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Effects of Lycopene and Sho-saiko-to on Hepatocarcinogenesis in a Rat Model of Spontaneous Liver Cancer

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Abstract: *The Long-Evans Cinnamon (LEC) rat is a well-characterized model of spontaneous hepatocarcinogenesis. It has been shown that dietary administration of lycopene or the herbal medicine Sho-saiko-to (TJ-9) has anticarcinogenic activity, although the mechanism by which these products protect against carcinogenesis is not well known. We investigated the outcome of administration of lycopene and TJ-9 on the occurrence of hepatic neoplasia in LEC rats. A diet containing 0.005% lycopene (originally the product of tomato oleoresin containing 13% lycopene) and 1% TJ-9 (crude extracts of 7 herbs: bupleurum root, pinellia tuber, scutellaria root, jujube fruit, ginseng root, glycyrrhiza root, and ginger rhizome) was administered from 6 weeks of age until the rats were sacrificed at 76 weeks of age, at which time most of the nontreated animals were known to have hepatocellular carcinoma (HCC). Development of HCC in treated groups was analyzed histologically by comparison with untreated controls. Glutathione* S*-transferase placental form (GST-P) was analyzed by an immunohistochemical method. Concentration of copper, iron, and zinc, which appear to play a role in hepatocarcinogenesis in LEC rats, was analyzed. The percent areas of HCC in the liver specimens of control, lycopene, and TJ-9 groups were 17.9 ± 17.1%, 27.2 ± 20.8%, and 27.6 ± 18.4%, respectively. These intergroup differences were not significant. The percent area, number of areas, and mean size of area staining positively for GST-P revealed no significant differences between the groups. The number of GST-P-positive areas within the HCC lesions was greater in the TJ-9 group than in the control or lycopene group (*p = 0.024 *and* p = 0.012, *respectively). The study also demonstrated a lower concentration of iron in livers of the lycopene group than the control group (*p = 0.019*). There were no differences in serum α-fetoprotein levels or the cumulative survival rates between the groups. In conclusion, long-term administration of lycopene or TJ-9 did not reduce the risk of hepatocarcinogenesis in LEC rats.*

Introduction

Epidemiological studies have suggested that increased vegetable intake lowers the incidence of cancer deaths in the elderly (1,2). Lycopene is one of the major carotenoids in tomatoes and is known to have a protective effect against cancer with its high single oxygen-quenching capability (3). Low plasma lycopene levels have been demonstrated in patients with hepatitis and cirrhosis (4). Sho-saiko-to (TJ-9) is a Chinese herbal medicine widely used in Japan. The active components of TJ-9, baicalin and baicalein, were reported to have an antioxidative activity (5). TJ-9 has been shown to prevent hepatic fibrosis (5) and growth of hepatocellular carcinoma (HCC) experimentally (6) and clinically (7). A high incidence of HCC in aging Long-Evans Cinnamon (LEC) rats has been reported (8). Approximately 30–40% of LEC rats die at around four months of age from acute liver failure; the remaining rats survive with chronic hepatitis and develop preneoplastic and neoplastic liver lesions (9). Almost 100% of these rats older than 1.5 years have liver cancer (8). LEC rats show a pattern of hepatocarcinogenesis similar to the Solt-Farber model (10), and glutathione *S*-transferase placental form (GST-P)-positive staining has been detected in LEC rats without administration of exogenous carcinogens (11). GST-P is a preneoplastic and neoplastic marker enzyme of rat hepatocarcinogenesis (12).

Inhibition of the development of HCC and cholangiofibrosis has been demonstrated in LEC rats with the use of the copper-chelating agent trientine dihydrochloride (13). Feeding the LEC rats a copper-deficient diet completely suppressed the manifestation of the chronic liver cell injury (14). Accumulation of copper may act as a promoting factor for the development of liver cancer in LEC rats (9). Iron deprivation also has been reported to inhibit liver tumor development, but not to inhibit the development of cholangiofibrosis (15). Accumulation of copper and iron, therefore, appears to play a role in hepatocarcinogenesis in LEC rats.

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To elucidate the effect of the heavy metals on hepatocarcinogenesis, copper, iron, and zinc in the liver, serum, and diet were analyzed. The present study aimed to clarify the effects of lycopene and TJ-9 on the spontaneous development of HCC in LEC rats.

