Inhibitory Effect of Coffee on Hepatoma Proliferation and Invasion in Culture and on Tumor Growth, Metastasis and Abnormal Lipoprotein Profiles in Hepatoma-Bearing Rats

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Summary We have already reported that instant coffee powder (ICP) and ICP-loaded rat sera could suppress proliferation and invasion of rat ascites hepatoma cell line of AH109A in vitro. In this report, we examined the mechanisms for suppression of tumor cell proliferation and invasion by ICP, and the effect of ICP on in vivo tumor growth, metastasis and abnormal lipoprotein profiles in hepatoma-bearing rats. ICP, when directly added to the culture media, induced cell cycle arrest (elongation of S phase) at a lower concentration (0.3 mg/mL) and apoptosis at a higher concentration (0.6–1.2 mg/mL). ICP and ICP-loaded rat sera showed reactive oxygen species (ROS)-scavenging property and canceled the enhancement of invasive activity of hepatoma cells induced by ROS in vitro. These results suggest that ICP suppresses the proliferation by inducing cell cycle arrest and apoptosis, and the invasion by scavenging ROS and that ICP could retain these properties after their gastrointestinal absorption. The hepatoma-bearing rats were fed with a 20% casein diet (20C) or 20C supplemented with 0.1% ICP for 14 d. Dietary ICP significantly reduced solid tumor growth and tended to reduce hepatoma metastases to lung and lymphatic nodes, suggesting that ICP could suppress tumor cell proliferation and invasion in vivo. In addition, dietary ICP significantly increased serum high-density lipoprotein (HDL)-cholesterol and tended to reduce very low-density and low-density lipoprotein (VLDL+LDL)-cholesterol, resulting in amelioration of abnormal lipoprotein profiles occurred in hepatoma-bearing rats. In conclusion, ICP has the ability to induce cell cycle arrest and apoptosis in hepatoma cells and to suppress tumor cell invasion by reducing oxidative stresses in vitro, and it could also exhibit these effects in vivo, leading to the inhibition of tumor growth and metastases.

Key Words coffee, tumor growth, metastasis, invasion, serum lipoproteins
Effect of Coffee on Hepatoma Growth and Metastasis

Tocoy growth factor (HGF), induced by endogenous and exogenous reactive oxygen species (ROS) (8). Some coffee components reportedly have anti-oxidative activities (9). Thus, we checked the possibility that coffee suppressed tumor cell invasion through its anti-oxidative property. In the present study, we found that coffee could suppress the proliferation of AH109A cells by inducing cell cycle arrest and apoptosis. Coffee also inhibited the invasion of AH109A cells by scavenging ROS, which augmented the invasive activity. In addition, these properties of ICP were retained after their gastrointestinal absorption, because ICP-loaded rat sera could efficiently scavenge ROS and significantly suppress the invasion of AH109A cells induced by ROS.

In the meantime, anti-proliferative and anti-invasive activities of coffee in vitro prompted us to investigate the in vivo effects of coffee on tumor cell growth and metastases. We also examined the in vivo effect of coffee on abnormal lipoprotein profiles, which inevitably occurred in hepatoma-bearing rats and is considered one of the symptoms of cancerous cachexia (2, 10). Feeding a coffee-supplemented diet proved to significantly reduce tumor size and weight in hepatoma-bearing rats, simultaneously showing the tendency to suppress tumor metastases. Moreover, dietary ICP significantly increased serum high-density lipoprotein (HDL)-cholesterol, and tended to reduce very low-density and low-density lipoprotein (VLDL + LDL)-cholesterol, resulting in amelioration of abnormal lipoprotein profiles that occurred in hepatoma-bearing rats. These results indicate the efficacy of coffee for the secondary prevention of tumors.

MATERIALS AND METHODS

Instant coffee powder (ICP), generously provided from Nestle Japan (Tokyo, Japan), was used throughout the experiments in this paper. Male Donryu rats were purchased from NRC Haruna (Gunma, Japan). They were fed a stock pellet diet (CE-2, CLEA Japan, Tokyo) and tap water ad libitum, unless otherwise described in the text. All procedures for animal experiments in this report were approved by the Animal Care and Use Committee of Tokyo Noko University. AH109A cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan and maintained in the peritoneal cavity of Donryu rats as described previously (4, 5, 6). The effect of ICP on spontaneous apoptosis in AH109A cells was examined by both Annexin V apoptosis detection kit (Beckman-Coulter, Hialeah, FL, USA) and detecting DNA ladder formation. The appearance of phosphatidylserine (PS) in the cell surface was thought to be a hallmark of early apoptosis. AH109A cells (2 × 10^5) were cultured in the medium containing 0.6 mg/mL of ICP for 3 or 6 h and analyzed according to the manufacturer's instructions. The DNA ladder formation was another hallmark of apoptosis. The level of DNA fragmentation in AH109A cells cultured in the presence of ICP (1.2 mg/mL) for 24 h was determined as described previously (11).

