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Epigallocatechin gallate induces telomere fragmentation in HeLa and 293 but not in MRC-5 cells

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Abstract

Telomeres are the tandem repetitive sequence at the end of chromosomes and its integrity is crucial for cell vitality. We studied the effect of (-)-epigallocatechin-3-gallate (EGCG), one of the major tea polyphenols, on telomeres in HeLa, 293 cells and MRC-5 fibroblasts. At concentrations of above 50 μM, EGCG was found to causes telomere fragmentation in HeLa cells as a result of single-strand breaks in a dose-dependent manner. Treatment of EGCG also caused telomere fragmentation in 293 cells but had little or only marginal effect on MRC-5 fibroblasts. The telomere fragments detected by electrophoresis showed a unique size distribution that seems to suggest that the strand breaks were not produced randomly, but with preference at some specific sites. We speculate that the differential effect of EGCG in inducing telomere fragmentation in HeLa and 293 verse MRC-5 cells might be relevant to the apoptosis-inducing effect of EGCG on cancerous cells but not on normal cells.

Keywords: DNA damage; Strand break; Apoptosis; Telomere; Tea

Introduction

Tea is a popular beverage in the Far East. Many studies in recent years have suggested that tea has potentially preventive effects against cancer (Ahn et al., 2003; Chung, 1999; Fujiki et al., 2001; Gupta et

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al., 1999a, 1999b; Li et al., 1999; Pianetti et al., 2002; Setiawan et al., 2001). Tea polyphenols, especially the (-)-epigallocatechin-3-gallate (EGCG), are believed to be involved in such cancer chemopreventive effects (Mukhtar and Ahmad, 2000; Stoner and Mukhtar, 1995). EGCG is a more effective scavenger of reactive oxygen species (ROS) than many other antioxidants (Lee et al., 2003) and has been shown to protect DNA against oxidative damage (Arimoto-Kobayashi et al., 2003; Johnson and Loo, 2000). EGCG may help to protect various cells from chemical or physical damage that leads to carcinogenesis (Chen et al., 2002; Ichihashi et al., 2000; Johnson and Loo, 2000; Katiyar et al., 2000; Wei et al., 1999).

An interesting property of tea polyphenols is their discriminative effects on normal and cancerous cells. EGCG, as the major component of tea polyphenols, has been found to completely inhibited the growth of SV40 transformed WI38 human fibroblasts but had little effect on the growth of the normal WI38 cells (Chen et al., 1998). A study on epithelial cells has revealed that EGCG at high concentrations lead to increase in ROS level in tumor cells and decrease in normal cells suggesting EGCG produced differential oxidative environments in tumor than in normal cells (Yamamoto et al., 2003). While many studies have demonstrated that EGCG induced apoptosis in cancer cells (Ahn et al., 2003; Chung et al., 2001; Gupta et al., 2000, 2004; Hastak et al., 2003; Hayakawa et al., 2001; Hibasami et al., 1998; Ishino et al., 1999; Islam et al., 2000; Kuo and Lin, 2003; Lu et al., 2002; Paschka et al., 1998; Yang et al., 1998; Yoo et al., 2002), the apoptosis-inducing effect was not seen in normal cells in a few comparative studies (Ahmad et al., 1997; Chen et al., 1998; Li et al., 2000). EGC, a minor component of tea polyphenols, has also been shown to induce apoptosis of human breast cancer cells but not of their normal counterparts (Vergote et al., 2002).

This work explored the effect of EGCG on the telomeres in HeLa cells. We separated telomere fragments from the bulk genomic DNA in EGCG-treated cells by alkaline denaturation and gel electrophoresis. The production of telomere fragments was caused by single-strand breaks and can be attributed to the oxidative stress created by EGCG at high concentrations. The size distribution of telomere fragments seems to suggest that the strand breaks might be produced at preferred sites. Interestingly, such effect of EGCG was also observed in the 293 transformed cells but not in normal MRC-5 fibroblasts. We speculate the ability to induce telomere fragmentation of EGCG in HeLa but not in normal cells may be relevant to the cytotoxic effect of EGCG on cancerous cells.

