Inhibitory Effects of Persimmon (Diospyros kaki) Extract and Related Polyphenol Compounds on Growth of Human Lymphoid Leukemia Cells

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We have investigated the effects of persimmon (Diospyros kaki) extract (PS) and related polyphenol compounds such as catechin (C), epicatechin (EC), epicatechingallate (EGC), epigallocatechin (EGG), and epigallocatechingallate (EGCG) on the growth of human lymphoid leukemia Molt 4B cells. We found that PS, ECG, EGC, and EGCG strongly inhibited the growth of the cells in a dose-dependent manner, while C and EC inhibited the growth of the cells only moderately. Ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine biosynthesis, was inhibited by 10–20% by these polyphenol compounds. The morphology of the Molt 4B cells indicated severe damage 3 days after treatment with PS, ECG, EGC, and EGCG. Irregular shape of the cells and DNA fragmentation were observed in PS, ECG, EGC, or EGCG-treated cells. These results suggest that PS, ECG, EGC, and EGCG induce apoptosis (programmed cell death) of Molt 4B cells.

Key words: persimmon extract; polyphenol compounds; apoptosis; leukemia

Recently the action mechanism of new compounds derived from natural products has been intensively investigated using neoplastic cells. It has been reported that EGCG inhibits carcinogenesis of the two-stage model that consists of initiation by 7,12-dimethylbenz[a]anthracene (DMBA) and promotion by 12-O-tetradecanoyl phorbol-13-acetate (TPA).1 Polyphenol compounds isolated from green tea suppressed induction of ornithine decarboxylase (ODC, EC 4.1.1.17), a rate-limiting enzyme of polyamine biosynthesis that was caused by topical application of TPA on mouse skin.2 We reported in our previous paper3 that persimmon extract inhibited carcinogenesis of the two-stage model that consists of initiation by DMBA and promotion by TPA. Beckwith et al.4 reported that dolastatins isolated from the marine sea hare Dolabella auricularia inhibit proliferation of human lymphoid cell lines by apoptosis (programmed cell death). It was described by Kaufmann5 that endonucleolytic DNA fragmentation (apoptosis) was induced by cytotoxic anticancer drugs. Moreover, it was reported that glycerolphospholipids induced apoptosis of human leukemia cell lines such as HL 60, Molt 4, and U 937.6 It was reported by Yanagihara et al.7 that two isoflavone derivatives, biochanin A and genistein, inhibited the growth of stomach cancer cell lines through activation of a signal transduction pathway for apoptosis. Martin et al.8 reported that the gross morphological change of HL-60 cells by treatment with retinoic acid resulted in apoptosis.

In this paper, we report that proliferation of human lymphoid leukemia cells was inhibited by persimmon extract (PS) and the related polyphenol compounds through apoptosis.

Materials and Methods

Chemicals. Catechin (C), epicatechin (EC), epigallocatechin (EGG), and epigallocatechingallate (EGCG) were purchased from KURITA Industrial Corp., Tokyo, Japan. DL-[1-14C]Ornithine (sp. act. 2GBq/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. RPMI 1640 medium was purchased from GIBCO laboratories, Grand Island, NY U.S.A. All other reagents were guaranteed grade.

Preparation of persimmon extract. Persimons (Diospyros kaki, astringent persimmon) was harvested in the experimental farm of Mie University. The edible portion of the persimmons was homogenized by a juicer. The persimmon juice obtained was centrifuged at 7000 x g for 20 min at 0°C, and the resulting supernatant was frozen and dried. One gram of the freeze-dried persimmon extract was dissolved in 7 ml of distilled water. This is called original persimmon extract.

Measurement of polyphenol content in persimmon extract. The polyphenol content in persimmon extract was measured by the vanillin hydrochloric acid method.9 Its content was estimated as catechin content and shown to be 27% in the persimmon extract. Kaki tannin has a molecular weight of about 10,000 as reported by Matsuo and Ito.10 Concentration of kaki tannin in the original persimmon extract was estimated to be about 0.3 mm.

Cell culture. Human lymphoid leukemia Molt 4B cells were provided by the American Type Culture Collection (ATCC), Rockville, MD, U.S.A. The cells were cultivated as described previously.11 Molt 4B cells were grown in RPMI 1640 medium with 10% fetal calf serum, penicillin G (50IU/ml) and streptomycin (50 μg/ml) at 37°C under humidified 95% air-5% CO₂ atmosphere and passed every 7 days. Mycoplasma testing was routinely negative.

Assay for growth inhibition. Exponentially growing cells were placed at the initial density of 3 x 10⁵ cells/ml in the culture flask, cultivated in the presence of vehicle or compounds for 3 days, and then the number of cells was counted using a hemocytometer.

Assay for DNA fragmentation. Exponentially growing cells were placed at the initial density of 3 x 10⁵ cells/ml in the culture flask. After cultivation in the presence of vehicle, PS, and the related polyphenol compounds for 3 days, the cells were pelleted by centrifugation. DNA was isolated from the cell pellets as described by Maniatis et al.12

Abbreviations: C, catechin; EC, epicatechin; EGC, epicatechingallate; EGG, epigallocatechin; EGCG, epigallocatechingallate; PS, persimmon extract; ODC, ornithine decarboxylase.

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Equivalent amounts of DNA (2 μg) were put into the well of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid (pH 7.5) containing 2 mM EDTA.