We have already reported that exogenously added ROS, which was generated by hypoxantine and xanthine oxidase, augmented the invasive activity of AH109A cells (12). The effects of ICP and ICP-loaded rat sera on ROS-augmented invasive activity of AH109A cells and the ROS scavenging property of ICP and ICP-loaded rat sera in those AH109A cells were examined using the invasion assay system mentioned above and by flow cytometry using 2',7'-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) as a fluorescence indicator, respectively, as described previously (8). ICP-loaded rat sera were prepared from blood collected 2 h after oral intubation with ICP (12.5 mg/0.5 mL/100 g body weight) to Donryu rats.

The in vivo effect of ICP on the growth and metastasis of AH109A was assessed as follows. After a 1 wk preliminary feeding, twenty male Donryu rats were subcutaneously implanted with 1 × 10^7 cells of AH109A in the back and divided into two groups. One group (11 rats) was fed with a control diet (20% casein diet formulated to AIN93G; 39.75% cornstarch (Nihon Nosan Kogyo), 3.5% mineral mixture (AIN-93 composition, Nihon Nosan Kogyo), 10.0% sucrose (Shin Mitsu Sugar, Tokyo, Japan), 7.0% soybean oil (Miyazawa Yukinori, Tokyo, Japan), 5.0% cellulose powder (Oriental Yeast, Tokyo, Japan), 3.5% mineral mixture (AIN-93G composition, Nihon Nosan Kogyo), 1.0% vitamin mixture (AIN-93 composition, Nihon Nosan Kogyo), 0.3% L-cystine (Ajinomoto, Yokohama, Japan), 0.25% choline bitartrate (Waiko Pure Chemical Industries, Ltd., Osaka, Japan) and the other group (9 rats) was fed with ICP diet (20% casein diet supplemented with 0.1% ICP at
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**Fig. 1.** Effect of ICP on cell cycle of AH109A cells. AH109A (2×10^5 cells) were seeded in the medium containing 0.3 mg/mL of ICP and cultured for 0, 12 and 24 h and analyzed as described in Materials and Methods. The distribution of cells in G1, S and G2/M phases were calculated and indicated in figures.

**Fig. 2.** Induction of apoptosis by ICP in AH109A cells. The effect of ICP on spontaneous apoptosis in AH109A cells was assessed (A) by Annexin V apoptosis detection kit and (B) by detecting DNA ladder formation as described in Materials and Methods.

the expense of cornstarch) for 2 wk. Body weight and food intake were measured daily. Tumor growth was monitored daily, and long and short diameters of solid tumors were measured by using calipers. After 2 wk, rats were decapitated and blood was collected. Primary tumors and livers were removed and weighed. Metastatic tumor foci in lung and inguinal and axillary lymphatic nodes were macroscopically examined, excised and the number of metastatic foci were recorded. Serum was prepared from blood and the concentrations of total, HDL- and (VLDL+LDL)-cholesterol were determined as described previously (2). Serum concentrations of triglyceride and thiobarbituric acid reactive substances (TBARS) were also determined by commercial kits (Wako Pure Chemical Industries, Ltd.).

Data were indicated as means±SEM. Statistical significance was inspected by ANOVA followed by Tukey's Q test (Fig. 3A and 3C) or by Student's t-test (Fig. 4 and Table 1). P value less than 0.05 was considered statistically significant.