Materials and methods

Materials

(-)-Epigallocatechin-3-gallate (EGCG), dichlorofluorescein diacetate (DCF-DA), N-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum was purchased from Hyclone (Utah, USA). Penicillin and streptomycin sulfate were purchased from Shijiazhuang Pharmacy Ltd. (Shijiazhuang, China). Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Glutathione (GSH, reduced) was purchased from Amresco (USA).

Cell culture and treatment

HeLa, 293 and MRC-5 cells were grown in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 units/ml streptomycin sulfate in humidified incubator at 37 °C under 95% air,

5% CO_2. Cells were harvested by trypsinization, washed with serum-free DMEM twice, made to 10^6 cells/ml and treated with EGCG in serum-free DMEM at 37 °C for 1 hour. Where indicated, cells were pre-incubated with 5 mM GSH or 5 mM NAC at 37 °C for 1 hour before being treated with EGCG.

Detection of telomere fragments

Cells were suspended in 0.8% low-melting agarose (Gibco, Grand Island, NY, USA) in 40 mM phosphate-buffered saline (PBS, pH7.4) and plugs made in 100 μl plastic mold. Following solidification at 4 °C, the plugs were immersed in either neutral (pH 7.5) or alkaline (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauroylsarcosine, pH 10.0, 10% DMSO and 1% Triton X-100 added immediately before use) lysis solution for 1 hour and 20 minutes. The plugs were then transferred to alkaline buffer (300 mM NaOH, 1 mM EDTA, pH > 13.0) and incubated for 1 hour and 20 minutes to denature the DNA (Digue et al., 1999; Gedik et al., 1992; Rapp et al., 2000). The two incubations were both conducted at room temperature in dark with gentle agitation.

To detect telomere fragmentation, the plugs were loaded onto 0.8% agarose gel and eletrophoresed at 5 V/cm for 1 hour and 40 minutes in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). After electrophoresis, telomere fragments were blotted onto Hybond-N^+ membrane (Pall Corporation, USA) and detected using the "*T*elo TAGGG Telomere Length Assay" kit (Roche, Germany) according to manufacturer's instruction. The signals were recorded and analyzed on ChemiImager 4400 (Alpha Innotech, USA).

Measurement of intracellular ROS level

Measurements were carried out as described (Yang et al., 2000). Cells (10^6) were incubated in serum-free DMEM containing 10-200 μM EGCG at 37 °C. After 1 hour incubation, cells were washed three times with PBS and then incubated in PBS containing 5 μM DCF-DA for 30 minutes at 37 °C. The distribution of cells with DCF fluorescence were measured by the FACSCalibur flow cytometry (BD, CA, USA) and the mean fluorescence per cell was calculated as an indication of intracellular ROS level.

Results

Detection of telomere fragments

Electrophoresis of cells embedded in agarose gel, known as comet assay, has long been used to assess both double- and single-strand breaks in individual cells (Cotelle and Ferard, 1999; Olive et al., 2001). In this work, a large number of cells were embedded in agarose plug and DNA denatured under alkaline condition. Telomere fragments produced as a result of both double- and single-strand breaks were separated by electrophoresis, blotted onto membrane and visualized by probe specific to telomere repeats. This method can be regarded as an ensemble version of the comet assay, which specifically detects telomere fragments. As shown in Fig. 1, after alkaline denaturation, telomere fragments produced by single-strand breaks via H_2O_2 treatment were separated by electrophoresis and reliably detected by Southern blotting as a smear over a wide size range. However, when plug was not denatured in alkaline

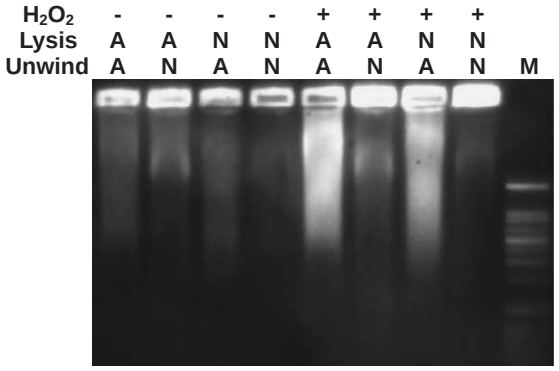

Fig. 1. Detection of telomere fragments by Southern blotting. HeLa cells (10^6) were cultured in the absence (−) or presence (+) of 300 μM H_2O_2, embedded in agarose plug, lysed in alkaline (A) or neutral (N) lysis buffer followed by unwinding in alkaline (A) or neutral (N) solution. M indicates molecular weight markers ranging from 21.2 to 0.9 kbp.