Materials and Methods

Male LEC rats (n = 105) bred at Charles River Japan (Kanagawa, Atsugi, Japan) were transported to our university at six weeks of age and housed at 24 ± 1°C and 55 ± 5% humidity. Rats were separated into three groups. The control group (n = 40) was fed a conventional basal diet (Charles River Formula-1, Charles River Japan, and Oriental Yeast, Tokyo, Japan). Composition of the diet per 100 g was as follows: 22.6 g protein, 5.6 g fat, 3.3 g fiber, 1.27 g calcium, 0.32 g sodium, 0.85 g potassium, 4.10 g other minerals, 53.8 g soluble nonnitrogen component, 8.1 g water, 16.6 mg iron, 0.92 mg copper, 6.9 mg zinc, 4.2 mg aluminum, 0.28 mg cobalt, 7.78 mg manganese, 3,783 IU vitamin A, 503 IU vitamin D_3, 21.2 mg vitamin E, 4.44 mg vitamin B-1, 3.06 mg vitamin B-2, 1.26 mg vitamin B-6, 12.2 µg vitamin B-12, 14 mg vitamin C, 0.31 g choline, 0.25 mg folic acid, and 356 kcal. The lycopene group (n = 40) was fed a prepared diet containing 0.005% (wt/wt) lycopene, which was manufactured as a 0.005% mixture of lycopene-enriched tomato oleoresin (product of concentrated tomato oleoresin containing 13% lycopene; Lyc-O-Mato, RicoRed, Natural Products Industries, Beer-sheva, Israel) with a powdered basal diet at <50°C (16). The TJ-9 group (n = 25) was fed a prepared diet containing 1% TJ-9 (Xiao-chai-hu-tang, Tsumura, Tokyo, Japan) (7). All rats were sacrificed at 76 weeks of age for evaluation of the incidence of HCC; pentobarbital sodium (0.04 mg/g body wt ip) was used for anesthesia. Histopathological examination of all animals was performed. Sera and part of each liver were frozen and stored at −80°C for further examination. Sections of liver were taken from the left and right lateral lobes. The numbers of liver specimens from the untreated, lycopene, and TJ-9 groups were 105, 74, and 85, respectively. Specimens were fixed in 10% formalin, embedded in paraffin, cut into 4-µm-thick sections, and stained with hematoxylin and eosin, silver stain, Azan stain, Victoria blue stain for copper-associated protein, or Berlin blue stain for iron and examined histologically by light microscopy.

For GST-P immunohistochemical staining, GST-P (12) was applied to the liver specimen for 30 minutes at room temperature. After several washes with phosphate-buffered saline, the sections were incubated with goat-anti-rabbit immunoglobulin-conjugated peroxidase-labeled dextran polymer (EnVision, DAKO, Carpinteria, CA) for 30 minutes at room temperature. After several washes, the sections were incubated with diaminobenzidine solution (DAB stain kit, Muto Chemical, Tokyo, Japan) for five to eight minutes. GST-P-positive lesions were measured using a computer-assisted morphometric analyzer (EIVASS, Zeiss, Oberkochen, Germany) without knowledge of the group of origin. Areas of cholangiofibrosis in each liver specimen were excluded from the analysis of the number and the area of GST-P-positive lesions.

Serum α-fetoprotein level at autopsy was measured by a sensitive sandwich enzyme immunoassay method using an affinity-purified antibody to rat α-fetoprotein (SRL, Tokyo, Japan) in 31 rats (17).

About 0.5 g of liver was digested with a mixture of perchloric acid and nitric acid (1:5), and the digest was diluted with deionized water. The copper, iron, and zinc contents were measured with an atomic absorption/flame emission spectrophotometer (type AA-630-01, Shimadzu, Kyoto, Japan). Serum and diet were assayed directly in the equipment after dilution with deionized water (18).

Values are means ± SD. Differences from the control group were assessed using one-factor analysis of variance. Intergroup differences were assessed with Fisher's protected least significant difference test. For comparison of the survival rate among the three groups, the Kaplan-Meier method (log rank Mantel-Cox test) was used; $p < 0.05$ was considered statistically significant.

