PROTECTIVE EFFECTS OF (−)-EPIGALLOCATECHIN GALLATE AND (+)-CATECHIN ON PARAQUAT-INDUCED GENOTOXICITY IN CULTURED CELLS

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ABSTRACT — The protective effects of green tea polyphenols on paraquat-induced genotoxicity in cultured cells were studied. (−)-Epigallocatechin gallate (EGCG), the major constituent of green tea, and (+)-catechin (CT), a minor constituent, equivalently decreased the frequencies of sister-chromatid exchanges (SCE) induced by paraquat (PQ), which is a generator of reactive oxygen species. These polyphenols were effective at concentrations of 1.0 μM and above. A reduction of the effect on the cell cycle rate caused by PQ was found when EGCG and CT were added at concentrations of more than 10.0 μM. These concentrations of EGCG and CT alone had no effect on cell cycle rate, which is used as index of cell proliferation. Decreases in the cell cycle rate were found at 200 μM EGCG and CT in the 24 hr exposure period. The equivalent effectiveness of EGCG and CT suggested the possibility of other mechanisms, apart from acting as reactive oxygen species scavengers, because it has been reported that EGCG is the most potent scavenger among tea catechins. From the present study, it was suggested that green tea and foods containing these polyphenols may be beneficial to human health by protecting against reactive oxygen species-induced genotoxicity.

KEY WORDS: Polyphenol, (−)-Epigallocatechin gallate, (+)-Catechin, Paraquat, Genotoxicity

INTRODUCTION

Green tea is a rich source of polyphenols and is one of the most widely consumed beverages in the world, especially in Japan and China. Several biological activities of green tea have been reported, including the possession of antioxidant (Roedig-Penman and Gordon, 1997; Yen et al., 1997), antimitagenic (Hour et al., 1999), and anticarcinogenic properties (Kuroda and Hara, 1999). These qualities have been attributed to the presence of the polyphenolic antioxidants as major constituents of green tea.

Clinical trials and many animal models have evaluated the effectiveness of green tea and its extracts as possible cancer-preventative agents. Imai et al. (1997) showed a potent preventive effect of green tea against cancer in people drinking more than 10 cups a day among a Japanese population. Nakachi et al. (1998) reported that increased consumption of green tea was correlated with decreased recurrence of stage I and II breast cancer. Sugumana et al. (1999) reported practical and effective cancer prevention both before cancer onset and after cancer treatment.

The mechanisms of these effects have been assumed to include the reactive oxygen species scavenging effects of polyphenols (Zhao et al., 1989; Yen and Chen, 1998; Yokozawa et al., 1998). Reactive oxygen species are known to damage DNA, and possibly to result in mutations and even cancer (Schwarz et al., 1984; Zimmerman and Cerutti, 1984; Nakamura et al., 1985). Thus, a reduction in the toxic effects of these reactive oxygen species by green tea may be beneficial to humans. However, little information has been reported on the preventive action of green tea polyphenols against reactive oxygen species-induced genotoxicity.

As a genotoxicity test, SCE analysis was used because it is very sensitive to DNA damage. SCE analysis has been advocated as an extremely sensitive test for detecting potentially mutagenic and/or carcinogenic substances (Perry and Evans, 1975; Wolff, 1977; Wolff, 1982).
1998). As a reactive oxygen species generator, paraquat (PQ) was used because a previous study revealed the induction of genotoxic effects, such as SCE and chromosomal aberrations (Tanaka and Amano, 1989), and the role of reactive oxygen species in the production of SCE and chromosomal aberrations was also demonstrated. Therefore, the effects of green tea polyphenols on PQ-induced genotoxicity were examined in this study using SCE analysis.

Additionally, the preventive effects of polyphenols on PQ-induced changes of cell proliferation were examined, because a previous study revealed that these changes were induced by PQ-generated reactive oxygen species (Tanaka and Amano 1989). The major constituent, (−)-epigallocatechin gallate (EGCG), and a minor constituent, (+)-catechin (CT), of green tea polyphenols were examined.

