Effects of (-)-Epigallocatechin Gallate (EGCG) on DNA Strand Breaks as Evaluated by Single-cell Gel Electrophoresis (SCG) in Human Lymphocytes

Yuquan LU, Tatsuya TAKESHITA and Kanehisa MORIMOTO

Department of Social and Environmental Medicine, Osaka University Graduate School of Medicine, Osaka

Abstract

(-)-Epigallocatechin gallate (EGCG), a catechin polyphenol component, is the main ingredient of green tea extract. Although the anti-carcinogenic and cancer inhibitory effects of EGCG have been widely reported, its genotoxicity is not clear and seldom reported. In this study, we examined the effects of EGCG on DNA strand breaks in the isolated lymphocytes and whole blood lymphocytes obtained from two smoking subjects and a nonsmoking healthy subject using a single-cell gel electrophoresis (SCG) assay. The results showed that after 2 hrs of treating the isolated lymphocytes from the smokers, EGCG induced a significant increase in DNA strand breaks at concentrations from 2.5×10^{-5} M to 2.0×10^{-4} M, while after 2 hrs of treating the whole blood obtained from the same smokers, EGCG suppressed the DNA strand breaks in the lymphocytes at concentrations of 1.0×10^{-4} M and 2.0×10^{-4} M. A similar suppressive result was also shown in the whole blood lymphocytes from the nonsmoker at nearly the same concentrations, while at concentrations of 1.0×10^{-3} M or 2.0×10^{-3} M, EGCG induced a significant increase in DNA strand breaks in the whole blood lymphocytes from the nonsmoker. This result suggests that EGCG is not only inhibitory against DNA strand breaks in whole blood, but also genotoxic to the isolated or whole blood lymphocytes at high concentrations. Thus, more research is needed to comprehensively assess the effects of EGCG on genetic materials.

Key words: (-)-Epigallocatechin gallate (EGCG); Single-cell gel electrophoresis (SCG); Green tea; Anti-carcinogenic; DNA strand breaks

1. Introduction

Tea is one of the most popular beverages consumed by over two thirds of the world's population. Recently, increasing attention has been given to the anti-carcinogenic effects of (-)-epigallocatechin gallate (EGCG), the main constituent of the polyphenols present in green tea leaves. It is reported that 1.0×10^{-4} M EGCG can significantly inhibit the growth of acute myeloblastic leukemia cells and induce apoptosis in human cancer cells¹⁻³⁾. Although the sensitivities of different cancer cell lines to EGCG differ⁴⁾, EGCG is more and more seen as a possible new cancer-inhibiting and anti-carcinogenic natural chemical.

Nevertheless, the effects of EGCG on normal cells are not clear. Although a high dose of green tea polyphenols can induce death in experimental animals⁵⁾ and inhaling green tea dust can increase the risk of pulmonary granulomatosis in human beings⁶⁾, there are few studies of the genotoxicity of EGCG on normal human cells. In this study, we examined the effect of EGCG on isolated lymphocytes and whole blood lymphocytes from two smokers and a nonsmoker with SCG assay. In a series of experiments, we found that EGCG had both inhibitory and stimulatory effects on DNA strand breaks in normal human lymphocytes depending on the dosage and extracellular environmental factors as evaluated by SCG assay.

2. Materials and methods

2.1. Materials

All reagents used in this experiment were of analytical grade. (-)-Epigallocatechin gallate (EGCG) was from Sigma. Before use, EGCG was dissolved in distilled water to prepare 5.0×10^{-2}

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Reprint requests to: Prof. Kanehisa MORIMOTO,

Department of Social and Environmental Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

TEL: +81(6)6879-3920 FAX: +81(6)6879-3929

e-mail: morimoto@envi.med.osaka-u.ac.jp

M of stock solution. When the stock solution was actually used, it was diluted with distilled water to make different concentrations of EGCG. In an examination of the effect of EGCG on the isolated or whole blood lymphocytes from the smokers, 0.02 ml of EGCG solution was added to 5 ml culture medium (containing 0.2 ml blood for whole blood culture), and the same volume of distilled water was added for control. In an examination of the effect of EGCG on the whole blood lymphocytes from the nonsmoker, 0.2 ml EGCG solution was added to 4.8 ml culture medium (containing 0.2 ml blood), and the same volume of distilled water was added for control.