Results

The 76-week survival rate was 50% (20 of 40) among control rats, 50% (20 of 40) among lycopene-treated rats, and 64% (16 of 25) among TJ-9-treated rats. Mean body weight decreased 16–20 weeks after birth as a result of fulminant hepatitis. The body weight of most of the rats surviving the fulminant hepatitis continuously increased until 76 weeks of age. The final body weight was not affected by long-term administration of lycopene or TJ-9. On gross examination of the liver, lesions of cholangiofibrosis showed irregularly shaped whitish nodules with firm consistency, whereas some large HCCs showed reddish tumors with unclear margins. Both lesions were scattered on the irregular surface of the liver.

Histopathological Observations

Histopathological observation at sacrifice disclosed that the livers in all rats were occupied by multiple macronodules of well-differentiated HCCs, hyperplastic nodules, and cholangiofibrosis. Some well-differentiated HCCs compressed surrounding hepatocytes and were demarcated from the chronic hepatitis lesion, but many of them invaded into the hepatic parenchyma. Hyperplastic nodules or foci were composed of compactly arranged small hepatocytes having clear cytoplasm and round nuclei with one or two nucleoli. The chronic hepatitis lesion showed fibrosis and many enlarged hepatocytes having large nuclei with infiltration of a few inflammatory cells. Iron and copper accumulation was observed diffusely in nonparenchymal and parenchymal cells. Areas of cholangiofibrosis, characteristic in aging LEC rats, were observed. These features were observed in

all three groups. The HCC in all three groups was predominantly of a thin, trabecular pattern with wide blood spaces or sinusoids. Occasional lesions demonstrated a thick trabecular pattern, compactly arranged small HCC cells with steatosis, or large HCC cells. Hyperplastic nodules showed various degrees of dysplasia in all groups. No effect of lycopene or TJ-9 administration on the livers was detected by histological examination.

All three groups of rats had HCC. The percent volume of HCC in the liver specimens of control, lycopene, and TJ-9 groups was 17.9 ± 17.1%, 27.2 ± 20.8%, and 27.6 ± 18.4%, respectively. These intergroup differences were not significant.

Immunohistochemical Staining of GST-P

GST-P-positive lesions in the liver specimen: GST-P-positive lesions were stained and irregularly scattered in the liver (Table 1). The percent area of GST-P-positive lesions in the liver specimens, the mean sizes of the GST-P-positive areas, and the number of lesions stained for GST-P per square centimeter of liver specimen were not significantly different among the three groups.

GST-P-positive lesions within HCC lesions: HCC lesions were partially GST-P positive in the control, lycopene, and TJ-9 groups. The nuclei of the HCC cells stained most intensely, although there was some cytoplasmic staining. The percentage of GST-P-positive area in HCC lesions was not significantly different among the three groups. The number of GST-P-positive areas per square millimeter of HCC was different among the three groups and was larger in the TJ-9 group than in the control or the lycopene group ($p = 0.024$, $p = 0.012$, respectively).

The percent area of cholangiofibrosis in the liver specimens of control, lycopene, and TJ-9 groups was 4.8 ± 4.5%, 12.2 ± 13.2%, and 5.3 ± 6.0%, respectively. The percent area of cholangiofibrosis in the liver specimens was not significantly different among the three groups.

Concentrations of Copper, Iron, and Zinc Ions

Concentrations of copper, iron, and zinc ions in the diet of control rats were 0.23, 2.93, and 4.14 ppm, respectively. Concentrations of copper, iron, and zinc ions in the diet of the lycopene group were 0.20, 2.91, and 4.41 ppm, respectively. Concentrations of copper, iron, and zinc ions in the diet of the TJ-9 group were 0.23, 2.48, and 3.75 ppm, respectively. These concentrations did not differ significantly among the groups.

Concentrations of copper, iron, and zinc ions in the liver are shown in Table 2. Concentrations of iron ion in the livers of animals in the lycopene group were lower than in the control group ($p = 0.019$).

Concentrations of copper, iron, and zinc ions in the serum are shown in Table 2. These concentrations did not differ significantly among the groups.

Serum α-fetoprotein levels of control, lycopene, and TJ-9 groups were 331.0 ± 249.1 ($n = 9$), 559.4 ± 1,211.4 ($n = 11$), and 588.3 ± 653.4 ($n = 11$) ng/ml, respectively. There were no significant differences in serum α-fetoprotein levels among the three groups.