MATERIALS AND METHODS

SCE analysis
Chinese hamster lung (CHL) cells were purchased from Dainippon Pharmaceutical Co. (CHL/1U, Osaka, Japan). These cells were seeded at a density of 10⁴ cells in rectangular bottles (50 cm²), and then incubated for about 20 hr at 37°C in Eagle’s minimum essential medium (Dainippon Pharmaceutical Co.), supplemented with 10% calf serum (Dainippon Pharmaceutical Co.). After changing the medium, the cells were exposed to EGCG (Kurita Industry Company, Tokyo, Japan), CT (Kurita Industry Company), paraquat (PQ, methyl viologen; Sigma Co., St. Louis, MO, USA) and 5-bromo-2′-deoxyuridine (final conc. 10 µM, BrdU; Sigma Co.) in the dark for 24 hr. EGCG and CT were added 30 min prior to PQ addition. PQ was used as a reactive oxygen species generator (Tanaka and Amano, 1989). 4 hr before the exposure period, colcemid (final conc. 0.2 g/ml; Gibco Laboratories, New York, USA) was added to each bottle. At the end of the incubation period, the cells were harvested by addition of trypsin (Merck, Darmstadt, Germany) and were subsequently centrifuged (112 × g, 5 min) to recover the cells. After treating the cells with 0.04 M KCl for 15 min at 37°C, chromosomes were obtained by centrifugation (175 × g, 20 min) and were fixed with methanol-acetic acid (3:1 v/v). These procedures were performed in a part-darkened laboratory (blinds were lowered), because BrdU is sensitive to light. The chromosomal preparations were stained with 2% Giemsa’s solution (Merck, Darmstadt, Germany), prepared in 0.3 M Na₂HPO₄ at pH 10.4, for 30 min (Ikushima and Wolff, 1974). With this technique, chromatids that had incorporated BrdU were darkly stained. The results were recorded as the frequency of SCE/metaphase cell for chromatids that had undergone two replication cycles (Tanaka and Amano, 1989).

Cell cycle rate
To determine the effect on cell proliferation, the cell cycle rate of the cells was determined. The CHL cells were seeded at a density of 10⁴ cells in rectangular bottles (50 cm²), and then incubated for about 20 hr at 37°C, as described above. After changing the medium, the cells were exposed to EGCG, CT, and PQ. The cells treated with colcemid for 16 hr were fixed as described above, and stained with Giemsa’s solution (pH 7.6). The cell cycle rate was evaluated by recording the number of mitotic cells.

Cell cycle rate (‰) = (number of mitotic cells recorded/number of total observed cells) × 100

Statistical analysis
All data are expressed as the mean ± S.E. Statistical significance was determined by using Student’s r-test (Gad and Weil, 1982).

RESULTS

SCE analysis
The SCE analysis was used as an index of genotoxic activity. The experiments were repeated 5 times and a representative example is presented in the figs. Fig. 1 shows the frequency of SCE in CHL cells treated with PQ, a superoxide anion generator. By exposure to PQ for 24 hr, increases in SCE were observed in a dose-dependent manner. The highest SCE frequency was found at 5.0 µM, Fig. 2 shows the effect of EGCG on PQ-induced SCE. When cells were incubated with each concentration of EGCG and 5.0 µM PQ, significant decreases in SCE frequencies were found at concentrations of EGCG above 1.0 µM. The effects of CT are similar to those of EGCG, as shown in Fig.3. CT was significantly effective at concentrations above 1.0 µM. The decreases in SCE by EGCG and CT were dose-dependent. EGCG and CT did not increase the SCE frequency compared with control (0 µM) at 1, 5, 10, 50, and 100 µM (data not shown).

