oxidation of a mutagenic metabolite, tetrachlorohydroquinone (TCHQ), is a factor in its tumorigenic activity (23–30). We previously found that giving mice PCP induced oxidative damage in hepatic DNA (28). Associated with the oxidative stress, we found PCP caused hepatic cell proliferation even at non-hepatotoxic doses (29) and had tumor-promoting activity in a two-stage mouse hepatocarcinogenesis model (30). These observations suggest that the cell proliferation/tumor promoting action of PCP may not be a consequence of a mutagenic event due to a PCP metabolite, but of an epigenetic event due to a non-mutagenic compound. Subsequently, we observed that a
non-cytotoxic dose of PCP, but not the mutagenic metabolite TCHQ, inhibited GJIC in non-tumorigenic rat liver epithelial (WB-F344) cells expressing Cx43, suggesting that PCP was acting by an epigenetic action to bring about non-cytotoxic dose-induced cell proliferation and tumor-promoting activity (31). During our screening of active antioxidants for preventing tumor development, we had previously found that EGCG effectively inhibited oxidative stress in the mouse liver caused by PCP (28). In addition, in in vivo studies we observed that EGCG prevented the down-regulation of GJIC by PCP in rat liver epithelial WB-F344 cells (unpublished data), suggesting that the potential anti-promoting activity of green tea was through prevention of the down-regulation of GJIC by PCP in vivo. To further explore the protective action of green tea as an anti-promoter, we examined the effects of green tea on GJIC in mouse liver expressing Cx32 after tumorigenic doses of PCP.

The present study was aimed at examining whether drinking green tea might prevent GJIC inhibition in the liver of mice, as well as cell proliferation, after feeding them tumorigenic doses of PCP for 2 weeks. We used our modified method of in vivo GJIC assay, incision loading/dye transfer (IL/DT) and first confirmed that the administration of PCP at carcinogenic doses actually inhibited GJIC in the liver. Next, we examined if co-treatment with green tea might prevent PCP-induced down-regulation of GJIC and cell proliferation. We found that this was supported by our results. This study is the first evidence demonstrating the prevention by green tea of GJIC inhibition by a tumor promoter in an in vivo system.

Materials and methods

Chemicals

PCP (purity >99%) was purchased from Aldrich (Milwaukee, WI) and lucifer yellow (LY) and rhodamine–dextran (mol. wt 10 000) (RhD) were obtained from Molecular Probes (Eugene, OR). 5-Bromo-2'-deoxyuridine (BrdUrd) was purchased from Sigma Chemical Co. (St Louis, MO) and anti-monocononal Cx32 from Chemicon International (Temecula, CA). The osmotic pump, Alzet model 1003D, was purchased from Alza Corporation (Palo Alto, CA). The fluorescein–avidin D and kit for immunostaining were from Vector Laboratories (Burlingame, CA).

Preparation of green tea

Medium grade green tea produced in Shizuoka, Japan, was obtained commercially. Green tea infusion was prepared according to the procedure described previously (32). Brieﬂy, 2% w/v green tea leaves were brewed with boiled water for 30 min (15 g green tea leaf in 750 ml boiled water). Bottles of the green tea, covered with aluminum foil, were given to mice as their only source of drinking water. Concentrations of catechins in green tea were determined with an HPLC system by UV detection (280 nm) as described previously (32). The compositional data of major catechins in the green tea used in this study were as follows (µg/ml): EGCg, 822 ± 28; (−)–epigallocatechin, 429 ± 10; (−)–epicatechin, 184 ± 11; (−)–epicatechin gallate, 141 ± 8.1; (+)–catechin, 30 ± 3.7 (the values represent the means ± SD from three infusions of green tea prepared separately).

Animals and treatments

Five-week-old male B6C3F1 mice (specific pathogen-free; SLC, Shizuoka, Japan) were housed at 23 ± 1 °C under 55 ± 5% humidity and given an F2 powder basal diet (Funabashi Farm Co., Chiba, Japan) and tap water freely. They were used after 1 week acclimatization. Thirty mice were randomly divided into six groups, each of five mice. PCP was mixed in the basal powder diet at concentrations of 0, 300 or 600 p.p.m. and fed to the mice ad libitum for 2 weeks. The doses of PCP selected were demonstrated to cause tumor development in a two-stage mouse hepatocarcinogenesis model. Green tea was administered to half of the groups of mice treated with PCP as the only source of drinking water from 1 week before PCP treatment and throughout the experiment. BrdUrd was continuously administered to the mice s.c. using an osmotic pump for 3 days before they were killed under anesthesia. Part of the hepatic tissue was excised and immediately used for IL/DT assay; the remaining tissue was quickly frozen in liquid nitrogen for immunostaining for Cx32.

