Inhibition of PhIP-induced mammary carcinogenesis in female rats by ingestion of freeze-dried beer

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Abstract

This study evaluated the modulating effect of non-alcoholic constituents of beer on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced mammary carcinogenesis. Female Sprague–Dawley (SD) rats at 6 weeks of age were divided into four groups ($n = 26$–30) and fed either a high fat diet or high fat diets containing 1, 2 or 4% freeze-dried beer (FD beer). One week after the start of feeding, rats received PhIP at a dose of 85 mg/kg by gavage four times weekly for 2 weeks. There were no differences in the body weights or diet intakes of rats between the control and the experimental groups. Weekly observation of palpable tumors indicated that tumor incidence and tumor multiplicity in the 2 and 4% FD beer groups were lower than in the control group throughout the experiment. Neoplastic lesions were pathologically examined at the end of the 22-weeks experiment. Tumor development was inhibited by FD beer intake in a dose-dependent manner. Tumor incidence (38.5%) and tumor multiplicity (0.8 ± 0.4) for the group fed with a diet containing 4% FD were significantly reduced as compared with the control group (73.3% and 1.8 ± 0.7). Supplementation with FD beer for 3 weeks together with the PhIP treatments resulted in increased liver GST activity, decreased liver CYP1A2 activity and a decrease in the number of DNA adducts in the mammary tissue, though these values were not significant. In conclusion, our results suggest that intake of FD beer may reduce the risk of carcinogenesis caused by heterocyclic amines.
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1. Introduction

Diet is considered to be one of the major environmental factors that cause human cancers. Heterocyclic amines (HCAs), found in cooked meat and fried fish [1], are the most common environmental mutagens and suspected human carcinogens. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is one of the most

abundant HCAs [2] and has been shown in rodents to cause colon, mammary and prostate cancers [3,4]. A high incidence of PhIP–DNA adducts in exfoliated ductal epithelial cells from human breast milk has indicated that humans are exposed to this mutagen daily [5]. PhIP is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals and supporting genotoxicity data by the US National Toxicology Program. Therefore, there is a growing interest in finding dietary factors with anti-carcinogenic effects in order to reduce the mutagenic and carcinogenic risks caused by HCAs [6–11].

Mammary cancer is the most commonly diagnosed malignancy and is the second leading cause of cancer death in women from western countries [12]. Although dietary factors affect the incidence of mammary cancer, there is a strong association of this type of cancer with endogenous hormones, especially estrogens [13]. Epidemiological studies suggest that alcohol consumption may increase blood estradiol levels in postmenopausal women, thereby providing a mechanism to increase the risk of hormone-induced breast cancer [14,15]. However, other studies have suggested that low-level consumption of alcohol does not increase breast cancer risk [16,17]. Although these studies found no association between cancer risk and the type of beverage consumed, there is an alternative report suggesting that ingredients in beer have anti-mutagenic effects against several HCAs in vitro and in vivo [18,19]. We recently demonstrated that intake of freeze-dried beer (FD beer) inhibits the formation of PhIP-induced aberrant crypt foci in rat colon [20]. In the present study, we evaluate the modulating effects of non-alcoholic components of beer on PhIP-induced mammary carcinogenesis in rats by feeding FD beer with a high fat diet. The results suggest that daily intake of non-alcoholic components of beer reduces the risk of mammary carcinogenesis caused by PhIP.

2. Materials and methods

2.1. Materials

PhIP–HCl was purchased from NARD Institute Ltd (Osaka, Japan). Corn oil, 10% neutral-buffered formalin, GSH and CDNB were purchased from Wako Pure Chemical Co. (Osaka, Japan). Methoxy resorufin was purchased from Sigma Chemical Co. (St Louis, MO). Commercially available pilsner type (all malt) beer was freeze-dried and used for the animal experiments.