2.2. Subjects

Two smoking and a nonsmoking volunteers were used as subjects, and samples of their peripheral blood were used in the experiments. Subject A was a 41-year-old male. He has smoked 15 cigarettes per day for 22 years and drank no alcohol. For 24 hrs before blood sampling, he drank neither alcohol nor tea. During the last 3 hrs before blood sampling, he smoked 3 cigarettes.

Subject B was a 33-year-old male. He has smoked 20 cigarettes per day for 17 years, and drank 2 shot glasses of whisky per time and 5-7 times per week. Ten hours before blood sampling, he drank 2 shot glasses (60 ml/glass, 40%) of alcohol. During the last 3 hrs before blood sampling, he smoked 3 cigarettes.

The nonsmoker was a 34-year-old male. He drank neither alcohol nor tea.

2.3. Effects of EGCG on human isolated lymphocytes

For each experiment, 5 ml of heparinized whole blood was collected by venipuncture from the smokers, and the lymphocytes were isolated with Ficoll-Paque Research Grade (Amersham Pharmacia Biotech AB, Sweden). The isolated lymphocytes were washed twice with RPMI 1640 (Niken Biomedical Laboratory, Japan) and cultured (1×10^5 lymphocytes/5 ml) with RPMI 1640 containing 10% fetal bovine serum (Biosciences Pty Ltd., Australia) and 1% penicillin-streptomycin (Life TechnologiesTM, N.Y., USA). After treatment with different concentrations of EGCG for 2 hrs at 37 °C and 5% CO₂, the cells were harvested for the SCG assay.

2.4. Effects of EGCG on human lymphocytes in whole blood culture

Five milliliters of heparinized whole blood was drawn from the two smoking and one nonsmoking volunteers. Then, 0.2 ml of whole blood was added to the 4.8 ml culture medium as described above. After treatment with different concentrations of EGCG for 2 hrs at 37 °C and 5% CO₂, the lymphocytes were isolated with Ficoll-Paque Research Grade, and the DNA damage in the lymphocytes was detected using SCG assay.

2.5. Single-cell gel electrophoresis (SCG)

At the end of the treatment, the lymphocytes were collected and adjusted to a 1.0×10^5 to 5.0×10^5 cells/ml cell suspension. We took 25 μ l cell suspension, mixed it with 75 μ l 0.75% low-melting agarose (Nusieve GTG, FMC BioProducts, Rockland, USA), and then placed it on pre-cleaned frosted micro slides (Matsunami Glass Ind., Ltd, Japan) that were first covered with 80 μ l 0.5% normal melting agarose (Sigma). We immediately covered the mixed cell suspension with a coverglass, and then kept the slides at 4 °C for 10 min to allow solidification of the

agarose. After gently removing the coverglass, we used the same method to prepare the third layer of gel and then treated the slides using the method described by Singh et al⁷⁾. The electrophoresis time was 20 min under 25 V and 300 mA using an electrophoresis compact power supply (ATTO Corporation, Japan).

After electrophoresis, we stained the slides with 20 μ g/ml ethidium bromide (Sigma), and measured the DNA strand breaks under a fluorescent microscope using a DNA SCG test system (Keio Electronic Ind., Co., Ltd, Japan).

2.6. Statistical analysis

Tail moment was used to represent the extent of DNA damage to the lymphocytes, and the Mann-Whitney U-test in SPSS statistical software was used for the result analysis.

3. Results

3.1. Effects of EGCG on isolated human lymphocytes

To examine the genotoxic effects of EGCG on the isolated human lymphocytes, we used the same concentrations of EGCG as previously reported to be inhibitory of cancer cells and noted the representative damage induced by EGCG in lymphocytes (Fig. 1). In the experiments, after treating the lymphocytes for 2 hrs with EGCG at concentrations from 2.5×10^{-5} M to 2.0×10^{-4} M, DNA strand breaks in lymphocytes either from subject A or subject B were both significantly increased ($P < 0.01$) (Table 1). This result showed that EGCG induced DNA strand breaks in normal human lymphocytes at the same concentrations reported to inhibit cancer cells.