Incision loading/dye transfer (IL/DT) procedure

The idea of this procedure originated from the in vitro scrape loading/dye transfer method of El-Fouly et al. (33) and the in vivo cut loading/dye transfer in skin of Goliger and Paul (34). After excising the hepatic tissue, a part of the left lobe was put on a plastic plate, slightly rounded at the top to keep the dye mixture in the incisions, and covered with a wet gauze. The mixture of fluorescent dyes containing 0.5% LY and 0.5% RhD in phosphate-buffered saline was dropped onto the tissue surface. Three to four incisions (7–8 mm long) were made on the surface of each specimen with a sharp blade (1 mm depth). Additional dye mixture was put into the incisions and kept for 3 min at room temperature. After incubation, the tissue was washed with phosphate-buffered saline three times and fixed in 10% buffered formalin overnight. Sections were quickly washed with water and then routinely processed for embedding in paraffin. Sections (5 mm) for GJIC analysis were prepared by cutting the paraffin block perpendicular to the incision line. Areas stained with LY alone or with RhD were detected by fluorescence emission using a confocal microscope (Fluoview; Olympus, Japan). The net area stained with LY alone and the length of incision was quantified by the analytical program included with the Fluoview. At least three of the incision sites per specimen were randomly chosen for analysis and the mean value was used as the data from one animal. The distance of dye transfer from the incision line (area/length) was represented as GJIC and values were expressed as a fraction of the control value.

Immunostaining of Cx32

For immunostaining Cx32, frozen sections were fixed with acetone at −20°C for 5 min and the procedure as described in the kit’s instructions followed. After blocking, the sections were treated with a monoclonal anti-Cx32 antibody (1:500) at 4°C overnight, followed by treatment with biotinylated anti-mouse IgG for 30 min and fluorescein–avidin D (1:200) for 45 min at room temperature. The fluorescence was viewed under a confocal microscope (Fluoview).

Quantification of cell proliferation

The paraﬁn block used in the IL/DT assay was also used to quantify cell labeling by BrdUrd. Immunostaining of BrdUrd was carried out as described previously (29); at least 3000 hepatocytes per animal were counted. The labeling index (LI) was deﬁned as a percentage derived from the number of labeled cells divided by the total number of cells counted.

Statistics

The value for each group was expressed as the mean ± SD of data derived from five animals. The signiﬁcance of differences in the results was evaluated by ANOVA, followed by Dunnett’s multiple comparison test.

Results

In the present experiment, we used a modiﬁed in vivo GJIC assay, the IL/DT method, applying a cocktail of fluorescent dyes: LY, which transfers through the gap junction channel, to monitor GJIC function (Figure 1A); RhD, which does not pass through the channels, to identify the incision sites or artiﬁcially damaged cells (Figure 1B). For the evaluation of GJIC function, the area stained only with LY, excluding the RhD stained region (Figure 1C), was analyzed, then normalized to the incision length to obtain the value of net distance of dye transfer through the gap junction.

In this study, we first explored whether GJIC in the mouse liver was inhibited during administration of PCP at carcinogenic doses, i.e. 300 [PCP(L)] and 600 p.p.m. [PCP(H)], which promoted tumor development after initiation with diethylnitrosamine (DEN) (30). The treatment with PCP at both doses (300 and 600 p.p.m.) for 2 weeks in the present study did not elevate the hepatotoxic index of serum aspartate aminotransferase (sAST) nor cause necrotic or apoptotic alterations in the liver by histopathological examination (data not shown). These observations were consistent with our previous study (28). The net transfer of LY was signiﬁcantly reduced in the liver after PCP(H) treatment for 2 weeks (Figure 2C) compared with the control (Figure 2A). A dose-dependent inhibition of
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GJIC was evident, as shown in Figure 3, being inhibited by 45 and 60% with PCP(L) and PCP(H), respectively, which corresponds to the dose dependency of tumor promoting activity observed in a previous study (30).

The administration of green tea infusion alone for 3 weeks, as a source of drinking water, did not affect transfer of LY (Figure 2B) compared with the control (Figure 2A). However, the combination of green tea treatment with PCP(H), 1 week of pre-treatment plus 2 weeks of co-treatment, apparently facilitated dye transfer (Figure 2D), compared with the PCP(H) treatment alone (Figure 2C). The inhibition of GJIC by PCP was only slightly reduced in the group co-treated with green tea, at ~90% of the control with both PCP(L) and PCP(H) (Figure 3).

