Effects of Dietary Powdered Green Tea and Theanine on Tumor Growth and Endogenous Hyperlipidemia in Hepatoma-bearing Rats

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The effects of dietary powdered green tea (PGT) and theanine on in vivo hepatoma growth and cancerous hyperlipidemia were investigated in rats that had been implanted with a rat ascites hepatoma cell line of AH109A cells. The hepatoma-bearing rats were fed with a 20% casein diet (20C), 20C containing 2% PGT, or 20C containing 0.1% theanine for 14 days. Dietary PGT significantly and time-dependently reduced the solid tumor volume and weight as did dietary theanine. The hepatoma-induced endogenous hyperlipidemia, which was characterized by rises in the serum cholesterol (hypercholesterolemia) and triglyceride (hypertriglyceridemia) levels, was significantly suppressed by PGT and theanine supplementation. Bile acid excretion into the feces was significantly higher in the PGT- and theanine-fed rats than in the control rats. This inhibition of hypercholesterolemia may have resulted from tumor growth suppression as well as increased excretion of steroids from the body. These results suggest that PGT had both anti-proliferative activity toward hepatoma cells and hypolipidemic activity in the hepatoma bearing rats. They also suggest that theanine was, at least in part, responsible for the PGT actions.

Key words: green tea; hepatoma; hyperlipidemia; theanine; tumor growth

Our previous in vitro and ex vivo studies have demonstrated that powdered green tea (PGT), powdered oolong tea, powdered black tea, and related tea components inhibited the proliferation of a rat ascites hepatoma cell line of AH109A and the invasion of the hepatoma cells across rat mesentery-derived mesothelial-cell monolayers. Further studies have indicated that the induction of apoptosis and cell cycle arrest in AH109A cells was an important mechanism for the action of the teas and related tea components. However, it is unclear whether or not teas and the relevant tea components could inhibit AH109A growth in vivo in rats. Hepatoma-bearing rats have shown cancerous hyperlipidemia, this type of endogenous hyperlipidemia often being produced in humans. AH109A-induced hyperlipidemia has been demonstrated to be reduced by such food components as fish oil, arginine, methionine, cystine and S-methyl-L-cysteine sulfoxide, a cysteine derivative present in cabbage. It has been reported that Pu-Erh tea and catechins had hypocholesterolemic activity and suppressed exogenous hyperlipidemia in cholesterol-fed rats.

The effects of tea and tea components on cancer-induced endogenous hyperlipidemia in rats have not previously been reported. In the present study, we investigate the effects of PGT and theanine on in vivo hepatoma growth and cancerous hyperlipidemia in hepatoma-bearing rats. Theanine, γ-glutamylethylamide, was employed as a PGT component, since it is one of the major amino acids in Japanese green tea, and little is known about its actions on cancer and hyperlipidemia as compared with tea cathekins. Theanine is also structurally related to glutamic acid that has shown hypocholesterolemic activity in humans and gerbils.

Materials and Methods

Animals and diets. Male Donryu rats (4 weeks of age) were obtained from NRC Haruna (Gunma, Japan). The animals were treated in accordance with guidelines established by the Animal Care and Use Committee at Tokyo Noko University. They were fed with a stock pellet diet (CE-2; CLEA, Tokyo, Japan) for 7 d and then with a basal diet for another 7 d in an air-conditioned room with an 8:00 a.m. to 8:00 p.m. light cycle. The basal diet (20C in Table 1) was prepared according to the AIN-93G formula.

Abbreviations: AI, atherogenic index; Ch, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PGT, powdered green tea; TBARS, thiobarbituric acid-reactive substance; T-Ch, total cholesterol; TG, triglyceride; VLDL, very-low-density lipoprotein
rats received a subcutaneous implantation of $5 \times 10^4$ AH109A cells suspended in Ca–Mg free phosphate buffered saline [PBS (−)] of 0.5 ml/rat in the back to produce a solid hepatoma.\(^9\) The rats were respectively fed with the 20C diet (control group), or the 20C diet supplemented with either 2% PGT (PGT group) or 0.1% theanine (theanine group) for 14 d (Table 1). Since the theanine content is more than 3% in dried green tea,\(^10\) the 2% PGT diet is roughly estimated to have contained 0.06% theanine. We therefore decided to add 0.1% theanine to the 20C diet for the theanine group. The food intake of each rat was measured every day. PGT was generously provided by Yamato Tea Co. Ltd. (Nara, Japan) and theanine (>98% pure) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Water and each diet were available ad libitum. The animals were deprived of their diet at 9:00 a.m. on the 14th day, but allowed free access to water until killing, which was performed 4 h later by decapitation. Blood was collected and left to clot at room temperature to obtain serum. The liver and solid hepatoma in the back were quickly removed, washed with cold 0.9% NaCl, blotted on filter paper, and weighed.

