

Figure 2. Effect of *Chlorella pyrenoidosa* on DEN- or MeIQx- induced GST-P-positive foci development in the liver. Group 2, DEN alone; group 3, DEN + Chl.; group 4, DEN + MeIQx; group 5, DEN + MeIQx + Chl.; group 6, MeIQx alone; group 7, MeIQx + Chl. (A) Number of GST-P-positive foci of ≥ 0.2 mm in diameter. (B) Number of GST-P-positive foci of < 0.2 mm in diameter. *Chl., *C. pyrenoidosa*. NS, not significant.

group 4 (DEN + MeIQx; basal diet), the average number and area of GST-P-positive foci were $8.05 \pm 2.90/\text{cm}^2$ and $0.56 \pm 0.20 \text{ mm}^2/\text{cm}^2$, respectively, which are about 3-fold higher than the corresponding values of group 2. In group 5 (DEN + MeIQx; *C. pyrenoidosa* diet), the average number and area of GST-P-positive foci were $4.46 \pm 2.68/\text{cm}^2$ and $0.29 \pm 0.19 \text{ mm}^2/\text{cm}^2$, respectively. Thus, *C. pyrenoidosa* significantly inhibited the development of GST-P-positive foci ($p < 0.01$, Fig. 2). The percentage inhibition of the number and area of GST-P-positive foci ≥ 0.2 mm in diameter was 67.6 and 74.2%, respectively. The difference between group 2 (DEN alone; basal diet) and group 3 (DEN alone; *C. pyrenoidosa* diet) indicated that *C. pyrenoidosa* did not have a marked suppressive effect. On the other hand, in DEN-non-treated groups, GST-P-positive foci ≥ 0.2 mm in diameter were observed in 1 rat in group 6 (MeIQx alone; basal diet) but there were no significant differences in the number or area of GST-P-positive foci ≥ 0.2 mm in diameter between groups 1 (DEN-non-treated; basal diet) and 6 (MeIQx alone; basal diet) or 7 (MeIQx alone; *C. pyrenoidosa* diet). Table III shows the quantitative data for GST-P-positive foci < 0.2 mm

Table III. Effect of *Chlorella pyrenoidosa* on the development of GST-P-positive foci (< 0.2 mm in diameter) in the liver, in DEN non-treated groups.

Group no.	Treatment	No. of rats	Size distribution of GST-P-positive foci (no./cm ²)			
			2-4 cells	5-10 cells	≥ 11 cells	Total
1	Basal diet	15	0.00 ± 0.00^a	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
6	MeIQx	15	5.36 ± 2.02^d	2.04 ± 0.85^d	0.20 ± 0.36^c	7.60 ± 2.59^d
7	MeIQx + Chl. ^b	15	2.85 ± 1.19^{dc}	0.69 ± 0.70^{dc}	0.11 ± 0.22^c	3.65 ± 1.55^{dc}

^aValue is mean \pm SD. ^bChl., *C. pyrenoidosa*. Significantly different from basal diet group (group 1 or 2) at ^c $p < 0.05$ and ^d $p < 0.01$, respectively.

^cSignificantly different from control group (group 6) at $p < 0.01$.

in diameter among DEN-non-treated groups. In group 1 (DEN-non-treated; basal diet), there were no GST-P-positive foci comprising > 1 cell. In group 6 (MeIQx alone; basal diet), the distribution of the number of cells that comprised GST-P-positive foci (2-4 cells, 5-10 cells and ≥ 11 cells) and the total number of GST-P-positive foci were significantly greater than those of group 1 (DEN-non-treated; basal diet). In group 7 (MeIQx alone; *C. pyrenoidosa* diet), the total number of GST-P-positive foci (mean \pm SD) was significantly less than that of group 6 (MeIQx alone; basal diet) ($3.65 \pm 1.55/\text{cm}^2$ vs. $7.60 \pm 2.59/\text{cm}^2$, $p < 0.01$; Fig. 2). Also, in group 7, there were significant decreases in the distribution of GST-P-positive foci cells (2-4 cells and 5-10 cells, but not ≥ 11 cells) ($p < 0.01$, Table III). The percentage of inhibition, calculated based on the total number of GST-P-positive foci < 0.2 mm in diameter, was 52%.