2.2. Treatment of animals

Five-week-old female Sprague–Dawley rats were purchased from Charles River, Japan. They were maintained at three rats per wire cage in an air-conditioned room under constant conditions of temperature ($22 \pm 2\,°C$) and humidity ($55 \pm 10\%$). The animals were allowed free access to a basal diet, AIN-76A (Dyets, Inc., Bethlehem, PA), and were randomized into experimental groups after 4 days of acclimatization. The animals were treated in accordance with Kirin Pharmaceutical's ethical guidelines for animal care, handling, and termination.

2.3. Carcinogenesis experiment

Carcinogenesis experiments were conducted according to the established method reported previously [10,21,22]. Briefly, 6-week-old female Sprague–Dawley rats were divided into four groups. They were fed either a basal high fat diet (control group, $n=30$) or basal high fat diets containing the non-alcoholic constituents of beer (freeze-dried beer; FD beer) at 1 (1% FD beer group, $n=30$), 2 (2% FD beer group, $n=30$) or 4% (4% FD beer group, $n=26$). The composition of the diets is shown in Table 1. Since most of the calories in the solid components of beer are derived from carbohydrates, an amount of sucrose equal to an amount of FD beer was subtracted from the experimental diets to adjust for the caloric content. Body weights of rats were measured weekly. After starting the experimental diets, the estimated food consumption was measured daily from days 70 to 100. One week after the start of feeding, rats received PhIP suspension in corn oil at a dose of 85 mg/kg by gavage four times weekly for 2 weeks. Palpable tumors were recorded weekly until 21 weeks. At 22 weeks, all rats were sacrificed, and neoplastic lesions were examined macroscopically and scored. Then the lesions were measured with calipers, and the tumor volume was calculated using

Table 1
The composition of each diet

Ingredient	Diet (g/kg)			
	Group 1 (control)	Group 2 (1% FD beer)	Group 3 (2% FD beer)	Group 4 (4% FD beer)
Casein	235	235	235	235
DL-methionine	3.5	3.5	3.5	3.5
corn starch	95	95	95	95
sucrose	317	307	297	277
cellulose	59	59	59	59
corn oil	235.2	235.2	235.2	235.2
salt mixture	41.1	41.1	41.1	41.1
vitamin mixture	11.8	11.8	11.8	11.8
choline bitartrate	2.4	2.4	2.4	2.4
FD beer	0	10	20	40

the formula (length)$\times$(width)$\times$(depth)$\times\pi/6$ [23]. After measuring the tumor size, they were immediately fixed with 10% neutral buffered formalin. The lesions were embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin (H&E) for histopathological examination according to the established criteria [24].

2.4. Assays for CYP1A2, GST and DNA adducts

Six-week-old female Sprague–Dawley rats were divided into five groups: PhIP($-$) control group ($n=2$) and PhIP($+$) control group ($n=4$) were fed with the basal high fat diet and three experimental groups ($n=4$) were fed with the basal high fat diets containing FD beer at 1, 2, or 4%. All groups were fed with the diets for 3 weeks. One week after the start of feeding, PhIP($+$) control group and three experimental groups received oral administrations of PhIP eight times following the same protocol as in the carcinogenesis experiment. Twenty-four hours after the last PhIP administration, livers and mammary glands with surrounding hypodermic fat tissue were promptly harvested, frozen in liquid nitrogen and stored at $-80\,^{\circ}\mathrm{C}$ until enzymatic or DNA adduct analyses were performed. We chose only one time point at 24 h after the last PhIP administration for the analysis of DNA adducts, since PhIP–DNA adduct levels at 3 h after the last PhIP administration were not different from those at 2 days after the last PhIP dosing in the previous report [25].