Determination of tumor growth. The body weight of each rat was recorded every day during the whole animal experiment. The tumor size was measured every day from the day after tumor incidence to the day of killing. The tumor volume was determined in the live animal by measuring the three-dimensional size (height, length and width) of each tumor and using the average of these three measurements as the diameter. The radius ($r = \text{diameter}/2$) was determined, and the volume was calculated as $4/3\pi r^3$.\(^17,18\) After the rats had been killed, the solid tumor in each rat was weighed.

Serum lipid analyses. Serum lipoproteins were separated into the very-low-density lipoprotein (VLDL) + low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions by the precipitation method.\(^4\) Total Ch of the unfractionated serum (VLDL+ LDL) and high-density lipoprotein (HDL-Ch) were enzymatically determined with commercial kits (Wako Pure Chemical Industries, Osaka, Japan), and the difference between T-Ch and HDL-Ch is regarded as (VLDL+LDL)-Ch. The atherogenic index (AI) was calculated as (T-Ch) and HDL-Ch. The concentrations of serum TG and lipid peroxide (thiobarbituric acid reactive substance, TBARS) were determined with commercial kits (Wako Pure Chemical Industries).

Fecal steroid excretion. Feces were collected for 2 d before sacrifice (days 12–14 after tumor implantation). Neutral sterols and bile acids were extracted according to the method of Yamanaka et al.,\(^19\) and then enzymatically determined with commercial kits (Wako Pure Chemical Industries) as previously described.\(^20\)

Statistical analysis. Each result is expressed as the mean ± standard error. Differences between the control (20C) and test (PGT and theanine) groups were compared by a one-way analysis of variance (ANOVA) and then by the Dunnett multiple-comparison test.

Results

As shown in Table 2, the body weight gain and food intake of the PGT and theanine groups over 14 d tended to be higher than those of the control group, although the difference was not significant. The liver weight was not significantly different between the control and tea treatment groups. The incidence of tumors in rats was on the 4th day in the control group and on the 5th day in both the PGT and theanine groups. Compared with the control group, the volume of the primary tumor in the

<table>
<thead>
<tr>
<th>Ingredient (g/kg)</th>
<th>Control (20C)</th>
<th>PGT (2%)</th>
<th>Theanine (0.1%)</th>
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<tr>
<td>PGT(^1)</td>
<td>0.000</td>
<td>20.000</td>
<td>0.000</td>
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<tr>
<td>Theanine(^2)</td>
<td>0.000</td>
<td>0.000</td>
<td>1.000</td>
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<td>Cornstarch(^3)</td>
<td>397.486</td>
<td>377.486</td>
<td>396.486</td>
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<td>Casein(^4)</td>
<td>200.000</td>
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<td>200.000</td>
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<td>α-Cornstarch(^5)</td>
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<td>132.000</td>
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<td>Sucrose(^6)</td>
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<td>Vitamin mixture(^10)</td>
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<td>l-Cystine(^11)</td>
<td>3.000</td>
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<tr>
<td>Choline bitartrate(^12)</td>
<td>2.500</td>
<td>2.500</td>
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<tr>
<td>tert-Butylhydroquinone(^13)</td>
<td>0.014</td>
<td>0.014</td>
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</table>

\(^1\) Yamato Tea Co., Nara, Japan.  
\(^2\) Tokyo Kasei Kogyo Co., Tokyo, Japan.  
\(^3\) Ajinomoto Co., Inc., Tokyo, Japan.  
\(^4\) Miyazawa Yakuhin Co., Tokyo, Japan.  
\(^5\) Nihon Nosan Kogyo, Yokohama, Japan.  
\(^6\) AIN-93G composition.  
\(^7\) AIN-93 composition.  
\(^8\) Oriental Yeast Co., Tokyo, Japan.  
\(^9\) Mitsui Sugar Co., Tokyo, Japan.  
\(^10\) Wako Pure Chemical Industries, Osaka, Japan.  