Discussion

This study was proposed to evaluate the inhibitory effect of *C. pyrenoidosa* on DEN-initiated and/or MeIQx-promoted hepatocarcinogenesis in rats, and we found that dietary *C. pyrenoidosa* had an inhibitory effect on hepatocarcinogenesis.

In modern living environments, humans are exposed to various environmental carcinogens and cancer promoters via food and other sources. MeIQx is a heterocyclic amine (HCA) and carcinogenic in the liver (18). HCA are ubiquitously present as food-derived compounds and are easily produced by cooking or heating meat or fish. Daily exposure of MeIQx in humans has been estimated to be 0.2-2.6 $\mu\text{g}/\text{person}/\text{day}$, which is the second highest level following 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) for this group of compounds (19). These findings suggest that MeIQx is involved in the induction and development of some human cancers. The present study demonstrated that dietary administration of *C. pyrenoidosa* can significantly inhibit the development of GST-P-positive foci induced by a combination of DEN and MeIQx (two-stage carcinogenesis model) or MeIQx alone.

The mechanism by which *C. pyrenoidosa* has inhibitory effects on hepatocarcinogenesis has not been clarified. However, there are several possible mechanisms. Firstly, chlorophyll in *C. pyrenoidosa* is thought to play a role in

its chemopreventive effects. Chlorophyll and chlorophyllin, a copper derivative of chlorophyll, form complexes with HCA (20,21). Formation of complexes between HCA and chlorophyll or their derivatives in *C. pyrenoidosa* can suppress the absorption of HCA from the intestine. Although there are many unknown aspects of the absorption of chlorophyll by the digestive tract, and the metabolism of chlorophyll in the body, *in vitro* studies have shown that chlorophyll and chlorophyllin suppress the genotoxicity of 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-p-2) (22), and that chlorophyllin inhibits the metabolism of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and forms complexes with IQ, thereby protecting against formation of IQ-DNA adducts (23). Furthermore, it has been reported that chlorophyllin inhibits NADPH-cytochrome P450 reductase in the electron transport system of cytochrome P450, thereby inhibiting the function of cytochrome P450 (24). Thus, chlorophyll and its derivatives in *C. pyrenoidosa* may suppress the bioactivation of HCA. Hasegawa *et al* reported that chlorophyllin inhibited the breast carcinogenesis induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), which is HCA (25). Secondly, proteins that are abundant in *C. pyrenoidosa* may also be involved in its anticarcinogenic effects. With regard to the physiological function of soybean proteins, there have been many studies that included chemopreventive effects (26,27). Kanamoto *et al* reported that the enterohepatic circulation of bile acids was closely involved in hepatocarcinogenesis induced by azoxymethane, and that soybean proteins inhibited the enterohepatic circulation of bile acids, thereby reducing the levels of serum bile acids and suppressing hepatocarcinogenesis (28). It appears that *C. pyrenoidosa* proteins play roles in suppressing hepatocarcinogenesis, and there is a need to further investigate the details of the mechanisms of their chemopreventive effects. Furthermore, *C. pyrenoidosa* is rich in some candidate chemopreventive agents such as carotene (29,30) and dietary fiber, with the latter promoting fecal excretion of bile acids (31). It has been reported that *Chlorella* activates the immune system, such as macrophages and NK cells (8,32). However, in the present study, the consumption of *C. pyrenoidosa* did not inhibit the development of GST-P-positive foci in the rats that received DEN but not MeIQx. Takaba *et al* found that chlorophyllin did not suppress the initiation activity of nitrosamines such as 2,2'-dihydroxy-di-n-propylnitrosamine and N-ethyl-n-N-hydroxyethylnitrosamine (33). Thus, *C. pyrenoidosa* has only weak suppressive effects against DEN-induced hepatocarcinogenesis or is less effective against carcinogens directly injected into the body than against carcinogens that are orally ingested.