For enzyme assays, a sample of the liver was homogenized in phosphate buffer (pH 7.5), and the cytosolic fractions were prepared by centrifugation at $9000\times g$. Glutathione S-transferase (GST) activity was determined using 1-chloro2,4-dinitrobenzene (CDNB) as a substrate. The reaction mixture (100 µl containing 1 mM glutathione (GSH), 1 mM CDNB, 0.2 M potassium phosphate, pH 6.5) was incubated with 10 µl of crude enzyme at 25 °C for 10 min. The formation of GSH conjugate was examined by measuring the optical density at 340 nm [26]. CYP1A2 activity was determined using methoxy resorufin as a substrate [27]. The reaction mixture (100 µl containing 0.1 mM Tris–Cl (pH 7.4), 2 mM MgCl2, 0.2 µM EDTA, 1 mM NADPH, 10 µM dicumarol, 16 µM methoxy resorufin) was incubated with 10 µl of crude enzyme at 37 °C for 1 h. The formation of resorufin was measured spectrofluorimetrically at 530 (excitation) and 590 nm (emission) [28,29]. Protein concentrations were determined by the Bradford method using BSA as a standard [30]. Relative enzyme activities of the samples were expressed as percentages of the PhIP-treated control group.

For DNA adducts analysis, DNA was prepared from the mammary tissue with a DNA isolation kit (ISOTISSUE, Nippon Gene Co., Japan) according to the manufacturer's directions. DNA adducts were analyzed according to the ^{32}P-postlabeling method using the intensification protocol described previously [19,31].

2.5. Statistical analysis

Data were expressed as the means $\pm$ SD or means $\pm$ SEM as indicated in each figure or table. The data were analyzed by one-way ANOVA followed by the Dunnett's test or the Kruskal–Wallis test followed by the Bonferroni Correction. Tumor incidence was compared using the χ^2-test and Fischer's exact probability test. Differences were accepted as statistically significant when the P-value was less than 0.05.

Table 2
Food intake, body, spleen, kidney and liver weights in carcinogenesis experiment

	Food intake (g)	Body weight (g)	Spleen weight (g)	Kidney weight (g)	Liver weight (g)
Control	11.9	344.6 ± 50.3^a	0.60 ± 0.16	1.86 ± 0.20	10.4 ± 1.55
1% FD beer	11.1	351.1 ± 42.0	0.55 ± 0.11	1.86 ± 0.27	9.90 ± 1.33
2% FD beer	11.4	349.1 ± 39.6	0.56 ± 0.16	1.91 ± 0.24	10.1 ± 1.18
4% FD beer	12.3	352.2 ± 62.7	0.52 ± 0.11	1.99 ± 0.24	9.87 ± 2.81

[a] Values are given as mean $\pm$ SD.

3. Results

3.1. General observations

There were no significant differences in body weight between the rats in the control groups and the rats in the FD beer-fed groups throughout the experimental period (Table 2). There were no rats that became moribund or died during the experimental period of 22 weeks. No significant differences in the weights of liver, spleen, and kidney were observed among the control and the beer-fed groups, indicating that chronic administration of FD beer was not associated with any gross changes or signs of toxicity (Table 2).

3.2. Time course changes of palpable tumors in rats

Palpable breast tumors were observed in all groups of rats 8 weeks after the administration of PhIP (experimental week 11). The time courses of tumor incidence (the percentage of rats with tumors) and multiplicity (the number of tumors per rat) are shown for each experimental group in Fig. 1. Tumor incidence in the groups fed with diets containing 2 and 4% FD beer was lower than in the control group throughout the carcinogenesis experiment (Fig. 1a). While tumor incidence in the group treated with 1% FD beer was higher than in the control group from 10 to 14 weeks, by 20 weeks it reached a similar value. The changes in tumor multiplicity mirrored those of tumor incidence. Feeding the rats with diets containing 2 or 4% FD beer greatly reduced the tumor multiplicity compared to the control group, while only a slight decrease was noticed in the group fed with the diet containing 1% FD beer after 16 weeks (Fig. 1b). Furthermore, there was a noticeable delay in the appearance of tumors in the group fed with the diet

containing 4% FD beer until 13 weeks, when only one palpable tumor was found in one rat (Fig. 1a and b).