solution, very faint signals were detected. The detection produced better results when combined with lysis in alkaline buffer. Therefore, this combination was used for the rest experiments.

EGCG induced telomere fragmentation as a result of single-strand break in HeLa cells

Using the method demonstrated above, the effect of EGCG on telomere was studied by treating cells with EGCG of various concentrations. The results in Fig. 2 show that EGCG at the concentrations within 20 μM did not cause visible damage to telomere. However, at concentrations of above 20 μM, telomere fragmentation began to appear and increased with increasing in EGCG concentration. The damage to telomere is mainly single-strand breaks because non-denatured samples showed only very faint signal (Fig. 3). The effect of EGCG was only seen in intact cells. When EGCG was supplied after cells were lysed, no telomere fragmentation was observed (Fig. 4) suggesting that the effect of EGCG depends on cellular metabolism. In contrast to the treatment of H_2O_2 that produced smear of telomere fragments over a wide size range, the signal of fragments produced by EGCG was concentrated within a narrower range on agarose gel. This distinct pattern of signal indicates that the single-strand breaks produced by EGCG is less random than those produced by H_2O_2, which seems to suggest that the telomere fragments might be produced by damages at preferred sites.

EGCG elevated ROS level in HeLa cells

The ROS assay (DCF assay) measures the intracellular ROS level. HeLa cells were incubated with various concentration of EGCG to examine whether EGCG affected intracellular ROS formation. The results in Fig. 5 show that EGCG at low concentrations (<50 μM) slightly lowered ROS level, but elevated ROS level at high concentration (100 μM and 200 μM). This result is in agreement with the report that EGCG at 10 μM significantly inhibited H_2O_2- and SIN-1 (3-morpholinosydnonimine, a peroxynitrite generator)-mediated oxidative damage to cellular DNA (Johnson and Loo, 2000), while at higher concentrations (100 μM or higher) induced cellular DNA damage in Jurkat T-lymphocytes (Johnson and Loo, 2000), HL-60 (Furukawa et al., 2003) and WIL2-NS cells due to the production of H_2O_2 (Sugisawa and Umegaki, 2002).

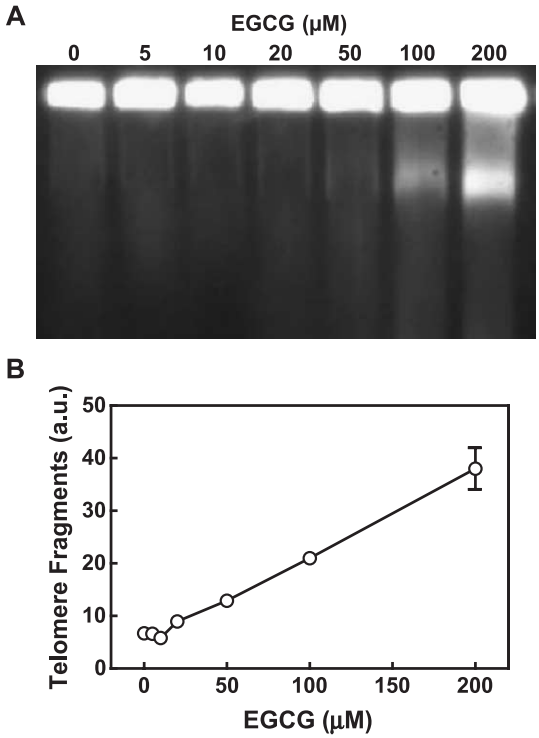

Fig. 2. Dose-dependence of EGCG-induced telomere fragmentation in HeLa cells. (A) A representative gel of detection of telomere fragments induced by EGCG at the concentrations indicated. (B) Plot of telomere fragment signal against EGCG dose. Data represent the mean of three independent measurements with standard deviation.