Table 1. Quantitative Value for GST-P-Positive Focal Lesions in the Liver and Hepatocellular Carcinoma Lesions at 76 Weeks of Age[a–c]

		GST-P-Positive Focal Lesions in Liver Specimen			GST-P-Positive Focal Lesions in HCC		
Group	*n*	No./cm^2	Mean area, mm^2	Area, %	No./mm^2	Mean area, mm^2	Area, %
Control	20	16.0 ± 6.5	1.2 ± 1.3	8.1 ± 6.6	0.048 ± 0.066*	1.1 ± 1.8	7.7 ± 15.8
Lycopene	20	12.1 ± 7.2	1.5 ± 2.3	8.3 ± 7.3	0.021 ± 0.038†	0.7 ± 1.6	3.1 ± 5.5
TJ-9	16	15.1 ± 4.1	1.3 ± 1.0	8.9 ± 7.2	0.063 ± 0.060*,†	1.2 ± 1.1	10.6 ± 13.1

a: Values are means ± SD; *n*, number of rats.
b: Intergroup differences are not significant. The glutathione *S*-transferase placental form (GST-P)-positive area within hepatocellular carcinoma (HCC) is greater in Sho-saiko-to (TJ-9) group than in control or lycopene group.
c: Statistical significance is as follows: *, $p = 0.024$; †, $p = 0.012$.

Table 2. Concentration of Metals in the Liver and Serum at 76 Weeks of Age[a–c]

	Concentration in Liver, ppm				Concentration in Serum, ppm		
Group	*n*	Copper	Iron	Zinc	*n*	Copper	Iron	Zinc
Control	14	4.84 ± 1.26	4.78 ± 1.32*	1.60 ± 0.27	10	0.28 ± 0.05	0.92 ± 0.12	0.83 ± 0.09
Lycopene	10	5.08 ± 1.31	4.14 ± 0.49*	1.60 ± 0.33	8	0.34 ± 0.32	0.96 ± 0.23	0.93 ± 0.27
TJ-9	8	5.61 ± 0.90	4.68 ± 0.38	1.83 ± 0.29	8	0.22 ± 0.05	0.97 ± 0.32	0.98 ± 0.23

a: Values are means ± SD; *n*, number of rats.
b: Concentration of iron ion is lower in livers of animals in lycopene group than in control group.
c: Statistical significance is as follows: *, $p = 0.019$.

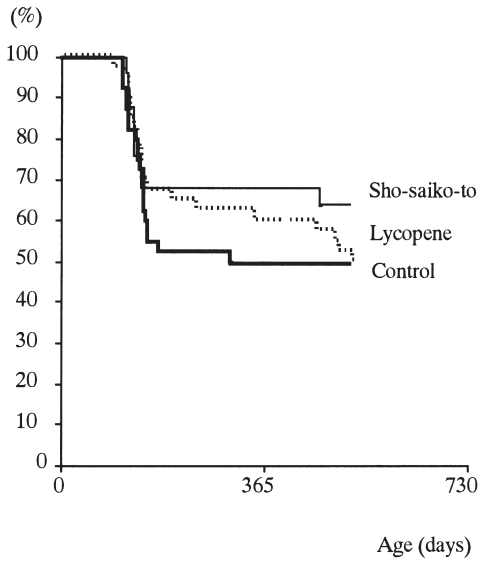

Figure 1. Cumulative survival of control, Sho-saiko-to (TJ-9), and lycopene groups. Intergroup differences are not significant.

The cumulative survival rates were better for the TJ-9 and lycopene groups than for the control group, but the difference was not significant (Figure 1).