In the present study, average *C. pyrenoidosa* consumption in group 5 was approximately 1.37 g/animal/day. On a body surface area basis (34), this level of *C. pyrenoidosa* consumption in rats corresponds to about 55.0 g/day for humans weighing 60 kg, and the level of chlorophyll consumption corresponded to about 1.3 g/day for humans weighing 60 kg. The minimal effective dose of *C. pyrenoidosa* against hepatocarcinogenesis was not investigated. The level of MeIQx consumption of the rats corresponded to about 0.000 times the typical human intake level (0.2-2.6 $\mu\text{g}/\text{person}/\text{day}$) on a body surface area basis. Guo *et al* reported that a

daily intake of 5 mg of chlorophyllin can theoretically complex all HCA ingested via food (35). Because it is known that the major structural requirement for complex formation with carcinogens is the tetrapyrrole macrocycle of chlorophylls, not the presence or type of metal located in the ring system (36), chlorophyllin and natural chlorophylls in human diet may have nearly the same functions. *C. pyrenoidosa* contains about 2% chlorophyll, indicating that about 0.25 g of *C. pyrenoidosa* is sufficient to provide 5 mg of chlorophyll. It is easy for humans to consume 0.25 g of *C. pyrenoidosa* in a dietary supplement.

The present results suggest that *C. pyrenoidosa* has chemopreventive effects against hepatocarcinogenesis in rats. The daily intake of *Chlorella* supplements, such as *Chlorella pyrenoidosa*, can protect against human hepatocarcinogenesis and lower the risk of HCA-induced human cancer.

References

1. Japan Ministry of Health, Labour and Welfare: Cancer Statistics in Japan 2003. Foundation for Promotion of Cancer Research (FPCR), Tokyo, 2003.
2. World Health Organization: World Cancer Report. International Agency for Research on Cancer, Lyon, 2003.
3. Doll R and Peto R: The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 66: 1191-1308, 1981.
4. Knekt P, Steineck G, Jarvinen R, Hakulinen T and Aromaa A: Intake of fried meat and risk of cancer: a follow-up study in Finland. *Int J Cancer* 59: 756-760, 1994.
5. Willett WC, Colditz GA and Mueller NE: Strategies for minimizing cancer risk. *Sci Am* 9: 88-95, 1996.
6. Fujikawa Y, Hirakawa K and Shinpo K: Effect of long-term administration of *Chlorella* tablets on hyperlipemia (in Japanese). *J Jpn Nutr Food Sci* 43: 167-173, 1990.
7. Tanaka K, Yamada A, Noda K, Shoyama Y, Kubo C and Nomoto K: Oral administration of a unicellular green algae, *Chlorella vulgaris*, prevents stress-induced ulcer. *Planta Medica* 63: 465-466, 1977.
8. Hasegawa T, Yoshikai Y, Okuda M and Nomoto K: Accelerated restoration of the leukocyte number and augmented resistance against *Escherichia coli* in cyclophosphamide-treated rats orally administered with a hot water extract of *Chlorella vulgaris*. *Int J Immunopharmacol* 12: 883-891, 1990.
9. Tanaka K, Konishi K, Himeno K, Taniguchi K and Nomoto K: Augmentation of antitumor resistance by a strain of unicellular green algae, *Chlorella vulgaris*. *Cancer Immunol Immunother* 17: 90-94, 1984.
10. Konishi F, Tanaka K, Himeno K, Taniguchi K and Nomoto K: Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): Resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. *Cancer Immunol Immunother* 19: 73-78, 1985.
11. Merchant RE, Rice CD and Young HF: Dietary *Chlorella pyrenoidosa* for patients with malignant glioma: effects on immunocompetence, quality of life, and survival. *Phytother Res* 4: 220-231, 1990.
12. Ito N, Tsuda H, Tatematsu M, Inoue T, *et al*: Enhancing effects of various hepatocarcinogenesis on induction of preneoplastic glutathione S-transferase placental form positive foci in rat - an approach for a new medium-term bioassay system. *Carcinogenesis* 9: 387-394, 1988.
13. Ogiso T, Tatematsu M, Tamano S, Hasegawa R and Ito N: Correlation between medium-term liver bioassay system data and results of long-term testing in rats. *Carcinogenesis* 8: 561-566, 1990.
14. Hirose M, Hasegawa R, Kimura J, Akagi K, Yoshida Y, Tanaka H, Miki T, Satoh T, Wakabayashi K, Ito N and Shirai T: Inhibitory effects of 1-O-hexyl-2,3,5-trimethylhydroquinone (HTHQ), green tea catechins and other antioxidants on 2-amino-6-methyldipyrido [1,2-a:3',2'-d]imidazole (Glu-P-1)-induced rat hepatocarcinogenesis and dose-dependent inhibition by HTHQ of lesion induction Glu-P-1 or 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). *Carcinogenesis* 16: 3049-3055, 1995.