Cell cycle rate
To examine the effect on cell proliferation, cell cycle rates were determined because CHL cells easily aggregate and it is difficult to count the number of cells...
accurately. The experiments were repeated 5 times, and a representative example is shown in the figs. Fig. 4 shows the cell cycle rates of CHL cells exposed to EGCG. When cells were exposed to EGCG for 24 hr, significant decreases in cell cycle rate were seen at concentrations of more than 200 μM, and at more than 100 μM for the 48 hr exposure period. These effects were dose-dependent. At 300 μM and 48 hr treatment with EGCG, no metaphase cells were observed. CT gave a similar result, as shown in Fig. 5. At concentrations of more than 200 μM for the 24 hr exposure period and 100 μM for 48 hr, significant decreases in the cell cycle rate were observed. However the decreases caused by CT were smaller than those by EGCG.

PQ caused changes in the cell cycle rate as shown in Table 1; at low concentrations, cell cycle rates were higher, and they were lower at higher concentrations. The effects of EGCG and CT on 0.5 μM PQ increased cell cycle rates, and on the 50 μM PQ decreased cell cycle rates were determined. Fig. 6 shows the effects of EGCG on the cell cycle rates of CHL cells exposed to PQ. EGCG was effective at concentrations of more than 10 μM on cells exposed to 0.5 μM and to 50 μM PQ. Similar results were found for CT, as shown in Fig. 7. CT was effective at concentrations of more than 10 μM on 0.5 μM and 50 μM PQ-exposed cells. The cell cycle rates were not affected by EGCG and CT alone at 10 and 50 μM, as shown in Fig. 4 and 5.

**DISCUSSION**

Leanderson et al. (1997) reported the inhibition of cigarette smoke- and H2O2-induced DNA breakage by green tea polyphenols in cultured human lung cells. Wei et al. (1999) demonstrated the potent scavenging of reactive oxygen species and blocking of UV-induced oxidative DNA damage by green tea extracts and the addition of EGCG to those. Hour et al. (1999) showed the effectiveness of green tea extracts and EGCG on eleven mutagens using the Ames test. Yonemitsu et al. (1999) reported the weight increase by green tea in PQ-poisoned mice, and revealed the possibility of green tea and EGCG as antidotes against PQ. The present study demonstrated the effect of EGCG and CT on PQ-induced SCE and changes in cell cycle rates in a dose-dependent manner. From these findings, a preventive effect of EGCG and CT on reactive oxygen species-induced cytotoxicity and genotoxicity were suggested. The promotion of tumors by both low and high concentrations of PQ may be protected by these polyphenols, as reactive oxygen species are thought to be involved in

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**Fig. 1.** Frequencies of sister-chromatid exchange (SCE) induced by paraquat (PQ). The CHL cells were exposed to PQ for 24 hr. Values are the mean ± S.E. The number of recorded metaphase cells was 50-60.

* P<0.001 vs. 0 μM PQ.

**Fig. 2.** Effects of epigallocatechin gallate (EGCG) on PQ-induced SCE. The CHL cells were exposed to EGCG and PQ for 24 hr. Values are the mean ± S.E. The number of recorded metaphase cells was 50-60.

**Fig. 3.** Effects of (+)-catechin (CT) on the PQ-induced SCE. The CHL cells were exposed to CT and PQ for 24 hr. Values are the mean ± S.E. The number of recorded metaphase cells was 50-60.
tumor formation (Schwarz et al., 1984; Zimmerman and Cerutti, 1984; Nakamura et al., 1985).