To explore the manner of inhibition of GJIC by PCP, we examined whether expression of Cx32, which is a major gap junction protein in the mouse liver, was reduced and/or if the localization of Cx32 in the plasma membrane was disturbed. Immunostaining of Cx32 showed that PCP treatment did not affect the localization of Cx32 in the plasma membrane, but reduced the density of Cx32 plaques (Figure 4D and E), compared with the high density of plaques observed in the control (Figure 4B) and green tea treatment alone (Figure 4C). The reduction in Cx32 plaques was very evident in the centrilobular region of the liver after PCP treatment (Figure 4D and E, CV), which corresponded to the decrease in dye transfer in the area (data not shown). The combination of green tea treatment significantly prevented the reduction in Cx32 plaques in the liver as against PCP treatment alone, although a slight reduction in plaques was still observed in the centrilobular region (Figure 4F).

We examined whether cell proliferation induced by PCP in the mouse liver, which was previously observed (28,30), might be associated with GJIC inhibition and if green tea treatment might prevent this cell proliferation. A dose-dependent increase in BrdUrd LI was observed in the liver of mice fed PCP for 2 weeks, being elevated 6- and 15-fold with PCP(L) and
Fig. 4. Effect of green tea on the expression of Cx32 in the plasma membrane in the liver of mice treated with PCP. PCP and green tea infusion were given to mice as described in the legend to Figure 2. A reduction in Cx32 spots was observed in the liver of mice fed 600 p.p.m. PCP (D and E), compared with the control (B) or green tea alone (C), and prevention of this reduction in Cx32 was observed on co-treatment with green tea (F). CV, central vein; PP, periportal region. Bar 50 µm.

Fig. 5. Effect of green tea on cell proliferation in the liver of mice treated with PCP. PCP and green tea infusion were given to mice as described in the legend to Figure 3. The values represent the means ± SD of data from five mice. *P < 0.05 versus control; **P < 0.001 versus control; †P < 0.001 versus PCP (300 p.p.m.) treatment alone; §P < 0.001 versus PCP (600 p.p.m.) treatment alone.

PCP(H), respectively (Figure 5). Pre- and co-treatment with green tea significantly prevented the increase in LI, inhibiting by 60 and 70% as against the elevated LI in PCP(L) and PCP(H), respectively (Figure 5).

Discussion

During the last decade of cancer research, oxidative stress has been implicated in tumor development and the preventive potential of antioxidants and green tea has received much attention (35). Since alterations in GJIC are known to contribute to tumor development and since in vitro studies have demonstrated that GJIC could be modulated by oxidative stress (36–39) and that this modulation of GJIC could be either prevented or ameliorated by antioxidants (40) and green tea (19–21), it seemed important to seek evidence for the prevention of down-regulation of GJIC in an in vivo system undergoing tumorigenesis to emphasize the preventive potential of those antioxidants.

Previously we reported that a non-cytotoxic dose of PCP, but not a mutagenic metabolite, inhibited GJIC in vitro, suggesting a critical role of PCP in tumor-promoting activity by an epigenetic mechanism (31). This idea was supported by the fact that PCP did not have initiating activity in a two-stage mouse hepatocarcinogenesis model, but promoted tumor development after initiation with DEN (30). In the present study we have used the IL/DT method to explore whether the administration of PCP at carcinogetic doses, which promote tumor development after initiation with DEN, may inhibit GJIC in liver tissue. The results showed that both low and high doses of PCP significantly inhibited GJIC in a dose-dependent manner, i.e. inhibiting by 45 and 60%, respectively,
which corresponded to the tumorigenic activity previously observed in the two-stage model (Figures 2 and 3). This finding supports our hypothesis that down-regulation of GJIC by PCP is a cellular event underlying its tumor-promoting action.

There is the possibility that cytotoxic effects due to severe oxidative stress induced by PCP/TCHQ might be involved in the GJIC inhibition by PCP in the present study and that regenerative cell proliferation may cause down-regulation of GJIC, as it is known that down-regulation of GJIC is also observed after partial hepatectomy (41) and wounding (34). However, since no significant hepatotoxic indices, such as elevation of sAST or a necrotic appearance of hepatocytes, were evident, which was compatible with a previous study (28), it is possible to assume that a non-cytotoxic mechanism induces signal transduction leading to GJIC inhibition. As a signal for GJIC inhibition, we assume the involvement of oxidative stress, which can be induced at non-cytotoxic levels but which may be enough to alter some signal transduction by epigenetic mechanisms. This speculation is supported by our in vitro observation that production of oxygen radicals in the mitochondria was detected in rat liver epithelial cells exposed to non-cytotoxic doses of PCP, while a significant activation of mitogen-activated protein kinases (MAPKs), which are known to be activated by oxidative stress, was associated with GJIC inhibition (unpublished data).