Table I. Final body weight and related liver weight.

Group no.	Treatment	No. of rats	Final body weight (g)	Related liver wt. (g/100g body wt)
1	Basal diet	15	276.1±11.9 ^a	2.35±0.08
2	DEN	15	263.8±10.8	2.31±0.06
3	DEN + Chl. ^b	15	269.5±12.2	2.31±0.06
4	DEN + MeIQx	15	256.3±10.6 ^c	2.55±0.09 ^d
5	DEN + MeIQx + Chl.	15	255.9±15.7	2.50±0.09 ^d
6	MeIQx	15	263.9±13.6 ^d	2.60±0.11 ^d
7	MeIQx+Chl.	15	268.5±10.6 ^c	2.58±0.05 ^d

^aValue is mean ± SD. ^bChl, *C. pyrenoidosa*. Significantly different from basal diet group (group 1 or 2) at ^cp<0.05 and ^dp<0.01, respectively.

hepatocellular carcinoma in long-term carcinogenicity studies (13). Hirose *et al* developed a bioassay system in which test substances are administered simultaneously with MeIQx, a known hepatocarcinogen, during the post-initiation stage of a rat liver medium-term bioassay, and have identified several substances that possess inhibitory activity against hepatocarcinogenesis (14,15).

Although several studies have demonstrated the antitumor effects of *Chlorella pyrenoidosa* and other *Chlorella* species (9-11), there have been relatively few studies on its chemopreventive effects. *Chlorella pyrenoidosa* has been shown to reduce the absorption of orally ingested dioxins and accelerate their fecal excretion (16), suggesting that *Chlorella* can prevent carcinogenesis by inhibiting the absorption of carcinogens via food. The purpose of the present study was to evaluate the modifying effects of dried *Chlorella pyrenoidosa* (*C. pyrenoidosa*) powder on the development and growth of GST-P-positive foci in rat livers using the tests created by Ito *et al* and Hirose *et al*.

Materials and methods

Chemicals and *Chlorella* sample. Diethylnitrosamine (DEN) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) was purchased from Nard Institute Ltd. (Osaka, Japan). The *C. pyrenoidosa* (SUN CHLORELLA strain) was manufactured by Sun Chlorella Corp. (Kyoto, Japan). The composition (g/100 g) of dried *C. pyrenoidosa* powder used in the present study was: moisture content, 5.3; chlorophyll, 2.3; protein, 59.7; dietary fiber, 9.8; lipid, 11.2; ash, 6.3; and carotene, 0.025. *C. pyrenoidosa* was administered by mixing it into a γ ray-irradiated (6.0 kGy) MF basal diet (Oriental Yeast Co., Tokyo, Japan).

Animals. Male F344/DuCrj rats were purchased at 5 weeks of age from Charles River Japan Inc. (Atsugi, Japan). The rats were kept in plastic cages (3 rats/cage) with hard woodchips for bedding in a room maintained at 23±2°C and 52±5% humidity, with a 12-h light-dark cycle. During the experimental period, the rats were given a diet and water *ad libitum*. Body weight and food consumption were measured once per week. Average MeIQx and *C. pyrenoidosa* consumption per rat were calculated based on food consumption.

Experimental protocol. Fig. 1 shows the experimental design. A total of 105 rats were randomly divided into seven groups of 15 rats. Rats in groups 2-5 were given a single intraperitoneal (i.p.) injection of DEN (200 mg/kg body weight; injection volume, 5 ml/kg body weight) dissolved in saline to initiate hepatocarcinogenesis. In groups 1, 6 and 7, saline only was injected instead of DEN. After 2 weeks, the rats in groups 1 and 2 received a basal diet (not containing MeIQx or *C. pyrenoidosa*), group 3 received 10% *C. pyrenoidosa*, groups 4 and 6 received 0.02% MeIQx, and groups 5 and 7 received 0.02% MeIQx and 10% *C. pyrenoidosa* for 6 weeks, respectively. Also, groups 3, 5 and 7 received a diet containing 10% *C. pyrenoidosa*, starting 3 days before the injection of DEN or saline. At 3 weeks after the injection of DEN or saline, all rats were subjected to a two-thirds partial hepatectomy under etherization. At 8 weeks after the injection of DEN or saline, all surviving rats in each group were etherized and sacrificed by bleeding from the aorta. After sacrifice, the liver was immediately excised and weighed, and the liver-to-body weight ratio was calculated for each rat using the final body weight.