Effect of free radical scavengers on EGCG-induced telomere fragmentation

To find out if the elevated ROS level is involved in EGCG-induced telomere fragmentation in HeLa cells, we examined both the telomere fragmentation and ROS production in the presence of free radical scavengers. Cells were preloaded with reduced GSH or NAC before they were exposed to

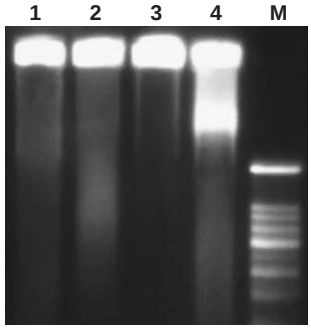

Fig. 3. EGCG induced telomere fragmentation as a result of single-strand break. HeLa cells (10^6) were cultured in the absence (lane 1, 2) or presence (lane 3, 4) of 200 μM EGCG. Lane 2 and 4 were subject alkaline unwinding before electrophoresis on agarose gel. M indicates molecular weight markers ranging from 21.2 to 0.9 kbp.

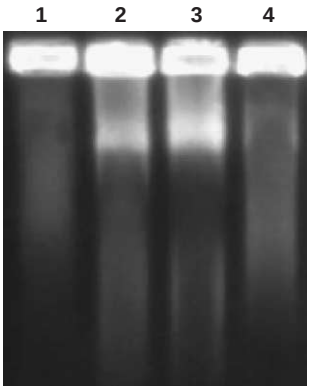

Fig. 4. Telomere fragmentation in HeLa cells (10^6) exposed to 200 μM EGCG at different stages. Lane 1: control; 2: exposed during cultivation in DMEM; 3: exposed in agarose plug; 4: exposed after cell lysis.

EGCG. The results in Fig. 6 indicate that both chemicals suppressed the telomere fragmentation suggesting that ROS was involved. The telomere fragmentation did not return to background level because the two scavengers themselves also caused damage to telomeres. This phenomenon is in agreement with the results previously reported that NAC induced oxidative damages to both cellular and isolated DNA (Oikawa et al., 1999) through generation of H_2O_2. It has also been reported that NAC and GSH (a metabolites of NAC) can interact with several commonly used cell culture media, including DMEM, to generate H_2O_2 (Hua Long and Halliwell, 2001), which in turn may damage DNA including telomere. The telomere damage produced by NAC and GSH may well be related to these properties of the two compounds.

Differential effect of EGCG on HeLa and 293 verse MRC-5 cells

To find out if EGCG has the same effect on other types of cells, we treated 293 cells (human embryonic kidney cell transformed by adenovirus 5 DNA) (Graham et al., 1977) and MRC-5 fibroblasts with 200 μM EGCG. The results in Fig. 7 show that both the HeLa and 293 cells exhibited considerable

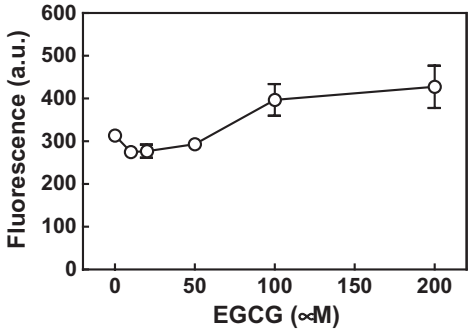

Fig. 5. Intracellular ROS formation in the presence of EGCG. HeLa cells (10^6) were incubated with EGCG of various concentrations and ROS levels determined by the DCF assay. Data represent the average of three independent measurements with standard deviation.