3.3. Effects of beer ingredients on mammary carcinogenesis in rats

The results of mammary tumor development in each group examined at week 22 are summarized in Table 3. Dose-dependent inhibition of tumor incidence and tumor multiplicity was observed following intake of FD beer. Tumor incidence in the control group and the groups fed FD beer at 1, 2 and 4% was
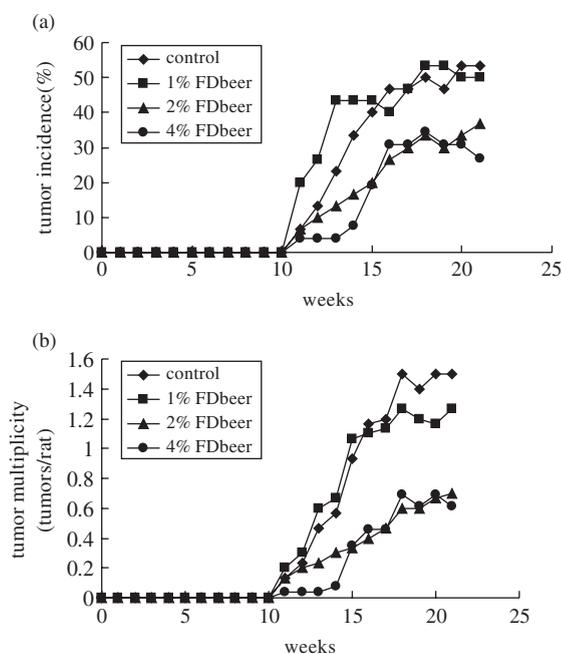

Fig. 1. Intake of FD beer has a dose-dependent effect on mammary carcinogenesis in rats. Tumor incidence (a) and tumor multiplicity (b) were measured in female rats treated with PhIP and fed experimental diets, control (rhombus), 1 (square), 2 (triangle) or 4% (circle) FD beer.

Table 3
Tumor volume, tumor incidence and tumor multiplicity in carcinogenesis experiment

	Animals (n)	Average tumor volume (cm³)	Tumor incidence (%)	Tumor multiplicity
Control	30	1.8 ± 0.7[a]	22/30 (73.3)	2.0 ± 0.4
1% FD beer	30	1.5 ± 0.5	19/30 (63.3)	2.0 ± 0.5
2% FD beer	30	0.9 ± 0.3	16/30 (53.3)	1.4 ± 0.3
4% FD beer	26	0.8 ± 0.4	10/26 (38.5)**	0.8 ± 0.4*

Significantly different from Group 1: *$P < 0.05$; **$P < 0.01$.

[a] Values are given as the mean ± SEM.

73.3, 63.3, 53.3 and 38.5%, respectively. As compared to the control group, the tumor incidence in the 4% FD beer-fed group was significantly reduced to 53% of the control value ($P < 0.05$). Tumor multiplicity in the control group and the groups fed with FD beer at 1, 2, and 4% was 2.0 ± 0.4, 2.0 ± 0.5, 1.4 ± 0.3 and 0.8 ± 0.4, respectively. The decrease in the 4% FD beer group was also significant as it was reduced to 40% of the control value ($P < 0.01$). Dose-dependent suppression was also observed in tumor volumes. The values were 1.8 ± 0.7, 1.5 ± 0.5, 0.9 ± 0.3, and 0.8 ± 0.4 cm³ for the control, 1% FD beer, 2% FD beer, and 4% FD beer groups, respectively. Most of the tumors found in all groups were diagnosed as invasive papillary adenocarcinoma (91%); some tumors were diagnosed as invasive tubular adenocarcinoma (6%) and the remaining were invasive cribriform adenocarcinoma and fibroadenoma (3%). The ratio of the papillary adenocarcinomas versus the tubular adenocarcinomas changed between the control and experimental groups. In the control group, 97% of the tumors were papillary adenocarcinomas, and there were no tubular adenocarcinomas. In the FD beer-fed groups, the percentage of tubular adenocarcinomas increased to 5.2, 9.8, and 13.6% for the 1, 2, and 4% FD beer groups, respectively.