3.2. Effects of EGCG on whole blood lymphocytes

We examined the effects of EGCG on the whole blood lymphocytes by treating the whole blood from the two smoking volunteers with the same method and EGCG concentrations as described above. As seen in Table 2, EGCG showed a suppressive effect ($P < 0.01$) against DNA strand breakage in the whole blood lymphocytes from smoker A and smoker B at concentrations of 1.0×10^{-4} M and 5.0×10^{-5} M, respectively.

To examine the effects of EGCG on the whole blood lymphocytes from the nonsmoker, we treated his whole blood with EGCG at concentrations from 3.1×10^{-5} to 2.0×10^{-3} M. As shown in Table 3, after 2 hrs of treatment, EGCG suppressed DNA strand breakage in whole blood lymphocytes at a concentration of 2.5×10^{-4} M. In contrast, at higher concentrations of 1.0×10^{-3} M and 2.0×10^{-3} M, EGCG increased DNA strand breaks in the lymphocytes.

4. Discussion

Tail moment is a sensitive biomarker to evaluate DNA damage as detected by SCG assay. It reflects both the amount of migrated DNA as well as the migration distance of the DNA fragments in the "cell tail" by a singular number. It is commonly defined through calculation of the distance of DNA fragment migration and the relative intensity of DNA fragments in the "cell tail." In the present study, we used a DNA SCG system to examine DNA strand breaks in lymphocytes from three healthy subjects. This system can measure tail moment according to the same definition as described above⁸⁾.

In daily life, since the human body is constantly exposed to

Fig. 1 The effect of EGCG on human isolated lymphocytes. After 2-h incubation at 37 °C and electrophoresis, the slides were stained with 20 μ g/ml ethidium bromide and examined with SCG assay. (A) control. (B) 5.0×10^{-5} M EGCG. (C) 2.0×10^{-4} M EGCG.

damaging agents, both outer (such as smoking, radiation and environmental pollutants, etc.) and inner (oxidative stress, genetic sensitivity etc.)^{8, 9}, DNA is constantly being damaged and repaired in the living cells. An SCG assay accurately reflects (mainly the strand breaks and alkali-labile points resulting from base damage etc.) this situation¹⁰. In our data you can also see the baseline damage in the lymphocytes from the controls.

After repeated experiments, our results showed that EGCG induced a significant increase in DNA strand breaks in normal human isolated lymphocytes at concentrations that reportedly induced cancer cell apoptosis. This seems contradictory to the widely believed protective effects of EGCG.

In fact, although EGCG is recognized to be an anti-carcinogen and antioxidant^{11, 12}, it does possess pro-oxidative activity. EGCG was reported to induce H₂O₂ in the human lung adenocarcinoma cell line NCI-H661 and the EGCG-induced H₂O₂ was suggested to mediate apoptosis or inhibit the growth of cancer cells in vitro¹³. Although the present study provided no direct evidence to prove that it is the EGCG-induced H₂O₂ that induced DNA strand breaks, the induction of DNA single-strand breaks by the hydroxyl radical generated from EGCG in supercoiled plasmid DNA may well be in line with our present findings¹⁴.

In the whole blood from the smokers and nonsmoker, contrary to the result in the isolated lymphocytes, EGCG showed suppressive effects against DNA strand breaks. Although different volumes of EGCG solution (0.02 ml for the smokers and 0.2 ml for the nonsmoker) were used to treat lymphocytes, which might result in a slight difference in concentrations of the culture medium, the results from both the smokers and nonsmoker showed a comparable effect.