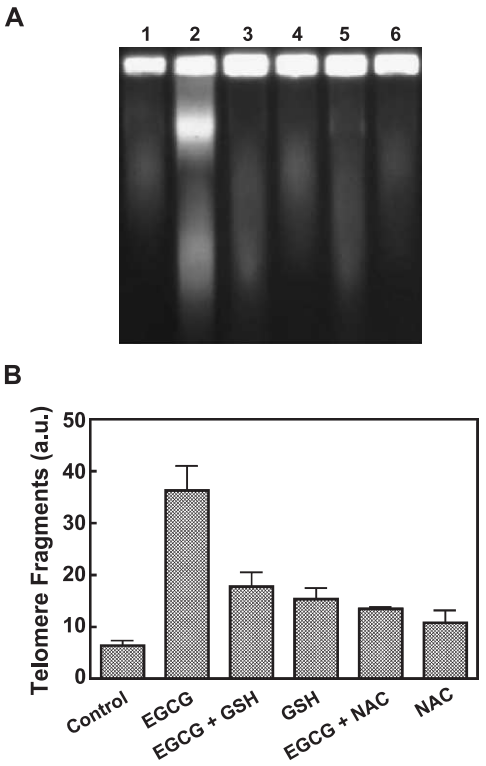

Fig. 6. Effect of free radical scavengers on EGCG-induced telomeric fragmentation. HeLa cells (10^6) were pretreated with 5 mM scavenger before exposing to 200 μM EGCG. Lane 1: control; 2: EGCG only; 3: GSH+EGCG; 4: GSH only; 5: NAC+EGCG; 6: NAC only.

telomere fragmentation while the MRC-5 cells showed much weaker signal. When the ROS production in HeLa and MRC-5 cells was examined by the DCF assay, it was found that HeLa cells produced 50% more ROS in the presence than in the absence of EGCG with a skewed distribution towards higher

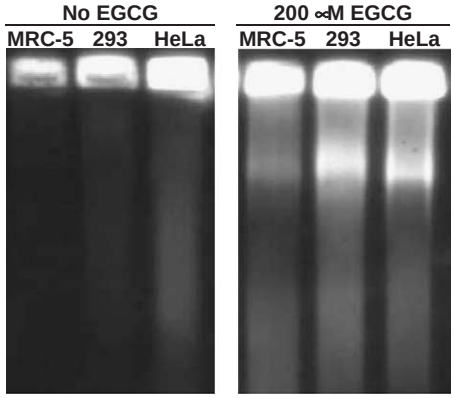

Fig. 7. Telomere fragments induced by EGCG in HeLa (cancer), 293 (transformed) and MRC-5 (normal) cells. Cells (10^6) were cultured in the absence or presence of 200 μM EGCG.

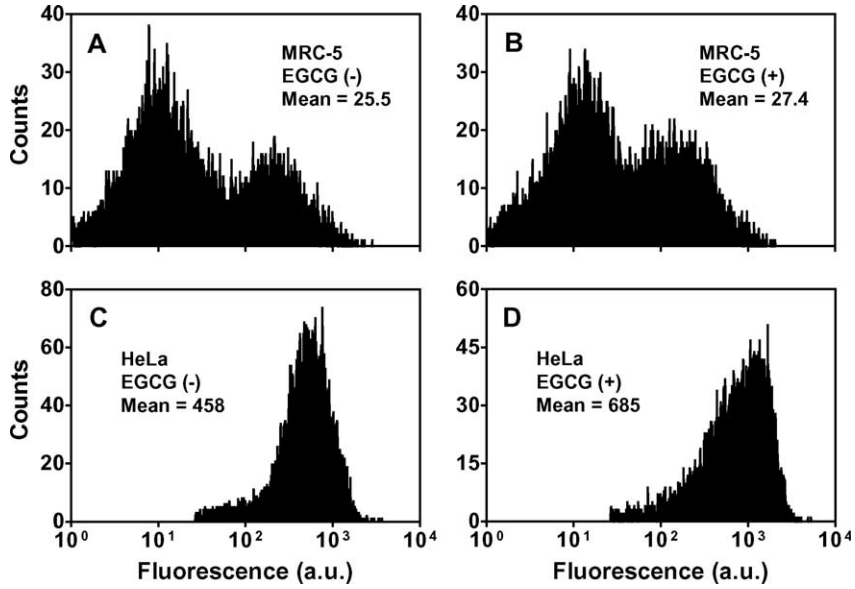

Fig. 8. Comparison of EGCG induced ROS production in HeLa (cancer) and MRC-5 (normal) cells. Cells (10^6) were incubated in the absence or presence of 100 μM EGCG and the distribution of cells with DCF fluorescence were given as an indication of intracellular ROS level.

fluorescence. In contrast, MRC-5 produced about 8% more ROS in the presence than in the absence of EGCG (Fig. 8).

