The effect of apple polyphenol extract (APE) on the proliferation and invasion of a rat ascites hepatoma cell line of AH109A was examined in vitro. APE suppressed both the hepatoma proliferation and invasion in a dose-dependent manner up to 200 μg/ml. Serum obtained from rats orally given APE also inhibited hepatoma proliferation and invasion when added to the culture medium. Subsequently, the effect of dietary APE on growth and the metastasis of AH109A hepatomas were investigated in vivo. APE reduced the growth and metastasis of solid hepatomas and significantly suppressed the serum lipid peroxide level in rats transplanted with AH109A. APE also suppressed the serum very-low-density lipoprotein + low-density lipoprotein (VLDL + LDL)-cholesterol level. These in vitro and in vivo findings suggest that APE has anti-hepatoma activities.

Key words: apple polyphenol extract; hepatoma; hyperlipidemia; metastasis; proliferation

Cancer cells often undermine the life of the affected host by their endless proliferation and metastasis, which are two biological characteristics of cancer cells. Metastasis is achieved in a series of complex and sequential steps. Invasion is the most important step in tumor metastasis. Tumor cells are known to produce a larger amount of reactive oxygen species (ROS) than do normal cells, and ROS perform critical signaling functions in proliferation. In our previous study, ROS were found to potentiate the invasive activity of the rat ascites hepatoma cell line of AH109A in the co-culture system with rat mesentery-derived mesothelial cells (M-cells), and some food factors, especially polyphenolic components possessing antioxidative properties, were found to inhibit the ROS-potentiated invasion of AH109A cells. Tea catechins were found to suppress the proliferation of AH109A cells as well as the invasion of the hepatoma cells. Apple polyphenol extract (APE) has antioxidative activity, anti-allergic activity, and anti-melanogenic activity. Recently, apple polyphenols have been found to induce apoptosis in cancer cells. So far, little is known about the effects of APE on the invasion and metastasis of cancer cells. Hence, one of the aims of this study was to investigate the effects of APE on the invasion and metastasis as well as the proliferation of AH109A cells.

Like other tumors, hepatomas often induce abnormal serum lipid metabolism in humans and animals. AH109A cells form solid tumors and also induce abnormal serum lipoprotein profiles when subcutaneously inoculated into rats. AH109A-induced hyperlipidemia has been found to be reduced by food components such as methionine, S-methyl-L-cysteine sulfoxide, a cysteine derivative present in cabbage, theanine, resveratrol, and pre-germinated brown rice. Hence, another purpose of this study was to examine the effects of dietary APE on hyperlipidemia as well as growth and metastasis of hepatomas in rats inoculated with AH109A cells, an in vivo model.

Materials and Methods

Apple polyphenol extract. Apple polyphenol extract (APE) was purchased from Nikka Whisky Distilling Co., Ltd. (Tokyo). It was prepared from unripe apples and contained about 40% condensed tannin, 20% chlorogenic acid, 7% procyanidin B2, 6% (−)-epicatechin, 4% phloridzin, 3% caffeic acid, 3% procyanidin B1, 1% p-coumaric acid, 1% phloretin, 0.5% (−)-catechin and unknown residual components.
Culture of AH109A hepatoma cells. AH109A cells were obtained from the Cell Resource Center for Biomedical Research of Tohoku University (Sendai, Japan). The cells were cultured as described previously in Eagle’s minimum essential medium (MEM, Nissui Pharmaceutical Co., Tokyo), containing 10% calf serum (CS, JRH Biosciences, Inc., Lenexa, KS).

Preparation of serum from APE-treated rats (ex vivo assay). APE was dissolved in water at a concentration of 600 mg/ml. APE aqueous solution was administered p.o. at a dose of 300 mg/100 g of body weight by stomach tube to 5-week-old male Donryu rats (NRC Haruna, Gunma, Japan), which had been fasted overnight. Prior to fasting, the rats were maintained on the AIN-93G basal diet for 5 d. Blood samples were collected 0, 1, 2, 3, 6, and 12 h after oral administration. To investigate the effects of serum samples with different metabolite concentrations on hepatoma cell proliferation and invasion, rats fasted overnight were orally given APE at 0, 10, 30, 100, and 300 mg/100 g of body weight. Blood was collected 2 h after oral intubation and clotted, and the serum was separated by centrifugation. Serum samples were sterilized by filtration and then added to the test medium at 10% concentration instead of CS.