Assay for activity of ornithine decarboxylase (ODC). ODC activity was measured by the release of $^{14}C\text{O}_2$ from $\text{L-[1-}\text{C}]\text{ornithine as described by Seely et al.}^{13}$ The assay mixture contained 0.1 mM $\text{L-[1-}\text{C}]\text{ornithine (7.4KBP}, 40 \mu\text{M pyridoxal-5-phosphate, 10 mM dithiothreitol, 50 mM Tris-HCl (pH 7.2), and ODC enzyme extract in a final volume of 0.2 ml.}$

Microscopic observation of morphological change of Molt 4B cells. Exponentially growing human lymphoid Molt 4B cells were placed at the initial density of 3 to $4 \times 10^5$ cells/ml. After cultivation for 3 days in the presence of vehicle, PS, and the related polyphenol compounds, the morphology of the cells was examined by an epifluorescence microscope with a cooled CCD camera digital imaging system and Fuji pietrography 3000 as described by Okumura et al.\textsuperscript{14}

Results and Discussion

The effects of PS and the related polyphenol compounds such as C, EC, ECG, EGC, and EGCg on the proliferation of human lymphoid leukemia Molt 4B cells have been examined. The results of the cell growth analysis are summarized in Table I. C, EC, and ECG inhibited the growth of cells by 34.9%, 10.4%, and 59.8%, respectively, at 0.2 mM, while EGC, EGCg, and PS suppressed the proliferation of Molt 4B cells by 100%, 93.1%, and 100%, at 0.1 mM, 0.1 mM, and 0.3 mM, respectively.

It is known that the level of ODC activity is high in proliferating neoplastic cells including cancer cells. Therefore, the effects of PS and the related polyphenol compounds on the ODC activity were examined. Table II shows that PS and the related polyphenol compounds are weak inhibitors of ODC activity. Moreover, the morphological changes of Molt 4B cells caused by treatment with PS and the related polyphenol compounds were examined by using an epifluorescence microscope. The morphology of the Molt 4B cells showed severe damages after 3 days of treatment with PS and EGCg (Fig. 1), but the PS and the related polyphenol compounds were examined by using a epifluorescence microscope. The morphology of the Molt 4B cells showed severe damages after 3 days of treatment with PS and EGCg (Fig. 1), but the morphological changes of the cells caused by C and EC were minimal (data not shown). To examine whether apoptosis is induced by PS and the related polyphenol compounds such as C, EC, EGC, EGCg, and EGCg, the Molt 4B cells were treated with these polyphenol compounds for 3 days, DNA was extracted from

Table I. Effects of Polyphenol Compounds on the Growth of Leukemia Cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>C</td>
<td>0.2</td>
<td>34.9</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>44.4</td>
</tr>
<tr>
<td>Epicatechin (EC)</td>
<td>0.2</td>
<td>10.4</td>
</tr>
<tr>
<td>Epicatechingallate (ECG)</td>
<td>0.1</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>59.8</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>87.5</td>
</tr>
<tr>
<td>Epigallocatechin (EGC)</td>
<td>0.025</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Epigallocatechin gallate (EGCG)</td>
<td>0.025</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>93.1</td>
</tr>
<tr>
<td>Persimmon extract (PS)</td>
<td>0.075</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Each value is the mean of duplicate experiments in which agreement was within <10% of the mean.

Table II. Inhibition of Ornithine Decarboxylase (ODC) by Polyphenol Compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.05</td>
<td>5</td>
</tr>
<tr>
<td>Epicatechingallate (ECG)</td>
<td>0.1</td>
<td>13</td>
</tr>
<tr>
<td>C</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>Epicatechin (EC)</td>
<td>0.05</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>6</td>
</tr>
<tr>
<td>Epigallocatechin (EGC)</td>
<td>0.05</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>Epigallocatechingallate (EGCG)</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>Persimmon extract (PS)</td>
<td>0.3</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>19</td>
</tr>
</tbody>
</table>

Each value is the mean of duplicate experiments in which agreement was within <10% of the mean.

Fig. 1. Morphological Change of Molt 4B Cells.

A: Non-treated Molt 4B cells. B: Molt 4B cells treated with 0.1 mM EGCg. C: Molt 4B cells treated with 0.3 mM PS. Molt 4B cells were cultivated with each compound for 3 days and then observed under an epifluorescence microscope as described in the text.
the treated cells, and then DNA fragmentation by these compounds was examined using agarose gel electrophoresis. As shown in Fig. 2, DNA fragmentation by PS, ECG, EGC, and EGCG was observed, but fragmentation by C and EC was not observed. These data suggested that the morphological changes (Fig. 1) appeared to be due to apoptosis resulting in DNA fragmentation (Fig. 2). On the other hand, it was reported that ECG, EGC, and EGCG, which are constituents of PS, strongly inhibited the activity of DNA polymerase.\(^{15}\) In this study, it might be considered that DNA fragmentation of Molt 4B cells treated with these compounds was induced by both the activation of endonuclease\(^{16}\) and the inhibition of DNA polymerase activity.\(^{15}\)

This study indicates the induction of apoptosis by polyphenol compounds in the leukemia cells. A strategy to selectively induce apoptosis of leukemia cells without altering healthy cells is a major goal for the development of new therapeutic techniques.

### References


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**Fig. 2.** DNA Fragmentation in PS- and Catechin Compounds-treated Cells.

Molt 4B cells were treated with test compounds for 3 days, and DNA in the cells was extracted as described in the text. Equivalent amounts of DNA (2 μg) were put into the wells of 2% agarose gel and electrophoresed as described in the text. (1) DNA digested with HindIII; (2) 0.1 mM EC; (3) 0.1 mM ECG; (4) 0.1 mM EGC; (5) 0.1 mM EGCG; (6) 0.1 mM C; (7) 0.3 mM PS; (8) distilled water.