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Polyphenol (−)-epigallocatechin gallate inhibits apoptosis induced by irradiation in human HaCaT keratinocytes

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Abstract

Green tea is a rich source of polyphenols, and (−)-epigallocatechin-3-gallate (EGCG) is a major constituent of green tea polyphenols. In the present study, we investigated the effect of EGCG on apoptosis induced by irradiation in the human keratinocytic cell line HaCaT. Irradiation by γ-ray induced apoptosis with concomitant cleavage of caspase-3 and its in vivo substrate poly(ADP-ribose) polymerase. Treatment of cells with EGCG inhibited irradiation-induced apoptosis as detected by Hoechst staining and internucleosomal cleavage of DNA, and prevented the cleavage of these proteins by irradiation. We also found that the treatment of cells with EGCG alone suppressed cell growth and induced apoptosis in these cells. Our results suggest that EGCG inhibits irradiation-induced apoptosis by inactivating the caspase pathway in HaCaT cells. Our study also indicates that EGCG has a dual effect on the survival of these keratinocytes.

Keywords: (−)-Epigallocatechin-3-gallate; Irradiation; Apoptosis; Keratinocyte; Caspase pathway

Tea is a popular beverage worldwide. Epidemiological studies have shown that its consumption is associated with health benefits including cancer prevention and heart disease [1]. However, the mechanisms for these functions are not clear. Among different teas, green tea has been chemically characterized; the water extractable fraction of green tea contains many polyphenols known as catechins. Catechin is one of the polyphenols, and (−)-epigallocatechin-3-gallate (EGCG) is a major catechin of green tea (>50% of polyphenolic fraction) [2]. Several other catechins, such as epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC), are also known as green tea polyphenols, although the effect of EGCG is the most potent among these catechin compounds [3–5]. Moreover, recent studies have revealed that EGCG has a variety of biological functions in vitro such as inhibiting cell proliferation, inducing cell cycle arrest or apoptosis, and influencing the activities of transcriptional factors [2,6]. It has also been reported that topical application of EGCG inhibits UV-induced apoptosis of keratinocytes, whereas EGCG is known to induce proliferation and also apoptosis of these cells [7,8]. Its antioxidant properties emerge as a potentially important mode of action. EGCG is known to protect against the genotoxic effects of UV-irradiation; the ability of EGCG to modify endpoints directly related to the carcinogenic process has been demonstrated in skin [9]. Thus, protective effects of EGCG from UV-irradiation in vitro and in vivo have been well documented. However, the effects of EGCG on irradiation in cells are not fully understood.

Irradiation causes DNA damage and induces neoplastic transformation in cells. There is growing evidence that radiation-induced oxidative DNA damage may play an important role in carcinogenesis of the skin [10]. When skin is exposed to high-dose radiation, complex pathophysiological reaction including severe inflammation, death of affected cells, and destruction of tissue occurs. This is the radiation burn or cutaneous radiation syndrome, the severity being dependent on the amount of energy deposited in the tissues and the rate of

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energy deposition. Several cases of exposure to high-dose irradiation have been reported including the Tokaimura criticality accident and others [11–13]. However, there are few studies regarding the treatment of radiation injuries since radiation accidents occur relatively rarely. On the other hand, in response to irradiation, cells induce genes or activate proteins that protect themselves from the external insult [14–16].

Keratinocyte proliferation is balanced by terminal differentiation in the granular layer that leads to nuclear fragmentation and caspase activation [17]. The caspase cascade has been thought to play a central role in death receptor-mediated signaling pathways that transduce apoptotic signals from the initiator to caspase-activated DNase (CAD) via the executioner caspases [18]. Identification of CAD, responsible for internucleosomal DNA fragmentation and its proteolytic activation by caspase-3, sheds new light on the molecular basis for the mechanisms of apoptosis [17,19], and the caspase pathways have been intensively studied over recent years [19]. Irradiation has been shown to induce apoptosis in normal keratinocytes as well as in cancer-prone cells [20]. In the present study, we investigated the effects of EGCG on apoptosis induced by irradiation in the human keratinocytes HaCaT and the signaling pathways through which EGCG acts in vitro. We found that EGCG inhibited apoptosis by irradiation via inactivation of the executor caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP) a substrate cleaved by caspase-3 during apoptosis in these cells.