**RESULTS**

**Induction of cell cycle arrest and apoptosis in AH109A cells by ICP**

The growth rate of cells was determined by the balance of cell proliferation rate and death rate. To clarify the mechanism(s) for the inhibition of AH109A growth by ICP, we first examined the effect of ICP on cell cycles of AH109A cells. As shown in Fig. 1, the fraction of cells in the S phase was found to increase at 12 or 24 h after the addition of ICP (0.3 mg/mL), when compared with 0 h control cells. Relative decreases in the fractions...
of cells in the G1 phase and G2/M phase were observed. This result suggests that ICP induces cell cycle arrest in AH109A by elongating S phase. Next, we examined whether or not ICP induced apoptosis in AH109A cells. Phosphatidylserine (PS), which can specifically bind to Annexin V, is one of the phospholipids in the cell membrane and exists predominantly in the inner leaflet of the cell membrane of normal cells. When apoptosis occurs, PS in the cell membrane immediately appears on the outer leaflet of the cell membrane. The cells which have their PS on their surface, therefore, are thought to be early apoptic cells. ICP, when directly added to the medium at the concentration of 0.6 mg/mL, increased the proportion of Annexin V-positive, PI-negative cells (Fig. 2A, 3h and 6h treatments, see quadrant 4). PI staining means the damage of cell membranes in these experiments, that is, necrosis. In addition, the higher concentration of ICP (1.2 mg/mL) clearly induced DNA fragmentation in AH109A cells, which is the other hallmark of apoptosis (Fig. 2B). These results clearly suggest that ICP affects both cell proliferation and death, causing strong inhibition of the growth of AH109A cells.

Inhibition of AH109A cell invasion by ICP via its ROS scavenging activity

We have already reported that ROS, generated both endogenously and exogenously, were deeply involved in AH109A cell invasion and ROS scavenging substances could suppress their invasion (8, 12). ICP is known to contain several antioxidative components (9). Therefore, we investigated whether the antioxidative property of ICP was involved in its anti-invasive activity. As aforementioned, ICP could suppress the proliferation of AH109A cells. To prevent that the suppressive effect of ICP on proliferation may bias against its effect on invasion, we first determined the dose and duration of ICP treatment, which do not affect AH109A cell proliferation. Although AH109A cells pretreated with 0.3 mg/mL ICP for 1h showed the comparable proliferative profile to the control cells (Control; 100.0 ± 2.5% vs ICP; 98.0 ± 1.5%, p>0.05), this pretreatment certainly reduced the intracellular peroxide level in AH109A cells and significantly reversed the intracellular peroxide level augmented by HX-XO treatment to the control level (Fig. 3P). Under the same condition, the invasive activity of AH109A cells significantly increased by the treatment with ROS and the simultaneous pretreatment with ICP (0.3 mg/mL, 1 h) significantly reduced it to the control level (Fig. 3A). These results suggest that ICP inhibits the invasion of AH109A cells via its ROS scavenging property. Furthermore, ICP-loaded rat sera (12.5 mg/0.5 mL/100 g body weight) showed no effect...
Table 1. Effect of an ICP-supplemented diet on food intake, body weight gain, liver and hepatoma weights, and serum and liver lipids levels in hepatoma-bearing rats.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>ICP</th>
</tr>
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<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>115.1±4.5</td>
<td>113.0±4.7</td>
</tr>
<tr>
<td>Food intake (g/14 d)</td>
<td>269.6±10.5</td>
<td>286.4±9.7</td>
</tr>
<tr>
<td>Body weight gain (g/14d)</td>
<td>111.9±5.1</td>
<td>123.2±5.1</td>
</tr>
<tr>
<td>Liver weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute (g)</td>
<td>10.3±0.6</td>
<td>11.1±1.0</td>
</tr>
<tr>
<td>Relative (g/100 g body wt.)</td>
<td>4.5±0.2</td>
<td>4.6±0.2</td>
</tr>
<tr>
<td>Hepatoma weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute (g)</td>
<td>5.2±1.1</td>
<td>2.3±0.7*</td>
</tr>
<tr>
<td>Relative (g/100 g body wt.)</td>
<td>2.4±0.5</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>Serum lipid levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBARS (nmol/mL)</td>
<td>13.1±0.8</td>
<td>10.3±0.6*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>90.6±7.9</td>
<td>92.3±9.3</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>104.6±6.8</td>
<td>112.4±6.8</td>
</tr>
<tr>
<td>HDL-Ch (mg/dL) A)</td>
<td>52.7±3.2</td>
<td>70.4±7.9*</td>
</tr>
<tr>
<td>(VLDL+LDL)-Ch (mg/dL) (B)</td>
<td>51.9±5.9</td>
<td>42.0±3.2</td>
</tr>
<tr>
<td>Atherogenic index (B/A)</td>
<td>1.02±0.14</td>
<td>0.67±0.09</td>
</tr>
<tr>
<td>Phospholipid (mg/dL)</td>
<td>158.7±9.3</td>
<td>181.7±14.7</td>
</tr>
<tr>
<td>Liver lipid levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride (mg/g liver)</td>
<td>16.4±2.8</td>
<td>18.0±2.9</td>
</tr>
<tr>
<td>Total-Ch (mg/g liver)</td>
<td>2.23±0.11</td>
<td>2.24±0.05</td>
</tr>
<tr>
<td>Phospholipid (mg/g liver)</td>
<td>26.5±0.7</td>
<td>26.8±1.1</td>
</tr>
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</table>