In vitro proliferation and invasion assays. The effects of APE and serum samples obtained from rats given APE on AH109A proliferation were studied by measuring the incorporation of [methyl-3H]thymidine (20 Ci/mmole, New England Nuclear, Boston, MA) into a DNA fraction, as previously described. Briefly, AH109A cells were exposed to test samples for 24 h. The proliferation of AH109A cells was evaluated by measuring the incorporation for the last 4 h of [methyl-3H]thymidine into the DNA fraction. The effects of APE and serum samples on AH109A invasion were investigated in a co-culture system of hepatoma cells with mesentery-derived mesothelial cells (M-cells). The invasion assay was based on the method of Akedo et al. with slight modifications, as described previously.

Hepatoma-bearing rats and their diets. All the animal experiments were conducted in accordance with guidelines established by the Animal Care and Use Committee of Tokyo Noko University and were approved by this committee. Male 4-week-old Donryu rats (NRC Haruna) were individually housed in stainless-steel cages with wire bottoms in an air-conditioned room at a temperature of 22 ± 2 °C, a relative humidity of 60 ± 5%, and an 8:00 to 20:00 light cycle. Rats were maintained on a stock CE-2 pellet diet (CLEA Japan, Tokyo). Prior to tumor cell injection, the rats were fed an AIN-93G basal diet containing 20% casein (20C) for 3 d. On the day of tumor cell inoculation, the rats were randomized to control and APE groups and fed the AIN-93G diet or AIN-93G supplemented with either 0.3% or 1.0% APE for 21 d. APE was added to the diet at the expense of β-cornstarch. Each rat was implanted s.c. on one site in the dorsal region with 1 × 10⁶ AH109A cells suspended in Ca²⁺, Mg²⁺-free phosphate buffered saline [PBS(−)] (0.5 ml/rat) to produce a solid tumor, as described previously. The size of each solid tumor was measured every day, starting from the day of tumor appearance until the day of sacrifice. The tumor size was expressed as the sum of three measured dimensions, viz. height, length, and width. On day 21, the rats were deprived of their diets at 9:00 but allowed free access to water until they were sacrificed at 13:00. Body weight was recorded, and blood was collected for serum separation. The liver and solid tumor were dissected, washed with 0.9% cold NaCl, blotted on filter paper, and weighed. Metastatic tumor foci in lung and inguinal and axillary lymphatic nodes were macroscopically examined and excised, and the numbers of metastatic foci were recorded. Body weight and food intake were measured daily.

Lipid and lipoprotein analyses. Concentrations of triglyceride and total cholesterol (T-Ch) in unfractioned serum and high-density lipoprotein cholesterol (HDL-Ch) were enzymatically determined with commercial kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan), as described previously. The difference between T-Ch and HDL-Ch was regarded as the very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol [(VLDL + LDL)-Ch]. The atherogenic index (AI) was calculated as (VLDL + LDL)-Ch over HDL-Ch, viz., (VLDL + LDL)-Ch/HDL-Ch. The serum lipid peroxide concentration was estimated as thiobarbituric acid reactive substances (TBARS) with a commercial kit (Wako Pure Chemical Industries, Ltd.).

Fecal steroid excretion. Feces were collected for 2 d before sacrifice. Neutral sterols and bile acids were extracted according to the method of Yamanaka et al., then enzymatically determined with commercial kits (Wako Pure Chemical Industries, Ltd.), as described previously.

Statistical analyses. Data were expressed as means ± SEM. Differences between group means were compared by Student’s t-test. Multigroup comparisons were carried out by one-way analysis of variance, followed by the Tukey-Kramer multiple comparisons test (Instat ver. 2.00, GraphPad Software Inc., San Diego, CA). Values of P < 0.05 were considered statistically significant.