This result is in line with the anti-carcinogenic effects of EGCG. The free radicals generated from cigarette smoke are believed to play important roles in inducing DNA strand breaks in living cells^{15, 16}, and the DNA strand breaks caused by cigarette smoking could be inhibited by EGCG through its antioxidant effect^{17, 18}. Nevertheless, as the process of the antioxidant effect of EGCG is rather complex¹⁹ and since there might be great individual differences, we need more samples to confirm the

Table 1 The effects of EGCG on the tail moment in human isolated lymphocytes

Concentrations (M)	Subject A				Subject B			
	n ^a	Exp. 1	Exp. 2	Total	n ^a	Exp. 1	Exp. 2	Total
Control	44	9.2 $\pm$ 1.9 ^b	7.2 $\pm$ 2.3	8.2 $\pm$ 2.1	42	22.5 $\pm$ 11.0	27.3 $\pm$ 14.1	24.9 $\pm$ 12.5
2.5×10^{-5}	42	77.2 $\pm$ 16.4	75.6 $\pm$ 17.1	76.4 $\pm$ 16.7*	40	52.6 $\pm$ 14.7	76.4 $\pm$ 22.2	64.5 $\pm$ 18.4*
5.0×10^{-5}	42	115.3 $\pm$ 21.0	139.1 $\pm$ 31.8	127.2 $\pm$ 26.4*	36	247.8 $\pm$ 68.0	275.8 $\pm$ 70.9	261.8 $\pm$ 69.4*
1.0×10^{-4}	36	227.6 $\pm$ 52.7	334.6 $\pm$ 66.3	281.1 $\pm$ 59.5*	44	334.2 $\pm$ 72.3	234.3 $\pm$ 60.9	284.3 $\pm$ 66.6*
2.0×10^{-4}	40	453.0 $\pm$ 90.6	478.2 $\pm$ 66.3	465.6 $\pm$ 78.4*	46	490.1 $\pm$ 115.9	466.7 $\pm$ 89.8	478.4 $\pm$ 102.8*

^anumber of lymphocytes

^bData are presented as Mean $\pm$ SE.

*Statistically significant difference as compared to control (Mann-Whitney U-test, $p < 0.01$).

Table 2 The effects of EGCG on the tail moment in human whole blood lymphocytes

Concentrations (M)	Subject A				Subject B			
	n ^a	Exp. 1	Exp. 2	Total	n ^a	Exp. 1	Exp. 2	Total
Control	46	8.6 $\pm$ 3.0 ^b	13.3 $\pm$ 3.5	10.9 $\pm$ 3.2	42	16.1 $\pm$ 5.0	22.9 $\pm$ 5.7	19.5 $\pm$ 5.3
2.5×10^{-5}	44	8.9 $\pm$ 2.6	14.8 $\pm$ 2.9	11.8 $\pm$ 2.7	46	11.3 $\pm$ 3.5	14.2 $\pm$ 3.8	12.7 $\pm$ 3.6
5.0×10^{-5}	48	9.7 $\pm$ 2.0	13.7 $\pm$ 3.8	11.7 $\pm$ 2.9	42	5.2 $\pm$ 1.3	2.4 $\pm$ 0.9	3.8 $\pm$ 1.1*
1.0×10^{-4}	48	2.4 $\pm$ 1.0	4.8 $\pm$ 1.7	3.6 $\pm$ 1.3*	44	1.2 $\pm$ 0.2	2.4 $\pm$ 0.5	1.8 $\pm$ 0.3*
2.0×10^{-4}	48	1.7 $\pm$ 0.4	1.5 $\pm$ 0.3	1.6 $\pm$ 0.3*	42	3.5 $\pm$ 0.5	4.3 $\pm$ 0.5	3.9 $\pm$ 0.5*

^anumber of lymphocytes

^bData are presented as Mean $\pm$ SE.

*Statistically significant difference as compared to control (Mann-Whitney U-test, $p < 0.01$).