Each value represents the mean±SEM for 11 (Control) and 9 (ICP) rats.

*Significantly different at p<0.05, compared to the control group by Student's t-test.

On AH109A proliferation, when cells were pretreated for 1 h (Control rat sera; 100.0±1.6% vs ICP-loaded rat sera; 100.3±1.6%, p>0.05), but they could also scavenge ROS and cancel ROS-induced hepatoma cell invasion under the same condition (Fig. 3C and 3D).

Effect of ICP on in vivo tumor growth and metastasis

Aforementioned results and our previous results (6) indicate the possibility that ICP may suppress the in vivo growth and metastasis of AH109A cells. We finally examined the effect of the ICP-supplemented diet on tumor growth and metastasis. The effect on abnormal lipoprotein profiles, which inevitably occurred in tumor-bearing rats, was also examined. In the previous ex vivo experiment (6), we found that serum obtained from rats which received single oral administration of ICP at doses more than 0.125 g/kg body weight, significantly inhibited both the proliferation and invasion of AH109A cells in culture. The lowest effective dose (approximately 0.1 g/kg) of ICP was calculated to be equivalent to a dietary content of 0.1%. Thus, we employed 0.1% as dietary ICP content. In fact, rats of the ICP group ingested approximately 0.1 g/kg of ICP per day (Table 1).

As shown in Fig. 4, tumor growth rate in rats fed with ICP-supplemented diet was slower than that in control rats. From day 9 to day 14 after tumor implantation, tumor sizes, indicated as the sum of long and short diameters. Asterisks indicate statistical significances at p<0.05 by Student's t-test.

During the 14-d feeding period, food intakes and body weight gains between the control and ICP groups were not significantly different (Table 1). There was no significant difference in absolute and relative weights of livers (Table 1). As expected from Fig. 4, final absolute hepatoma weight of ICP-fed rats was significantly lower than that of control rats (Table 1). These results suggest that ICP in the diet could show a suppressive effect on the in vivo growth of AH109A cells without any effect on the food intake and the body weight gain. Table 2 summarizes the result of AH109A cell metastases. Although three of eleven control rats exhibited some metastatic foci (six for three rats), none of the ICP-fed rats exhibited any metastatic foci. These results also suggest that ICP could show a suppressive effect on the in vivo invasion of AH109A cells, leading to suppression of metastasis.

While the serum concentration of total cholesterol tended to increase in ICP-fed rats compared to control rats, the concentration of HDL-cholesterol significantly increased and that of (VLDL+LDL)-cholesterol tended to decrease by feeding an ICP-supplemented diet, causing the decrease in atherogenic index (Table 1). These results mean that dietary ICP could ameliorate abnormal lipoprotein profiles that occurred in hepatoma-
bearing rats. Dietary ICP had no effect on the concentrations of serum triglyceride and phospholipid (Table 1). However, dietary ICP significantly decreased serum concentration of TBARS, suggesting that ICP also showed anti-oxidative activities in vivo. Dietary ICP had no effect on liver lipid contents (Table 1).

**DISCUSSION**

In our previous paper (6), we reported that ICP could suppress the proliferation and invasion of rat ascites hepatoma cells in culture and that sera prepared from ICP-loaded rats also retained the suppressive effects on their proliferation and invasion. This suggested that ICP contained anti-proliferative and anti-invasive components and that these effective components could be absorbed from gastrointestinal tracts or that their metabolites showed anti-proliferative and anti-invasive activities. However, the precise mechanisms for coffee’s effects on proliferation and invasion remained unknown. In this report, we have investigated these mechanisms in vitro.