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>PGT</th>
<th>Theanine</th>
</tr>
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<tr>
<td>Initial body weight (g)</td>
<td>166.9 ± 3.9</td>
<td>167.0 ± 3.2</td>
<td>167.0 ± 3.0</td>
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<tr>
<td>Body weight gain (g/14d)</td>
<td>68.6 ± 4.7</td>
<td>79.0 ± 4.2</td>
<td>76.9 ± 4.9</td>
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<tr>
<td>Food intake (g/14d)</td>
<td>207.0 ± 4.3</td>
<td>219.7 ± 5.9</td>
<td>221.3 ± 10.4</td>
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<tr>
<td>Liver weight (g/rat)</td>
<td>9.9 ± 0.3</td>
<td>10.2 ± 0.3</td>
<td>9.9 ± 0.4</td>
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</table>

Each value is expressed as the mean ± SEM of 10 rats in each group.
Fig. 1. Inhibitory Effects of Dietary PGT and Theanine on the Growth (A, Volume; B, Weight) of Solid Tumors in Hepatoma-bearing Rats.

A. Tumor volume was determined in live animals by measuring the three-dimensional size (height, length and width) of each tumor and using the average of the three measurements as the diameter. The radius (r = diameter / 2) was determined, and the volume was calculated as described in the Materials and Methods section (volume = 4/3πr³). B. After the rats had been killed, the primary tumors in each rat were respectively weighed as described in the Materials and Methods section. Each value and bar represents the mean and SEM of 10 rats in each group.* Values are significantly different at P < 0.05 / P < 0.01 by the Dunnett multiple-comparison test when compared to the control group.

Fig. 2. Effects of Dietary PGT and Theanine on the Serum Cholesterol Level and Atherogenic Index (A), and Serum Triglyceride and Lipid Peroxide Levels (B) in Hepatoma-bearing Rats.

Serum lipid analyses were carried out as described in the Materials and Methods section. Each value and bar represents the mean and SEM of 10 rats in each group.* Values are significantly different at P < 0.05 / P < 0.01 by the Dunnett multiple-comparison test when compared to the control group.

rats was significantly and time-dependently less in both the PGT and theanine groups, although the effect of theanine was less in the later stage (Fig. 1(A)). The absolute weight of solid tumor was significantly lower in the PGT group by 27.2% and lower but not significantly so in the theanine group by 19.2% (Fig. 1(B)) than in the control group; the relative weight (g/100 g of body weight) of the primary tumor was significantly lower by 30.7% in the PGT group and by 19.7% in the theanine group (data not shown). The size (diameter) of solid tumors in the PGT and theanine groups was in proportion to the volume of primary tumors (data not shown).

As shown in Fig. 2(A), the serum levels of T-Ch and (VLDL + LDL)-Ch in the rats with the tea treatment were significantly lower and serum HDL-Ch was significantly higher in both the PGT and theanine groups than in the control group. These changes resulted in noticeably lower AI in the two groups. Serum TG was significantly lower by 42.3% and 46.2%, respectively, in both the PGT and theanine groups than in the control group (Fig. 2(B)). The serum lipid peroxide (TBARS) level was significantly lower by 17.4% in the theanine group and tended to be lower by 14.1% in the PGT group than in the control group (Fig. 2(B)).

Compared with the control group, the weight of dried feces was significantly higher in the PGT group
but similar in level in the theanine group (Fig. 3(A)).

The bile acid excretion was significantly higher in both the PGT and theanine groups than in the control group (Fig. 3(B)); the excretion of enzymatically assayed neutral sterols was significantly higher in the PGT group than in the control group, but similar in level in the theanine group (data not shown).