Results

Effect of APE on proliferation and invasion of AH109A cells in culture

Figure 1 shows the inhibitory effect of APE against the proliferation and invasion of AH109A cells in the in vitro and ex vivo experiments. APE dose-dependently
**Fig. 1.** Effect of APE and APE-Loaded Rat Serum (Time and Dose Tests) on the Proliferation and Invasion of AH109A Cells.

A and B, APE was directly dissolved in culture medium at the concentrations indicated in graphs. The control value for the proliferation assay (A) was $5.90 \pm 0.23 \times 10^{-2}$ dpm/well. C and D, Rats fasted overnight were given oral intubation of APE at a dose of 300 mg/100 g of body weight. Blood was collected 0, 1, 2, 3, 6, and 12 h after oral administration, and serum from the blood was subjected to the proliferation and invasion assay systems with medium containing 10% of each rat serum. The control value for the proliferation assay (C) was $1.04 \pm 0.04 \times 10^{-2}$ dpm/well. E and F, Blood was collected 2 h after oral intubation, and serum prepared from the blood was subjected to the proliferation and invasion assay systems with a medium containing 10% of each rat serum. The control value for the proliferation assay (E) was $1.03 \pm 0.03 \times 10^{-2}$ dpm/well. The proliferation of AH109A cells was evaluated by measuring the incorporation of [methyl-3H]thymidine into the DNA fraction. Each value represents the mean ± SEM for 6 wells (proliferation) or 10 areas (invasion). Values not sharing a common letter are significantly different at $P < 0.05$ by the Tukey-Kramer multiple comparisons test.
and significantly inhibited both the proliferation and the invasion of AH109A cells when added to the culture medium (Fig. 1A and B). To determine whether APE would retain its inhibitory effects on AH109A proliferation and invasion when orally given to rats, the effect of APE-loaded rat serum on the proliferation and invasion of AH109A cells was investigated. First, the time-dependent effect of APE on the proliferation and invasion of AH109A cells was evaluated. Rats fasted overnight were orally given APE at a dose of 300 mg/100 g of body weight, and blood was obtained at 0, 1, 2, 3, 6, and 12 h after oral intubation of APE. The sera were prepared and added to the culture media instead of CS at a concentration of 10%. As Fig. 1C and D show, the serum obtained at 2 and 3 h after oral intubation of APE significantly suppressed hepatoma proliferation. Likewise, APE-loaded rat serum obtained at 1, 2 and 3 h after oral intubation significantly inhibited AH109A invasion. These anti-proliferative and anti-invasive effects diminished 6 h after oral intubation. Secondly, we investigated the dose-dependent effect of APE on the proliferation and invasion of AH109A cells. Blood was collected 2 h after oral intubation of APE at doses of 0, 10, 30, 100, and 300 mg/100 g of body weight, and proliferation and invasion assays were carried out. The serum inhibited both the proliferation and the invasion of AH109A cells in a dose-dependent manner (Fig. 1E and F). Although APE suppressed cancer cell proliferation, it had no toxicity for confluent normal M-cells (data not shown).

**Effect of dietary APE on tumor growth, metastasis, and cancerous hyperlipidemia in hepatoma-bearing rats**

As shown in Fig. 2, the incidence of tumors in rats was on the 5th day in the control and the two APE groups. Compared with the control group, the primary tumor size in the rats was significantly and time-dependently less in the two APE groups (Fig. 2A). The absolute weight of solid tumor was significantly lower in the two APE intake groups by 64.2% (0.3% intake group) and 58.3% (1.0% intake group), than in the control group (Fig. 2B). Six of the 10 rats (60%) were metastasized in the control group, whereas 1 of 10 rats (10%) in the two APE intake groups were. The total number of metastasized tumors was 17 per 10 rats in the control group and 1 per 10 rats in the two APE intake groups (Table 1). As shown in Fig. 3, the serum T-Ch and (VLDL + LDL)-Ch levels were significantly increased by hepatoma bearing, and these rises were significantly suppressed by 0.3% (T-Ch) and 1.0% APE [T-Ch and (VLDL + LDL)-Ch] intake. APE did not exert any significant influence on the HDL-Ch level. These changes resulted in the reduction of the AI, which was significant and noticeable in the 1.0% APE intake group. Fecal neutral sterol excretion significantly increased in the APE intake groups and fecal bile acid excretion tended to increase in the APE intake groups (Table 1). As shown in Fig. 4, the serum lipid peroxide (TBARS) level was significantly lower in the two APE intake groups than in the control group, and the serum triglyceride level tended to be lower in the two APE intake groups than in the control group.