Previous in vitro studies reported that green tea and its components protected against the inhibition of GJIC caused by toxicants or tumor promoters. In our in vitro study we observed that EGCG, a major constituent of green tea, prevented the down-regulation of GJIC by PCP, suggesting that green tea might have potential protective effects against GJIC inhibition in vivo (unpublished data). In fact, the present study has shown that giving mice green tea significantly prevented down-regulation of GJIC in the liver after simultaneously administrating PCP at tumorigenic doses. This observation suggests that green tea may inhibit the promoting action of PCP in mouse hepatocarcinogenesis. Future experiments are being performed to test this hypothesis in a two-stage mouse hepatocarcinogenesis model.

GJIC is known to be regulated by transcriptional, translational, and post-translational processes for Cxs. Understanding the molecular mechanisms for the modulation of gap junction function is important in order to characterize the manner of action of tumor promoters/anti-promoters. In a previous study, we observed that PCP did not change the phosphorylation of Cx43, in contrast to the action of 12-O-tetradecanoylphorbol-13-acetate, which hyperphosphorylated Cx43 via protein kinase C activation, however, PCP reduced the total level of Cx protein localized on the plasma membrane (31). We further observed that EGCG treatment preserved Cx43 plaques in the plasma membrane after PCP treatment (unpublished data). The present experiment also demonstrated that PCP administration markedly reduced the numbers of Cx32 spots on the hepatic plasma membrane, especially in the centrilobular region, and that green tea prevented this reduction in plaque formation (Figure 4), supporting the observation of an in vitro study (31).

The increase in cell proliferation in the liver after PCP administration, reflected in an elevation in LI, was also inhibited by green tea (Figure 5). This observation supports the hypothesis that inhibition of GJIC might not only release cells from contact inhibition but also trigger growth stimuli (2), which is consistent with our recent observation that GJIC inhibition by carcinogens was preceded by activation of MAPK, a mitogenic signal pathway (42). These facts suggest that inhibition of GJIC by PCP might stimulate growth in the liver and that up-regulation of GJIC by green tea may protect against tumor development by preventing such cell proliferation.

As a mechanism for preventing down-regulation of GJIC, previous reports suggested that antioxidative properties might involve scavenging of oxygen radicals which may contribute to the closure of GJIC channels (19–21,40). Since we previously found that cell proliferation in the liver is associated with oxidative stress, such stress may be involved in GJIC inhibition and the antioxidants in green tea may be responsible for the protection against such inhibition. This speculation is supported by our observation that PCP generates oxygen radicals in mitochondria and that the antioxidant component of green tea, EGCG, prevents the inhibition of GJIC by PCP in WB-F344 rat liver epithelial cells (unpublished data). Since green tea has been reported to induce antioxidant enzymes, such as catalase and glutathione peroxidase, enhancement of antioxidant status might protect against oxidative stress due to PCP action. Alternatively, since EGCG is known to act not only as an antioxidative factor but also as an inhibitor of some enzymes (14,15) or as an inducer of gene expression (18), EGCG might inhibit enzymes which degrade Cxs or induce transcription of Cx. In our in vitro observations, EGCG protected against a marked decrease in Cx43 protein level rather than up-regulating Cx43 mRNA level (unpublished data). This observation suggests that prevention of post-translational degradation of Cx is a major action of green tea components, either by scavenging of oxygen radicals which might facilitate the degradation of Cx or by inhibiting the activity of enzymes involved in Cx degradation. Lastly, since green tea has been demonstrated to induce detoxifying enzymes (18), such as glutathione S-transferase and sulfotransferase, the possibility that green tea facilitates excretion of PCP cannot be excluded.

As shown by other authors using a microinjection method for GJIC assay in vivo (9), our IL/DT method illustrates the potential benefit of detecting and predicting effective doses for tumorigenesis. We believe that these methods have many advantages for studying tumor-promoting activity in vivo because, even though the Cx expression level recovered at an early stage after treatment with the tumor promoter, a low level of GJIC persisted for a longer time (9), indicating that an analysis of the expression level of Cx, such as for protein, mRNA or its localization, does not always reflect gap junction function in vivo. With respect to devising a much simpler and quicker procedure compared with the microinjection method, IL/DT may be useful for rapidly screening tumor promoters and for setting doses for studies of carcinogenesis.

In conclusion, the present study has shown that green tea prevents GJIC inhibition in the mouse liver after tumorigenic doses of PCP, suggesting a potential anti-promoting action of green tea against PCP tumorigenesis. This result is the first evidence showing the protective effect of green tea on GJIC inhibition in vivo, which may be a factor characterizing the biological action of green tea as an anti-promoter. In addition, these results provide further evidence for the role of modulated GJIC in the carcinogenic process (43).

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