3.4. Enzyme and DNA adduct analyses

Results of the enzyme assays are shown in Fig. 2. CYP1A2 activity in the liver increased 5.4 times with oral administration of PhIP (Fig. 2a). Groups that were fed with diets containing 2 and 4% FD beer demonstrated approximately 40% decreases in CYP1A2 activity. Although these decreases were not statistically significant, it was suggested that

the beer ingredients might inhibit the major CYP responsible for metabolic activation of PhIP. On the other hand, glutathione S-transferase (GST) activity in liver was induced by 25% with oral administration of PhIP, and increased a further 20% after intake of the 4% FD beer diet. However, these increases were not significant (Fig. 2b). Analysis of DNA adducts revealed that PhIP treatment induced the formation of DNA adducts in mammary tissue (Table 4). Intake of FD beer decreased the number of DNA adducts in the mammary tissue, although the decreases in the FD beer-fed groups were not significant and not dose-dependent, probably due to too few animals per group. The group that was fed with the 4% FD beer diet showed the greatest reduction in DNA adducts (Table 4).

4. Discussion

Previous studies have suggested that beer contains components with anti-mutagenic effects against HCAs, food-derived mutagens and putative human carcinogens [19,32]. We recently demonstrated that several types of commercial beer showed anti-mutagenic effects on HCAs by in vitro and in vivo studies [20]. We documented that intake of FD beer reduced the number of PhIP-induced putative preneoplastic lesions (aberrant crypt foci) in the colons of male rats. It has been shown that PhIP induces mammary tumors in female rats, and this carcinogenic model has been used to study anti-carcinogenic effects of many dietary factors [7,10, 33–35]. Therefore, in this study we evaluated the modulating effects of FD beer components on PhIP-induced mammary carcinogenesis.

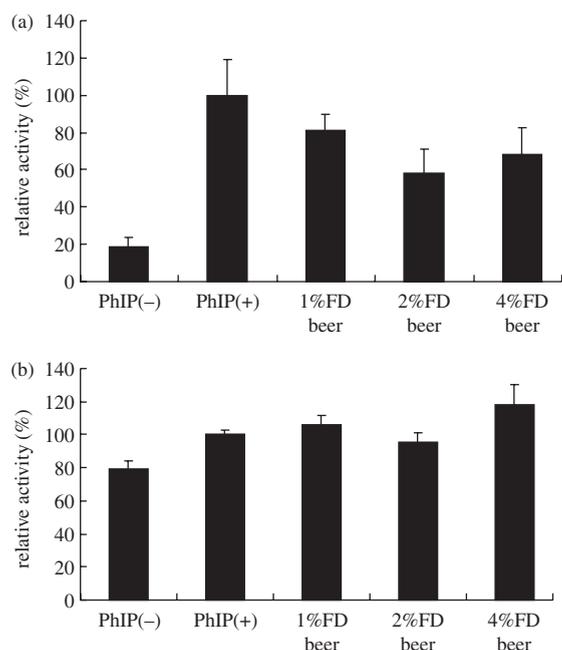We used a high fat diet for the present study to effectively induce mammary tumors [10,21].

Fig. 2. Effects of FD beer intake on liver enzyme activity from PhIP-treated rats. CYP1A2 activity (a) and GST activity (b) were measured from the livers of female SD rats treated with PhIP and fed experimental diets containing FD beer. Results are given as mean relative activity $\pm$ SEM.