Immunohistochemical staining. Slices (n=3) of 5 mm thickness from the right lateral and caudate lobes were fixed in 10% buffered formalin for immunohistochemical examination of GST-P-positive foci. The avidin-biotin-peroxidase complex (ABC) method was used to determine GST-P-positive foci in the liver section (anti-rabbit GST-P antibody; Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) (17). The number and area of GST-P-positive foci ≥0.2 mm in diameter and total area of liver sections examined, were measured using a video image processor (IPAP-WIN; Sumika Technoservice Corp., Osaka, Japan). In groups without DEN treatment, the number and area of GST-P-positive foci ≥0.2 mm in diameter were measured in addition to the number of GST-P-positive foci <0.2 mm in diameter per cm² of the liver section and number of cells that comprised those foci, using a microscope. The inhibition percentage of *C. pyrenoidosa* was calculated based on the average number or area of GST-P-positive foci per cm² of the liver section, using the formula (14): (MeIQx value - basal diet value) - [(MeIQx + *Chlorella*) value - basal diet value] x 100 / MeIQx value - basal diet value.

Statistical analysis. Body and liver weights were compared between DEN-treated groups and DEN-non-treated groups.

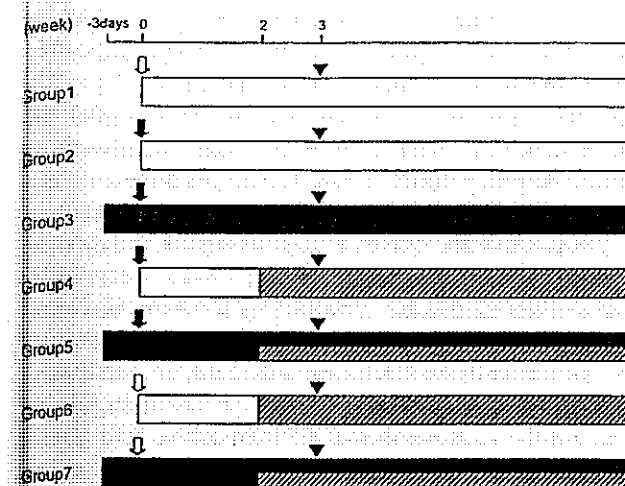


Figure 1. Experimental design.

Legend for Figure 1:
 ↓ : Diethylnitrosamine (DEN), 200mg/kg i.p.
 □ : Saline, i.p.
 ▼ : Two-thirds partial hepatectomy
 ■ : *Chlorella* 10% in basal diet
 ▨ : 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 0.02% in basal diet
 ▩ : MeIQx 0.02% + *Chlorella* 10.0% in basal diet
 □ : Basal diet

Figure 1. Experimental design.

Group 1 (DEN-non-treated; basal diet) and group 2 (DEN alone; basal diet) as controls, respectively. The modifying effects of *C. pyrenoidosa* were evaluated by comparing groups 1 (DEN alone; basal diet) and 3 (DEN alone; *C. pyrenoidosa* diet), groups 4 (DEN + MeIQx; basal diet) and 5 (DEN + MeIQx; *C. pyrenoidosa* diet), and groups 6 (MeIQx alone; basal diet) and 7 (MeIQx alone; *C. pyrenoidosa* diet). Statistical analysis was performed using a Student's t-test or Welch's test. A p-value of <0.05 or <0.01 was considered significant.

Results

The mean food consumption for groups 2, 3, 4 and 5 (DEN-treated groups) was 13.22, 13.74, 13.11 and 13.67 g/animal/day,

respectively. Mean food consumption for groups 1, 6 and 7 (DEN-non-treated groups) was 13.75, 13.71 and 14.10 g/animal/day, respectively. Average MeIQx consumption for groups 4 and 5 (DEN-treated) was 12.59 and 13.11 mg/kg body weight/day, respectively. Average MeIQx consumption for groups 6 and 7 (DEN-non-treated) was 12.57 and 12.83 mg/kg body weight/day, respectively. Average *C. pyrenoidosa* consumption for groups 3, 5 and 7 was 6960.16, 7189.53 and 6903.02 mg/kg body weight/day, respectively.