First, we have clarified that ICP could induce cell cycle arrest in AH109A cells by elongating S phase and that ICP could induce apoptosis in AH109A cells. These results indicate that ICP suppressed the growth of hepatoma cells by affecting both their proliferation and death. Some compounds or stimuli were reported to induce cell cycle arrest by decreasing the rate of S phase progression (13, 14). These effects seem to be attributable to their inhibitory effects on DNA polymerase α. Some components in coffee, that could inhibit the activity of DNA polymerase α, might be involved in anti-proliferative activity of coffee. At present, we have not identified the effective component(s) in coffee that could induce cell cycle arrest or apoptosis in AH109A cells. Mazzuca et al. reported that trigonelline induced cell cycle arrest in plant cells (15). Jang et al. (16) and Jiang et al. (17) respectively reported that caffeine and chlorogenic acid induced apoptosis in human tumor cell lines. However, we have already confirmed that these coffee components had no effect on the proliferation of AH109A cells (data not shown). To characterize the effective components in coffee on proliferation of AH109A cells is important and related studies are now in progress in our laboratory.

Tumor cell invasion is the most important and characteristic step in tumor metastasis. In this report, we have clarified that the antioxidative property of coffee was deeply involved in its anti-invasive effect. We also have clarified that sera prepared from ICP-loaded rats show similar antioxidative properties and could cancel ROS-induced invasion of hepatoma cells. These results suggest that effective components in ICP could be absorbed from gastrointestinal tract and they or their metabolites retain the effectiveness in blood, although the possibility that orally administered-ICP induced antioxidative factors originated from the host could not be excluded.

Caffeic acid (18, 19) was reported to show anti-invasive activities via its anti-inflammatory activity. However, we have already found that caffeic acid, chlorogenic acid and quinic acid (7) and trigonelline (20) could show both anti-oxidative and anti-invasive effects on AH109A cells. These compounds proved to retain their effectiveness after oral intubation (20). Although a possibility that some components in coffee inhibit tumor cell invasion by inhibiting some metastasis-relating molecules or enzymes cannot be excluded, our results indicate that the anti-invasive effect of coffee may be due to anti-oxidative properties of these components.

Coffee itself or some coffee components, that is, caffeic acid or chlorogenic acid, are reportedly known to prevent carcinogenesis in rodent models (21–23). However, little is known about the effect of coffee on implanted tumor growth and their metastasis. In the present study, we have examined the effect of a coffee-supplemented diet on solid tumor growth and their metastases. The results showed that tumor growth in rats fed with the ICP-supplemented diet decreased as compared with that in control rats. As mentioned above, ICP could suppress AH109A cell proliferation in vitro and ex vivo. These results also suggest that ICP and/or metabolites of ICP components can suppress tumor growth in vivo. Although we have not yet characterized the effective components in ICP that inhibit AH109A cell proliferation, Miller et al. reported that feeding with the diet containing kahweol and cafestol, ingredients of coffee beans, prevents carcinogen-induced tumor growth in hamsters (24). The effects of these compounds should be investigated in the future. ICP also tended to suppress in vivo metastases of AH109A cells. ICP could show their anti-oxidative properties after oral intubation, because serum TBARS concentration was significantly lower in ICP-fed rats than that in control rats. These in vivo anti-oxidative properties would be involved in the inhibitory effect of ICP on metastases, by inhibiting the invasion of AH109A cells as mentioned above.

We have also found that ICP could ameliorate abnormal lipoprotein profiles in hepatoma-bearing rats (Table 1). We have already reported that rats implanted with AH109A cells show the elevated (VLDL+LDL)-Ch level and decreased HDL-Ch level as compared to control rats (2, 25). By feeding the ICP-supplemented diet, serum HDL-Ch concentration significantly increased, resulting in the decreased atherogenic index. Nicotinic acid was reported to increase serum HDL-Ch concentration (26) and ICP contained a significant amount of nicotinic acid (21–47 mg/100 g ICP powder). Thus, nicotinic acid would be one of effective components in ICP that increase HDL-Ch concentration, although possibilities of the involvement of other components cannot be excluded.

Coffee was epidemiologically known to prevent carcinogenesis (27, 28). In this paper, we have found that coffee has the ability to prevent tumor growth and metastasis and have clarified their mode of actions. These results suggest that ICP could become the promising food for both primary and secondary prevention of
tumors. Further studies are needed and will benefit for the creation of new functional foods based on the effect of coffee.

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REFERENCES