For experimental samples, FD beer was mixed in the high fat diet and fed to the animals throughout the study. Feeding the rats with diets containing FD beer up to 4% did not affect the ingested amount or growth of rats, as shown in Table 2. There were slight changes in the body weights between 2 and 4 weeks due to the toxic effect of the PhIP treatments (data not shown), which is consistent with previous observations [20,36, 37]. The tumor incidence and tumor multiplicity observed in the control groups in the present study were similar to those reported previously [10,21], and these parameters were inhibited by intake of FD beer in a dose-dependent manner (Fig. 1 and Table 3). The values for tumor incidence and tumor multiplicity increased from week 21 when palpable tumors were observed to week 22 when anatomical tumors were observed. Two reasons may account for these differences. First, the number of neoplastic lesions simply may have increased during that 1-week period. Second, small lesions most likely were undetected during palpation but were macroscopically diagnosed

as neoplastic lesions during necropsy. Significant inhibition of tumor incidence and multiplicity were observed in the group fed with the diet containing the highest amount of FD beer (4%).

Our previous study has shown that anti-carcinogenic effects of FD beer, such as inhibition of PhIP–DNA adducts and PhIP-induced aberrant crypt foci formation in rat colon, were observed in the PhIP-treated rats fed with the standard diet [18,20]. Therefore, it is likely that these inhibitory effects of FD beer were caused by direct influence on PhIP-induced carcinogenesis but not by merely inhibition of high fat-induced enhancement of mammary carcinogenesis. We examined the effect of FD beer on the activities of hepatic enzyme CYP1A2 and GST, an enzyme involved in the activation of HCAs and a multifunctional enzyme involved in the detoxification of xenobiotic compounds, respectively [38,39]. Supplementing the diet with FD beer for 3 weeks showed decreasing trend for the PhIP-induced elevation of CYP1A2 activity and showed increasing trend for the GST activity in the liver (Fig. 2). A decrease in the number of PhIP-induced DNA adducts from the mammary tissue was also observed in the FD beer-fed groups (Table 4). These observations suggest that beer components may exert their anti-mutagenic effects by inhibiting the activation of PhIP and inducing the detoxification of the activated PhIP–NHOH, thereby inhibiting the formation of PhIP–DNA adducts in mammary tissue. The average tumor volume was also suppressed by intake of FD beer in a dose-dependent manner. The average volume from the 4% FD group was approximately 44% of the control group (Table 3). This may indicate that beer components also possess anti-tumor promotion activity.

Beer contains many components, the anti-mutagenic and anti-carcinogenic properties of which have been suggested from in vitro and in vivo experiments

Table 4
DNA adducts in short-term experiment

	Animals (n)	DNA adducts/10^8 nucleotides
PhIP($-$)	2	0
PhIP($+$)	4	561.9 ± 187.4[a]
1% FD beer	4	264.1 ± 150.5
2% FD beer	4	472.1 ± 121.8
4% FD beer	4	148.8 ± 57.3

[a] Values are given as mean $\pm$ SEM.

[19,32,40–43]. Most of these compounds are derived from hops and are unique to beer. Phytoestrogenic compounds including prenylnaringenins and iso-xanthohumol might exert anti-estrogenic action and affect hormone-dependent carcinogenesis in vivo [44–47]. Prenylchalcones and humulones suppress the expression or activity of COX-2 and iNOS, which facilitate tumor-associated angiogenesis and tumor growth [40,43,48,49]. Malt also contains several phytochemicals that may have cancer-preventive activity such as ferulic acid, catechins, proanthocya-nidines, folate, and soluble or insoluble dietary fiber [50–53]. Previous studies have suggested that the components providing the anti-mutagenic effects on HCA activation were derived from both malts and hops [20].

In conclusion, intake of FD beer inhibited PhIP-induced mammary tumor incidence and multiplicity in female SD rats. The anti-mutagenic effects of beer constituents on HCAs can be at least partly attributed to the inhibition of tumor formation. It should be noted that the results presented in this study were obtained using non-alcoholic constituents of beer. Therefore, it is difficult to speculate about the anti-carcinogenic effects of beer on humans due to the uninvestigated aspect of alcoholic content. Further study is necessary to clarify the functional compounds in beer and the underlying mechanisms inhibiting carcinogenesis. The discovery of such compounds could lead to the development of chemopreventive agents.

Acknowledgements

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