Table I shows the final body and relative liver weights. In DEN-treated groups, the average final body weight (mean ± SD) of group 4 (DEN + MeIQx; basal diet) was significantly lower than that of group 2 (DEN alone; basal diet) (256.3±10.6 vs. 263.8±10.8 g). There was no significant difference in the average final body weight between groups 4 (DEN + MeIQx; basal diet) and 5 (DEN + MeIQx; *C. pyrenoidosa* diet). On the other hand, in the DEN-non-treated groups, the average final body weight of group 6 (MeIQx alone; basal diet) and group 7 (MeIQx alone; *C. pyrenoidosa* diet) was significantly lower than that of group 1 (DEN-non-treated; basal diet) (263.9±13.6 and 268.5±10.6 vs. 276.1±11.9 g, respectively). The average relative liver weight (mean ± SD) of group 4 (DEN + MeIQx; basal diet) and group 5 (DEN + MeIQx; *C. pyrenoidosa* diet) was significantly greater than that of group 2 (DEN alone; basal diet) (2.55±0.09 and 2.50±0.09 vs. 2.31±0.06 g/100 g body weight, respectively). There was no significant difference in the average relative liver weight between groups 4 and 5. In DEN-non-treated groups, the average relative liver weight of groups 6 (MeIQx alone; basal diet) and 7 (MeIQx alone; *C. pyrenoidosa* diet) was significantly greater than that of group 1 (DEN-non-treated; basal diet) (2.60±0.11 and 2.58±0.05 vs. 2.35±0.08 g/100 g body weight, respectively). There was no significant difference in the average relative liver weight between groups 6 and 7. Thus, *C. pyrenoidosa* did not have a marked effect on liver weight.

Quantitative data for GST-P-positive foci ≥0.2 mm in diameter in the liver are summarized in Table II. In group 1 (DEN-non-treated; basal diet), no GST-P-positive foci were seen. In group 2 (DEN alone; basal diet), the average number and area of GST-P-positive foci (mean ± SD) were 2.75±2.26/cm² and 0.20±0.18 mm²/cm², respectively. In

Table II. Effect of *Chlorella pyrenoidosa* on the development of GST-P-positive foci (≥0.2 mm in diameter) in the liver.

Group no.	Treatment	No. of rats	GST-P-positive foci	
			No. (no./cm ²)	Area (mm ² /cm ²)
1	Basal diet	15	0.00±0.00 ^a	0.00±0.00
2	DEN	15	2.75±2.26	0.20±0.18
3	DEN + Chl. ^b	15	3.17±2.16	0.19±0.11
4	DEN + MeIQx	15	8.05±2.90 ^d	0.56±0.20 ^d
5	DEN + MeIQx + Chl.	15	4.46±2.68 ^{c,e}	0.29±0.19 ^c
6	MeIQx	15	0.04±0.04	0.00±0.01
7	MeIQx + Chl.	15	0.00±0.00	0.00±0.00

^aValue is mean ± SD. ^bChl, *C. pyrenoidosa*. Significantly different from basal diet group (group 1 or 2) at ^cp<0.05 and ^dp<0.01, respectively. ^eSignificantly different from control group (group 4) at p<0.01.

- Suzuki, S., Yamamoto, A. and Ogawa, H. (1975) Inhibitory effect of a streptococcal preparation (OK-432) on induction of splenomegaly by Friend leukemia virus. *Gann* 66, 455-456.
- Yoshida, J., Takamura, S. and Suzuki, S. (1987) Cell growth-inhibitory action of SAGP, an antitumor glycoprotein from *Streptococcus pyogenes* (Su strain) *Japan Journal of Pharmacology* 45, 143-147.
- Woodruff, M.A. and Warner, N.C. (1977) Effect of *Corynebacterium* on tumor growth in normal and athymic mic. *Journal of National Cancer Institute* 58, 111-116.