**Discussion**

In the present study, we first investigated the *in vitro* effect of APE on hepatoma proliferation and invasion at APE concentrations of 0 to 200 µg/ml. APE dose-dependently inhibited both the proliferation and the invasion of AH109A cells (Fig. 1A and B). Up to the highest concentration tested (200 µg/ml), APE had no effect on the viability of non-cancerous M-cells (data not shown). These effects of APE were similar to those of tea catechins, which suppressed both the proliferation
Hepatoma-Bearing Rats

Some previous studies indicate that APE showed antioxidative activity.10,11) From these reports and the invasion of AH109A cells.8,9) Hepatocyte growth factor (HGF) has been reported to be identical to the scatter factor and to be deeply involved in tumor cell invasion.30) It is known that tumor cells produce large amounts of ROS,4) and we have recently reported that ROS induce the HGF expression and stimulate the autocrine action of HGF in invading AH109A cells.25) Some previous studies indicate that APE showed antioxidative activity.10,11) From these reports and the present results, it is likely that APE depresses the synthesis and/or secretion of HGF in AH109A cells by their antioxidative property and inhibit AH109A invasion as resveratrol does.31)

To confirm the bioavailability of APE and the in vivo possibility of its inhibitory effect on the malignancy of AH109A, we examined the effect of APE on proliferation and invasion using rat serum obtained at different times and doses after oral intubation of APE. In the time-course test, APE-loaded rat serum suppressed AH109A proliferation 2–3 h after APE administration. Likewise, APE-loaded rat serum obtained 1–3 h after administration showed significant inhibitory effects on AH109A proliferation 2–3 h after APE administration.

Table 1. Initial Body Weight, Food Intake, Body Weight Gain, Liver Weight, Number of Metastases, Liver Lipid Levels, and Steroid Excretion in Hepatoma-Bearing Rats

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Normal (20C)</th>
<th>Control (20C)</th>
<th>APE (0.3%)</th>
<th>APE (1.0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g/rat)</td>
<td>73.6 ± 1.8</td>
<td>74.8 ± 2.4</td>
<td>73.8 ± 2.1</td>
<td>73.7 ± 2.3</td>
</tr>
<tr>
<td>Food intake (g/21 days)</td>
<td>399.0 ± 8.5</td>
<td>238.3 ± 21.8</td>
<td>303.4 ± 18.0</td>
<td>273.4 ± 13.4bc</td>
</tr>
<tr>
<td>Body weight gain (g/21 days)</td>
<td>190.9 ± 5.6</td>
<td>89.2 ± 11.9b</td>
<td>105.6 ± 12.7b</td>
<td>98.9 ± 9.1b</td>
</tr>
<tr>
<td>Liver weight (g/rat)</td>
<td>12.7 ± 0.3a</td>
<td>7.2 ± 0.8b</td>
<td>7.4 ± 0.6b</td>
<td>7.3 ± 0.5b</td>
</tr>
<tr>
<td>Number of metastases (number/rat)</td>
<td></td>
<td></td>
<td>1.7 ± 0.7b</td>
<td>0.1 ± 0.1b</td>
</tr>
<tr>
<td>Liver lipid level (μmol/g liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>18.6 ± 3.7</td>
<td>24.1 ± 3.9</td>
<td>27.4 ± 5.3</td>
<td>26.0 ± 4.1</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>5.4 ± 1.2</td>
<td>8.9 ± 1.0</td>
<td>9.5 ± 1.5</td>
<td>10.1 ± 1.5</td>
</tr>
<tr>
<td>Steroid excretion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fecal dry weight (g/2 days)</td>
<td>2.6 ± 0.2a</td>
<td>1.2 ± 0.1b</td>
<td>1.5 ± 0.3b</td>
<td>1.8 ± 0.2b</td>
</tr>
<tr>
<td>Neutral sterols (μmol/2 days)</td>
<td>41.1 ± 1.7a</td>
<td>9.5 ± 1.0b</td>
<td>14.6 ± 3.5c</td>
<td>20.9 ± 2.7c</td>
</tr>
<tr>
<td>Bile acids (μmol/2 days)</td>
<td>41.7 ± 5.9a</td>
<td>8.0 ± 1.4b</td>
<td>14.6 ± 3.0c</td>
<td>15.3 ± 2.3b</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM for 6 (normal) or 10 (control, 0.3% APE and 1% APE) rats. Values not sharing a common letter are significantly different at P < 0.05 by the Tukey-Kramer multiple comparisons test.