As shown in Fig. 2, 3, 6, and 7, the activities of CT and EGCG were equivalent. Nanjo et al. (1999) reported the radical scavenging activity of tea catechins and showed that the presence of at least an ortho-dihydroxy group in the B ring and a galloyl moiety at the 3-position was important in maintaining the effectiveness of the radical scavenging ability. This report also showed that the radical scavenging activity of EGCG was stronger than that of CT (Nanjo et al., 1999). The equivalent activities of EGCG and CT in the present study suggest perhaps that other mechanisms involving polyphenols may be present in addition to that of radical scavenging. EGCG is very soluble in water, while CT is moderately soluble; whether the solubility of the polyphenol influences the intensity of the effect has not yet been established. EGCG and CT reduced the frequencies of PQ-induced SCE at concentrations of 1.0-10.0 μM (Figs. 2, 3). At these concentrations, EGCG and CT alone did not affect the cell cycle rates (Figs. 4, 5), suggesting that green tea polyphenols may protect cells from reactive oxygen species-induced cancers at nontoxic concentrations.

On the other hand, it is unclear whether decreases in cell cycle rates caused by EGCG and CT contribute to their anticancerous effects. Recently, several studies concerning apoptosis and cell cycle arrest by EGCG as a mechanism of its anti-cancerous activities have been

Fig. 4. Effects of EGCG on cell cycle rates in cultured CHL cells. The cells were exposed to EGCG for 24 hr or 48 hr. Cell cycle rate is the percentage of metaphase cells to the total number of recorded cells treated with colcemid for 16 hr. Values are means ± S.E. n=5.

Fig. 5. Effects of CT on cell cycle rates in cultured CHL cells. The cells were exposed to CT for 24 hr or 48 hr. Cell cycle rate is the percentage of metaphase cells to the total number of recorded cells treated with colcemid for 16 hr. Values are means ± S.E. n=5.

Fig. 6. Effects of EGCG on cell cycle rates in PQ-treated cells. The CHL cells were exposed to EGCG and PQ for 24 hr. Cell cycle rate is the percentage of metaphase cells to the total number of recorded cells treated with colcemid for 16 hr. Values are means ± S.E. n=5.

Fig. 7. Effects of CT on cell cycle rates in PQ-treated cells. The CHL cells were exposed to CT for 24 hr. Cell cycle rate is the percentage of metaphase cells to the total number of recorded cells treated with colcemid for 16 hr. Values are means ± S.E. n=4.
Green tea polyphenols protect paraquat-induced genotoxicity.

were equivalent to those of EGCG in the present study. CT is a minor constituent in green tea. Therefore, foods containing CT, other than green tea, may also be beneficial to health. The present study demonstrates that green tea and EGCG- and CT-containing foods may be beneficial to human health as a result of their ability to reduce reactive oxygen species-induced cancer risk, as demonstrated using cultured cells.

REFERENCES


Table 1. Effects of paraquat on cell cycle rates in cultured CHL cells.

<table>
<thead>
<tr>
<th>Paraquat (µM)</th>
<th>Cell cycle rate (%)†</th>
<th>24 hr ±</th>
<th>48 hr ±</th>
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<tr>
<td>0</td>
<td>30.4 ± 2.0</td>
<td>27.7 ± 1.4</td>
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<tr>
<td>0.1</td>
<td>52.8 ± 2.9*</td>
<td>28.6 ± 2.3</td>
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<tr>
<td>0.5</td>
<td>53.4 ± 2.4**</td>
<td>32.8 ± 1.9</td>
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<tr>
<td>1.0</td>
<td>42.5 ± 1.2*</td>
<td>25.9 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>35.4 ± 2.2</td>
<td>14.5 ± 1.8**</td>
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<tr>
<td>10.0</td>
<td>37.0 ± 3.1</td>
<td>11.7 ± 0.9**</td>
<td></td>
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<tr>
<td>50.0</td>
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<td>0 ± 0.0**</td>
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<tr>
<td>70.0</td>
<td>10.1 ± 1.5**</td>
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<tr>
<td>100.0</td>
<td>8.0 ± 1.3**</td>
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†: Metaphase cells as a percentage of recorded total cells after colcemid treatment (16 hr).
‡: Incubation period with paraquat. Values are mean±S.E. n=4.
* : P<0.01 vs. 0 µM, **: P<0.001 vs. 0 µM