Discussion

Based on the palpation in the back of the rats, the incidence of solid tumors was delayed for one day in the PGT and theanine groups when compared with the control group without the tea treatment. The volume of tumors in the rats was significantly and time-dependently lower in both the PGT and theanine groups. The weight of solid tumors per rat on the 14th day was significantly lower in the PGT group and a little lower in the theanine group than in the control group. PGT caused much stronger inhibition of solid tumor growth than did theanine throughout the whole experimental period (Fig. 1). This inhibition of tumor growth by PGT may have been due to its anti-proliferative activity by inducing apoptosis and cell cycle arrest in the AH109A cells, since our previous in vitro and ex vivo results confirmed that PGT and the sera from PGT-treated rats induced apoptosis and cell cycle arrest in AH109A cells. Our separate experiment indicated that, like carotenoids, chlorogenic acid and curcumin, theanine suppressed the in vitro invasion of AH109A in a cell culture system at concentrations at which the amino acid did not suppress the in vitro proliferation of AH109A. Nonetheless, theanine could inhibit the in vivo growth of hepatoma cells in rats. Although the reason for this phenomenon is not clear at present, there is a possibility that theanine might have suppressed in vivo a certain target other than tumor cells; for instance, theanine might have suppressed angiogenesis in the tumor tissue like glycine, and indirectly inhibited the tumor growth by intercepting the supply of nutrients and oxygen. The total weight of metastasized tumors in the body was significantly lower in the PGT and theanine groups than in the control group (data not shown). This effect was not only due to growth inhibition, but also due to the suppression of some steps of metastasis by PGT and theanine. Further studies are required to clarify these aspects.

The rats that had received a subcutaneous implantation of the ascites hepatoma cell line of AH109A displayed cancerous hyperlipidemia characterized by increased serum cholesterol (hypercholesterolemia) and triglyceride (hypertriglyceridemia) levels. The hypercholesterolemia in the hepatoma-bearing rats showed a highly atherogenic lipoprotein profile, that is, an enormous increase in the VLDL + LDL fraction and significant decrease in the HDL fraction. Increased cholesterogenesis in the host liver and decreased fecal bile acid excretion lead to hypercholesterolemia. Tea and tea catechins are well documented to improve exogenous hyperlipidemia. However, the effects of other components in green tea on hyperlipidemia are not well understood. Theanine is the most abundant amino acid in Japanese green tea, having been synthesized from glutamic acid and ethylamine in the tea root and then transferred to the tea leaves. After an intragastric administration to animals, theanine is transported through the intestinal brush-border membrane, incorporated into the serum, liver and brain, and excreted unchanged into the urine or after being degraded into glutamic acid and ethylamine. In the present study, PGT and theanine significantly reduced the levels of serum T-Ch, (VLDL + LDL)-Ch and AI, but increased the level of HDL-Ch in hepatoma-bearing rats, when compared with the control group (Fig. 2(A)). A positive correlation has been found between the hepatoma weight and (VLDL + LDL)-Ch concentration, and a negative correlation between the hepatoma weight and HDL-Ch. Thus, the hypocholesterolemic effects of PGT...
and theanine may be, at least in part, attributed to their inhibitory action on tumor growth (Fig. 1). This inhibition of hypercholesterolemia may also have resulted from the increase in fecal bile acid excretion by rats ingesting PGT and theanine (Fig. 3), besides the suppression of tumor growth by PGT and theanine. Decreased triglyceride clearance from the circulating blood is considered to be the cause of hepatoma-induced hypertriglycerideremia. PGT and theanine significantly reduced the serum triglyceride level (Fig. 2B). The inhibition of tumor growth by PGT and theanine may have partially resulted in the decreased hepatoma-induced hypertriglycerideremia, although this decrease may be attributable to other mechanisms such as the activation of lipoprotein lipase in the adipose tissue. Further studies are needed to clarify the precise modes of hypolipidemic actions of PGT and theanine.

In summary, dietary PGT had both an anti-proliferative action on tumor growth and hypolipidemic activity in hepatoma-bearing rats. Theanine was at least partly responsible for the PGT action. PGT and its constituent theanine were thus demonstrated to simultaneously suppress endogenous hyperlipidemia as well as tumor growth.

References

23) Kozuki, Y., Miura, Y., and Yagasaki, K., Inhibitory