Discussion

In this work, we show that EGCG at concentrations of above 50 μM induced telomere fragmentation in HeLa cells. The telomere fragmentation induced by EGCG appears to be different than those produced by H_2O_2 treatment in that the telomere fragments produced by EGCG treatment are more homogeneous in size. This phenomenon seems to suggest that there might be preference in the attacking sites on DNA. A recent work has shown that EGCG binds to a 67-kDa laminin receptor (Tachibana et al., 2004) suggesting possible function of EGCG in receptor-mediated pathways. It is possible that the telomere damage produced by EGCG may involve mechanism in addition to the general oxidative stress. In contrast to the effect on HeLa (cancer) and 293 (transformed) cells, EGCG produced little or only marginal telomere fragmentation in MRC-5 fibroblasts. This phenomenon seems can be explained by the difference in ROS production by EGCG in the cancerous verse normal cells. Yamamoto et al. (2003) reported that EGCG at high concentrations lead to increase in ROS level in tumor cells but decrease in normal cells. In our case, the HeLa cells produced, as judged by increase in DCF-DA fluorescence, 50% more ROS in the presence than in the absence of EGCG. However, the MRC-5 cells produced only 8% more ROS in the presence than in the absence of EGCG. The differential effect of EGCG on telomere fragmentation between HeLa and 293 verse MRC-5 cells may have possible relevance to the ability for EGCG to induce cellular apoptosis in cancer cells but not in normal cells (Ahmad et al., 1997; Chen et al., 1998; Li et al., 2000).

Several lines of evidences suggest that telomere plays a role in cellular apoptosis. Human telomere is composed of tandem repeats of (TTAGGG/CCCTAA) and stretches for about 2-20 kb long. In normal somatic cells, telomeres are shortened by about 50-200 bp per cell division. Shortening of telomere length to a threshold or disrupt of telomere structure is believed to have relation with senescence and cancer (Campisi et al., 2001). Telomeres in most cancer cells are maintained by telomerase that add telomere repeat to telomere ends to compensate telomere shortening (Kim Sh et al., 2002). Telomere shortening in telomerase inhibited cancer and immortalized cells can lead to apoptosis (Herbert et al., 1999; Nakajima et al., 2003; Zhang et al., 1999). Disruption of telomere structure has been shown to cause apoptosis (Karlseder et al., 1999; Multani et al., 2000; Ramirez et al., 2003; Zhang et al., 1999). Exogenous telomere nucleotide fragments introduced into Jurkat cells have been shown to induce apoptosis (Eller et al., 2002). Apoptosis induced by certain factors is accompanied by telomere loss (Ichiyoshi et al., 2003; Ramirez et al., 2003; Ren et al., 2001).

The work of Sitte et al. shows that cells kept in resting status accumulate single-strand breaks in telomeres (Sitte et al., 1998). When released into cell cycle, the cells experienced an abrupt shortening in telomere length indicating that telomere fragments produced by strand breaks are lost during DNA replication. This phenomenon suggests that the strand breaks occurred in the telomere sequences are expected to have two consequences for proliferating cells. First, the telomere fragment that stretches from the point of break to the terminus of the telomere may not be replicated as part of the chromosome if the lesion is not repaired, which may result in a shortening of the telomere. Second, the telomere fragment may be detached from the chromosome and released into the cell, which should also have the same or similar effect as the synthetic telomere oligonucleotides introduced into cells. Based on the effect of telomere shortening (Herbert et al., 1999, 2002; Nakajima et al., 2003; Zhang et al., 1999) and exogenously introduced telomere nucleotides (Eller et al., 2002) in inducing apoptosis, we speculate that the telomere fragmentation induced by EGCG may contribute to cellular apoptosis through the same mechanisms.

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