Fig. 3. Effect of APE on Serum Cholesterol Levels and the Atherogenic Index in Hepatoma-Bearing Rats.

Each value represents the mean ± SEM for 6 (normal) or 10 (control, 0.3% APE and 1% APE) rats. Values not sharing a common letter are significantly different at P < 0.05 by the Tukey-Kramer multiple comparisons test.
recently reported that apple procyanidin concentrations in rat plasma reached a maximum 2 h after administration and decreased thereafter. These previous findings concerning the main components of APE are not inconsistent with those of the present study. In the dose-test, the inhibition of both AH109A proliferation and invasion by APE-loaded rat serum was almost dose-dependent in the range of 0–300 mg/100 g of body weight (Fig. 1E and F). Further studies are needed to clarify the active ingredient(s) of APE and APE-loaded rat serum, which suppress the tumor proliferation and invasion of AH109A cells.

We also investigated the effect of APE on in vivo hepatoma growth, metastasis, and cancerous hyperlipidemia in Donryu rats that had been implanted with AH109A cells. Gosse et al. reported that polyphenol-enriched fractions isolated from apples inhibited human colon cancer cell growth and reduced the number of preneoplastic lesions in Wistar rats. According to our ex vivo results, the inhibitory effects of APE on the in vivo growth and metastasis of AH109A cells were to be anticipated. Since the minimum effective dose of APE was 30 mg/100 g of body weight in the ex vivo assay, we fed APE to rats by adding 0.3% and 1.0% to the 20C (AIN-93G) diet. The volume and weight of tumor in the rats were time-dependently and significantly lower in the two APE intake groups than in the control group (Fig. 2). There was only one metastasized animal in the two APE intake groups (Table 1). Since ex vivo results have demonstrated that serum obtained after intubation of APE could decrease the AH109A invasion in this study (Fig. 1), the suppressive effect of dietary APE on the metastasis was due not only to growth inhibition, but also to suppression of the invasive step of metastasis.

The rats that received a subcutaneous implantation of AH109A cells 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, specifically, an enormous increase in (VLDL + LDL)-Ch and slight decrease in HDL-Ch. In this study, dietary APE decreased the serum T-Ch level, the (VLDL + LDL)-Ch level, and AI in hepatoma-bearing rats as compared with the control rats (Fig. 3). A positive correlation has been found between hepatoma weight and (VLDL + LDL)-Ch concentration, and a negative correlation between hepatoma weight and HDL-Ch. Hence, the hypocholesterolemic effect of dietary APE might, at least in part, be attributed to their inhibitory action on tumor growth (Fig. 2). Treating the hepatoma-bearing rats with APE stimulated the excretion of neutral sterols (Table 1). Hence, the inhibition of hypercholesterolemia might also have resulted from the increased excretion of neutral sterols in the APE-fed rats, besides the suppression of tumor growth by APE. The serum lipid peroxide (TBARS) concentration was significantly lower in the two APE intake groups than in the control group (Fig. 4). These results also suggest that the antioxidative properties of APE or its metabolites are available both in vitro and in vivo.

In summary, we found that not only APE itself but also APE-loaded rat serum effectively inhibited the proliferation and invasion of AH109A cells. Moreover, dietary APE also inhibited proliferation, metastasis, and cancerous hypercholesterolemia in hepatoma-bearing rats. It is conceivable that dietary APE possesses potential therapeutical effects against liver cancer.

Acknowledgment

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