**Note**

Protecting Effect of a Green Tea Percolate and Its Main Constituents against Gamma Ray-induced Scission of DNA

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Gamma-ray-induced scission of pUC18 plasmid DNA prepared from *E. coli* was examined in the presence of a green tea percolate and its main constituents, L-ascorbic acid (used as the sodium salt) and (−)-epigallocatechin gallate. Each of these showed a protecting effect against DNA scission. The relationship between the protecting effect against DNA scission and the scavenging effect of the hydroxyl radical was examined, and is discussed from the viewpoint of interaction with DNA.

**Key words:** gamma-ray-induced DNA scission; green tea percolate; L-ascorbic acid; (−)-epigallocatechin gallate (EGCG); spin trapping

It has been reported that a green tea percolate and its constituents, catechins, showed anti-mutagenic or anti-carcinogenic effects. For example, (−)-epigallocatechin gallate (EGCG) reduced the high spontaneous mutations due to altered DNA polymerase III in a mutator strain of *Bacillus subtilis*, while EGCG and other tea catechins containing a pyrogallol moiety reduced the mutation of *E. coli* caused by UV irradiation. It has also been recently shown that EGCG suppressed the metastasis of cancer cells.

It is well known that radiation causes mutation or carcinogenesis, the main factor for these events being attributed to radiation-induced scission of DNA molecules. If tea constituents are also effective in this case to suppress mutation or carcinogenesis, it may be conceivable that the tea constituents would inhibit radiation-induced DNA scission. This concept can be investigated by *in vitro* experiments, so we tried to examine whether tea constituents would show a protecting effect against γ-ray induced scission by using pUC18 plasmid DNA from *E. coli*. In addition, we applied the ESR spin trapping method to elucidate the protecting mechanism.

**Preparation of the samples and the reagents.** pUC18 plasmid DNA (2686 bp, 1.6 × 10⁹ dalton) was prepared from *E. coli* by the method described. Four g of commercially available Japanese green tea (leaves of *Camellia sinensis*) was percolated with 50 ml of water at 75°C for 3 min, this being used as the original percolate solution. EGCG (Wako) and sodium L-ascorbate (Na-Asc, Wako) were dissolved in an SSC buffer solution (0.15 M NaCl and 0.015 M sodium citrate at pH 7) at a concentration of 1 × 10⁻³ M just before the experiment, and was subsequently diluted to the required concentrations. A specially purified spin trapping agent, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), was obtained from LABOTEC Co., Ltd. (Tokyo). All the other reagents used were of guaranteed grade.

**Gamma-irradiation experiment.** Four μl each of the SSC buffer solution of DNA (0.019 μg/μl) were put into micro-tubes. To each of these, 6 μl of the tea percolate diluted to various concentrations with the SSC buffer solution or with the buffer solutions containing various amounts of Na-Asc and EGCG were added. These samples were then γ-irradiated with a 60Co source for 2 h at different dose rates, which were determined with a Fricke dosimeter, by changing the distance from the source. After finishing this irradiation, closed circular (CC) and open circular (OC) DNA were separated by a mini-gel electrophoresis system. Each DNA band stained with ethidium bromide was photographed, and the degree of darkening of the band on the film was measured by a densitometer. The residual percentage of the CC form of DNA, which was used as an index of the protecting effect, was obtained according to ref. 13.

**Spin trapping experiment.** The hydroxyl (OH) radical was generated by the Fenton reaction (Fe²⁺ + H₂O₂) and trapped with DMPO by the method of Mizuta et al. Seventy five μl each of an aqueous FeCl₃ (1 × 10⁻³ M) solution was placed in a number of micro-tubes, and 15 μl of DMPO was added then. Next, 35 μl of water or of an aqueous solution containing various amounts of Na-Asc and EGCG was added and vortex-mixed. Finally, 75 μl of an aqueous H₂O₂ solutions (1 × 10⁻³ M) was added to each of these mixtures and quickly blended. Time measurement started with this mixing, each resulting mixture being sucked into a glass capillary tube (Drummond 75-μl micropipette, 0.8 mm i.d.) before one end of the tube was sealed with a teflon cap. ESR spectra were then measured with a JEOL RE3X spectrometer (X-band), using 100 kHz field modulation.

**Protection by the tea percolate.** Figure 1 shows the

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<thead>
<tr>
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<th>4</th>
<th>3</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample No.</strong></td>
<td><strong>CC form</strong></td>
<td><strong>OC form</strong></td>
<td></td>
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Fig. 1. Electrophoretic Pattern of γ-Ray Irradiated DNA.

Sample numbers are the same as those in the Table. Radiation dose, 120Gy.

Abbreviations: EGCG, (−)-epigallocatechin gallate; Na-Asc, sodium OH radical, hydroxyl radical; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; L-ascorbate; CC form, closed circular form; OC form, open circular form.
electrophoretic pattern of the samples γ-irradiated in the presence of the tea percolate. Only the CC-form band was observed in sample 5, which was non-irradiated and did not contain the tea percolate, while only the OC form was observed in sample 4, which contained no tea percolate. This shows that all the original CC-form DNA was completely decomposed into the OC form under these irradiation conditions (60 Gy h⁻¹ 2 h). However, the CC form still remained in samples 1-3 containing various amounts of the tea percolate, the residual ratio depending on the quantity of the tea percolate. In the samples containing the same amounts of tea percolate as those in samples 1-4 and having been stood for 2 h without γ-irradiation, no DNA scission was observed. These results indicate that the tea percolate has suppressed the γ-irradiation-induced scission of DNA. The residual CC-form DNA (%) is tabulated in the Table.