Table 3 The effects of EGCG on the tail moment in human whole blood lymphocytes from the nonsmoker

Concentration (M)	n ^a	Mean $\pm$ SE		
		Exp. 1	Exp. 2	Total
Control	89	14.9 $\pm$ 2.8	11.9 $\pm$ 3.6	13.4 $\pm$ 3.2
3.1 $\times 10^{-5}$	87	11.7 $\pm$ 2.9	16.4 $\pm$ 4.8	14.0 $\pm$ 3.8
6.2 $\times 10^{-5}$	89	15.9 $\pm$ 3.9	12.1 $\pm$ 3.1	14.0 $\pm$ 3.5
1.3 $\times 10^{-4}$	86	9.6 $\pm$ 2.1	8.8 $\pm$ 1.9	9.2 $\pm$ 2.0
2.5 $\times 10^{-4}$	94	4.5 $\pm$ 1.1	5.0 $\pm$ 1.5	4.7 $\pm$ 1.3 ^{b, **}
5.0 $\times 10^{-4}$	89	20.5 $\pm$ 3.4	16.8 $\pm$ 3.8	18.6 $\pm$ 3.6
1.0 $\times 10^{-3}$	86	34.2 $\pm$ 7.5	31.5 $\pm$ 7.7	32.8 $\pm$ 7.6 ^{c, *}
2.0 $\times 10^{-3}$	92	52.8 $\pm$ 12.7	69.7 $\pm$ 15.7	61.2 $\pm$ 14.2 ^{c, **}

^anumber of lymphocytes^bDNA strand breaks were inhibited. ^cDNA strand breaks were increased.*, **Statistically significant difference as compared to control (Mann-Whitney U-test, * $p < 0.05$, ** $p < 0.01$).

present findings in future studies.

However, extensive differences in tail moment, and thus in the extent of DNA damage were observed among cells exposed to EGCG (Fig. 1). There are several possible explanations for the differential response observed for EGCG. Individual cells may vary in their sensitivity to EGCG, their radical scavenging capabilities, and other mechanisms which either enhance or diminish the effects of EGCG. Whatever may be the mechanism underlying this differential response to EGCG, our data demonstrate the stimulating effects of EGCG on DNA strand breaks in isolated lymphocytes and its suppressive effects on the DNA strand breaks in whole blood lymphocytes.

As for the opposite effects of EGCG between isolated lymphocytes and whole blood lymphocytes, we suppose it was the extracellular environmental factors that played a role. First, in human plasma, EGCG exists mainly in the conjugated form²⁰. The change of its structure after exposure to other contents, the existence of chelating metals (Fe²⁺/Fe³⁺ etc.) and free radicals of different sizes and sources in plasma could all influence the antioxidant effects of EGCG^{19, 21}. Secondly, because of the pro-oxidative activity of EGCG, H₂O₂ and EGCG semiquinone free radicals might be formed in the process of scavenging free radicals like O⁻₂^{13, 19}. The existence of other contents like catalase and

superoxide dismutase (SOD) in whole blood might possibly inhibit their effects and make it more difficult for EGCG to induce DNA strand breaks in whole blood lymphocytes than that in isolated lymphocytes. This might explain, at least partially, why EGCG increases DNA damage only when treated at a sufficiently high concentration in the whole blood like that in the isolated lymphocytes treated at a much lower concentration.

From Table 2, it seems that the whole blood lymphocytes from the two smokers have a different sensitivity to EGCG. In fact, just as different cell lines have a different sensitivity to EGCG⁴, different individuals may show a different response to EGCG. Previous studies suggest that EGCG in the blood of different individuals might show different distributions among conjugated forms and free forms, and different individuals have different sensitivity to EGCG²².

Although we showed that EGCG induced DNA strand breaks in the isolated lymphocytes and in whole blood, this does not necessarily mean that drinking tea would be harmful to one's health in daily life.

This is because EGCG can be easily metabolized in the body²³, and it is difficult for EGCG to reach a concentration as high as 1.0 $\times 10^{-3}$ M in blood only through ordinary drinking. If a single cup of tea contains 150 mg of EGCG, and some tea drinkers consume 10 cups a day²³, the concentration of EGCG would be less than 1.0 $\times 10^{-4}$ M in the peripheral blood and would do no harm to lymphocytes as assessed by SCG assay.

To conclude, in our present study, as we found that EGCG is not only inhibitory against DNA strand breaks in whole blood, but also genotoxic to isolated lymphocytes or whole blood lymphocytes at high concentrations, more research is needed to comprehensively assess the effects of EGCG on genetic materials.

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