Suppression of glutathione S-transferase placental form-positive foci development in rat hepatocarcinogenesis by *Chlorella pyrenoidosa*

HIDEO TAKEKOSHI¹, TORU MIZOGUCHI¹, YOKO KOMASA¹, HIROFUMI CHUBACHI¹, YUKARI INOUE¹, HIDEYO IMANISHI² and MASUO NAKANO^{3,4}

¹Sun Chlorella Corp., 369 Osaka-cho, Karasuma-dori Gojo, Shimogyo-ku, Kyoto 600-8177;

²Apios, Inc., 70-19 Imajuku 2-chome, Asahi-ku, Yokohama 241-0817; ³Hokkaido Medicinal Plant Research Institute, 1-4 Shimoaikappu, Ashoro-cho, Ashoro-gun, Hokkaido 089-3707;

⁴Rakuno Gakuen University, 582 Bunkyo-dai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan

Received January 17, 2005; Accepted March 21, 2005

Abstract. The modifying effects of dietary administration of dried *Chlorella pyrenoidosa* powder (*C. pyrenoidosa*) on development of glutathione S-transferase placental form-positive foci (GST-P-positive foci), which are putative preneoplastic lesions, in male F344 rats were investigated using a medium-term liver bioassay system. In rats given *C. pyrenoidosa* in a basal diet, the number and area of GST-P-positive foci in the rat livers, which diethylnitrosamine (DEN) initiated and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) promoted, were significantly decreased compared with those fed a basal diet not containing *C. pyrenoidosa*. The inhibition percentage of the number and area of GST-P-positive foci ≥ 0.2 mm in diameter was 67.6 and 74.2%, respectively ($p < 0.01$). Furthermore, *C. pyrenoidosa* significantly decreased the number of GST-P-positive foci induced by MeIQx alone. The inhibition percentage of the number of GST-P-positive foci < 0.2 mm in diameter was 67.6% ($p < 0.01$). These results suggest that *C. pyrenoidosa* has chemopreventive effects against hepatocarcinogenesis in rats. *Chlorella pyrenoidosa* appears to be a promising chemopreventive agent for human liver neoplasia and carcinogenesis induced by heterocyclic amines such as MeIQx.

Introduction

Since 1981, cancer has been the leading cause of death in Japan. The Ministry of Health, Labor and Welfare of Japan predicts that the number of cancer patients will continue to increase and nearly double from the current figure of 300,000 by 2015 (1). The number of cancer patients is also increasing worldwide and the WHO estimates that this population will increase 1.5-fold (15 million) by 2020 (2). Cancer can be viewed as a lifestyle-related disease, and epidemiological studies have shown that changes in lifestyle, particularly diet, play an important role in the etiology of cancer (3,4). Willett *et al* reported that 30% of cancer deaths can be prevented by improving adult diets and reducing obesity (5). Chemoprevention is currently a topic of intense interest in cancer medicine, and research into functional foods that help prevent cancer has produced some promising results.

Chlorella pyrenoidosa is a unicellular green algae that grows in fresh water. Compared to other plants, *Chlorella pyrenoidosa* is much richer in proteins and chlorophylls and contains large quantities of vitamins, minerals, dietary fibers and nucleic acids. The proteins in *Chlorella pyrenoidosa* include all essential amino acids for human growth and health. *Chlorella pyrenoidosa*, other species of *Chlorella*, and *Chlorella* extracts have been shown to lower cholesterol (6), prevent stress-induced ulcers (7), improve immunity against infection (8) and exhibit antitumor effects (9-11). At present, *Chlorella pyrenoidosa* is widely available as supplements and in health foods in many regions of the world.

A rat liver medium-term bioassay system for the detection of carcinogenic potential of chemicals has been developed by Ito *et al* (12). This bioassay system, based on the initiation-promotion concept of carcinogenesis, allows the evaluation of promoting activity of test substances using the development of glutathione S-transferase placental form-positive foci (GST-P-positive foci), which are putative preneoplastic lesions of the liver, as an endpoint marker. Quantitative data of obtained GST-P-positive foci using medium-term bioassays have been shown to closely correlate with the incidence of

Correspondence to: Professor Masuo Nakano, Rakuno Gakuen University, 582 Bunkyo-dai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan.
E-mail: htakekoshi@sunchlorella.co.jp

Abbreviations: DEN, diethylnitrosamine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; GST-P, glutathione S-transferase placental form; HCA, heterocyclic amine

Key words: chemoprevention, hepatocarcinogenesis, *Chlorella pyrenoidosa*, rat