**Protection by EGCG.** Figure 2A shows the protecting effect of EGCG, in which the residual percentage of the original CC form is plotted against the radiation dose under various EGCG concentrations. The protecting effect increased with increasing EGCG concentration at all radiation doses, although DNA scission continued to increase with increasing radiation dose in all the samples containing different amounts of EGCG.

**Protection by Na-Asc.** In the case of co-existing Na-Asc, a remarkable difference from the cases of the tea percolate and EGCG was observed as shown in Fig. 2B. DNA scission took place even without γ-irradiation only in the presence of Na-Asc. The degree of DNA scission increased with increasing Na-Asc concentration. This result suggests that there may be some species derived from Na-Asc which decompose DNA. Similarly to this result, Murata et al.¹⁵ have reported that the reactive oxygen species was produced from ascorbate, and Scarpa et al.¹⁶ have reported that the superoxide anion radical was produced as an active intermediate in the autoxidation of ascorbate by molecular oxygen. Our result is, therefore, not unsuspected. On the other hand, a protecting effect against scission was apparent in the larger dose region, indicating that Na-Asc had antipodal, dual properties.

**Spin trapping experiment.** It is generally accepted that the main factor for radiation-induced scission of DNA molecules is attributable to the OH radical formed as a result of decomposition of the surrounding water molecules. Therefore, it is natural to consider that the protecting effect of EGCG and Na-Asc may be derived from the scavening reaction of the OH radical. We tried to examine the scavenging ability with EGCG and Na-Asc by using the spin trapping method, although similar experiments have already been performed.¹⁷ - ¹⁹

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Relative concentration of green tea percolate</th>
<th>Residual CC-form DNA (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>1.0*</td>
<td>95 (irrad.)</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>77 (irrad.)</td>
</tr>
<tr>
<td>3</td>
<td>0.01</td>
<td>45 (irrad.)</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0 (irrad.)</td>
</tr>
<tr>
<td>5 (control)</td>
<td>0</td>
<td>100 (non-irrad.)</td>
</tr>
</tbody>
</table>

* The concentration of sample 1 was the same as that of the original percolated solution (see the Materials and Methods section). Samples 2 and 3 were respectively 10- and 100-times diluted from the original.

The ESR intensity of the second line of the quartet signal characteristic to the spin adduct of the OH radical (DMPO-OH) was measured. As the intensity decreased with the progress of time, the intensity 1 min after mixing the solution was used for the comparison. Figure 3 shows the relationship between the ESR intensity and the concentrations of EGCG and Na-Asc. The curves are similar to those reported by Makino et al.¹⁷ and it is clear that the scavenging ability of Na-Asc to the OH radical was larger than that of EGCG. This result is consistent with that reported by Yamada et al.¹⁸ in which L-ascorbic acid showed a larger value than that of a polyphenol compound, gallic acid.

**Relationship between the protecting ability against DNA scission and the scavenging ability to the OH radical.** We have considered so far that the protecting ability against DNA scission is
attributable to the OH radical scavenging effect of EGCG and Na-Asc. In order to confirm this point, the relationship between the protecting and the scavenging abilities was compared.

Figure 4 shows a comparison of the protecting ability of EGCG and Na-Asc against DNA scission. Taking into account the effect of scission by Na-Asc itself, it is concluded that EGCG had stronger protecting ability than that of Na-Asc. Therefore, the order of the scavenging and protecting abilities was reversed. We can explain this apparent contradiction by the fact that the lifetime of the OH radical is generally very short, so that DNA scission is caused only by those OH radicals formed near the DNA molecules. In order to scavenge such OH radicals, scavengers must be located close to DNA molecules, and it seems optimal that the scavengers interact with the DNA molecules. In such a case, the local concentration of the scavenger would be raised compared with that in the bulk solution, and it would act more effectively. Na-Asc is very hydrophilic, so it has the tendency to be located in the bulk water. On the other hand, the EGCG molecule is more hydrophobic than that of Na-Asc; although it is soluble in water, so it seems to prefer interacting with DNA molecules. In addition, the EGCG molecule has a condensed aromatic ring and is likely to be intercalated in the DNA helix. This speculation is supported by the experimental finding that staining with ethidium bromide became difficult when DNA had been treated with concentrated EGCG for a long time. This result can be explained by the concept that EGCG previously intercalated would prevent the intercalation of ethidium bromide. We postulate from these results that EGCG may have had a higher local concentration near the DNA molecules and thus showed a more effective protecting effect against DNA scission.

We consider that DNA scission is mainly caused by the OH radical, although the possibility of another mechanism must be checked carefully.

References

11) S. Kondo, in “Molecular Radiation Biology,” Gakkai Shuppan Center, Tokyo, 1972, Chapter 5.