Discussion

To analyze the effect of lycopene or TJ-9 on hepatocarcinogenesis in LEC rats, we sacrificed all rats at 76 weeks of age, when most of the animals were known to have HCC (19). Hepatocarcinogenesis in the three groups was similar with respect to the incidence of HCC, serum α-fetoprotein level, and histological grade of HCC. Oxygen radicals play an important role in the development of hepatitis and subsequent liver cancer in the rat (20). However, neither agent influenced hepatocarcinogenesis in this study. Antioxidative activities of lycopene (3) and TJ-9 (5) to prevent HCC may be insufficient for preventing hepatocarcinogenesis in the LEC rat. Lycopene, as well as α- and β-carotene, has been shown to inhibit the production of chemically induced transformed foci of 10T1/2 cells in a dose-dependent manner (21). Lycopene inhibits endometrial (Ishikawa), mammary (MCF-7), and lung (NCI-H226) cancer cell growth in culture more effectively than α- or β-carotene but did not inhibit the growth of human fibroblasts effectively (22). The anticarcinogenic activity of intraperitoneal injection of lycopene-enriched tomato oleoresin has been reported in 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumors (23). The anticarcinogenic activity of lycopene toward lung tumors after combined initiation treatment with diethylnitrosamine, *N*-methyl-*N*-nitrosourea, and 1,2-dimethylhydrazine in male B6C3F_1 mouse has been reported (24). Tomato juice exerts an inhibitory effect on the development of urinary bladder carcinoma induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine treatment in male Fischer 344 rats (25). A prospective cohort study suggested an association of dietary intake of tomato-based foods and the reduction of prostate cancer risk (26). Tomato juice rich in lycopene, but not purified lycopene, has a protective effect against colon carcinogenesis in rats (27). Vainio and co-workers (28) reported that, pending further research, lycopene should not be recommended for cancer prevention in the general population. In experimental hepatocarcinogenesis, it is generally accepted that the size of GST-P-positive lesions correlates with the intensity of the promotion effect, whereas the number of lesions in rat livers correlates with the intensity of the initiation effect (14). The mean number of GST-P-positive lesions per square centimeter in male LEC rats continuously increased until 80 weeks of age (29). Although the method of counting GST-P-positive lesions is useful for the short-term study (30), this method is also practical to use for assessment of initiation of hepatocarcinogenesis in a long-term follow-up study. We utilized the GST-P assay for evaluating initiation and promotion (31) in this study, because this assay is one of the most widely used methods for the quantitative study of hepatocarcinogen-associated preneoplastic lesions (32). Twelve weeks of administration of 0.0025% lycopene in drinking water did not decrease the incidences of bladder carcinomas in male rats after treatment with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (33). Dietary lycopene (0.03%) has been shown to decrease the initiation of preneoplastic foci in the liver by administration of diethylnitrosamine in rats (34). Forty weeks of administration of 50 ppm lycopene in drinking water decreased the incidence of liver carcinomas in male C3H/He mice after combined treatment with 4-nitroquinoline-1-oxide and glycerol (16). Also, administration of 50 ppm lycopene in drinking water decreased the incidence of lung adenomas plus carcinomas in mice (24). We decided to use a diet containing 0.005% lycopene in these earlier rodent studies of the chemopreventive efficacy of lycopene. Another report showed that β-carotene was protective against aflatoxin B_1-induced initiation of hepatocarcinogenesis, whereas lycopene was not (35).

In our study, HCC lesions were partially GST-P positive in all three groups. GST-P is a marker of preneoplastic and neoplastic cells (36,37), not only at the protein level but also at the mRNA level (38). GST-P tends to decrease with cellular dedifferentiation in HCC (36). TJ-9 inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G_0/G_1 phase (39), and it also prevents liver fibrosis and development of preneoplastic lesions in choline-deficient diet-induced liver cirrhosis in male Wistar rats (40).

In our study, concentrations of copper in the livers of the rats in all three groups were similar, and the promoting effect of copper in the liver may have occurred equally. The concentration of copper, iron, and zinc ions in the diet did not differ among the groups. The reason for the low concentration of iron ion in the livers of the animals in the lycopene group is unknown. Kinase activity of $pp60^{c\text{-}src}$ has been reported to increase proportionally with the development of

HCC in LEC rats (41), but the mechanisms of hepatocarcinogenesis in LEC rats have not been well delineated.

In conclusion, no influence on the incidence of HCC or serum α-fetoprotein levels in LEC rats was noted after the administration of lycopene or TJ-9. The concentration of iron ion was lower in the livers of animals in the lycopene group than in the control group. No statistical difference was found in the volume, mean size, or number of GST-P-positive areas in the liver specimens. The number of GST-P-positive areas in HCC lesions was larger in the TJ-9 group than in the control and lycopene groups.

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