Materials and methods

Chemicals. EGCG (MW 458.4) from green tea was purchased from Sigma–Aldrich (St. Louis, MO) and was >95% pure. Polyclonal rabbit antibody against PARP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal anti-caspase-3 antibody from BD Biosciences (Transduction Laboratories, Lexington, KY), and polyclonal antibody against phosphorylated p53 at Ser15 from Cell Signaling Technology (Beverly, MA). Anti-human $p21^{WAF1}$ and Bax antibodies were purchased from Santa Cruz Biotechnology. Hoechst 33258 (Bisbenzimide H 33258 Fluorochrome, Trihydrochloride) was obtained from Calbiochem–Novabiochem (La Jolla, CA).

Cells and culture. Human keratinocytic HaCaT cells were grown in α-MEM supplemented with 7% heat-inactivated fetal calf serum (FCS) (Intergen, Purchase, NY), penicillin (50 U/ml), streptomycin (50 mg/L), and $NaHCO_3$ (2 g/L) in a humidified atmosphere containing 5% CO_2.

Analysis for determination of apoptosis. Apoptosis was determined by microscopic examination of nuclear morphology. Cells were fixed in 1% glutaraldehyde overnight and chromatin was stained with 10 μg/ml Hoechst 33258 for 30 min. Cells with condensed chromatin were counted under an Axioplan 2 microscope (Carl Zeiss, Jena, Germany). The percentages of apoptotic cells were determined from 10 fields chosen randomly (>200 cells counted).

Western blotting. The cells were lysed in an ice-cold solution containing 40 mM Tris–HCl (pH 8.0), 0.1% NP-40, 120 mM NaCl, and protease inhibitors (Complete, Boehringer–Mannheim GmbH, Mannheim, Germany). The lysates were heated to 100 °C for 5 min. These denatured samples were loaded onto SDS–polyacrylamide gel. After electrophoresis the proteins were electrotransferred onto a Nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Residual binding sites on the membrane were blocked by incubation in TBS with 0.1% Tween 20 and 5% skim milk. The membranes were then incubated with the primary antibody in TBS with 0.1% Tween 20 and 5% skim milk for 1 h at room temperature. The primary antibody was removed and the blots were washed three times in TBS with 0.1% Tween 20. To detect the antibody reactions, the blots were incubated for 1 h with horseradish peroxidase-labeled antibody (IgG) diluted 1:4000 in TBS with 0.1% Tween 20 and 5% skim milk. After rinsing intensively in TBS with 0.1% Tween 20, the blots were placed in ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) for 1 min at room temperature. The blots were then removed from the working solution and exposed to film.

Determination of DNA fragmentation. The detection of internucleosomal DNA fragmentation was performed using Suicide-Track (Oncogene Research Products, San Diego, CA), based on separation of apoptotic DNA from high molecular weight, intact, genomic DNA. After removing the culture media, cells were washed with PBS and scraped with a rubber policeman. The cell pellets were suspended in DNA extraction buffer and rocked gently. DNA was also extracted from apoptotic cells in the removed media. After degradation of contaminated RNA and treatment with proteinase K, the fragmented DNA was precipitated in isopropanol with sodium acetate (pH 5.2). The precipitates were pelleted by centrifugation for 5 min at 15,000–16,000*g*, rinsed with 70% ethanol, air-dried, and resuspended in buffer containing 1 mM EDTA and 10 mM Tris–HCl, pH 7.5. The samples were heated to 65 °C for 10 min and electrophoresis was performed with a 1.5% agarose gel. Finally, the gels were stained with ethidium bromide.

Results

Effects of EGCG on growth and cell death of human HaCaT keratinocytes

EGCG has been shown to induce G1 arrest and apoptosis [6]. In the initial experiments, we studied the effects of EGCG on cell growth and death in human HaCaT keratinocytes. These cells were spread at an initial concentration of 3×10^5 per plate and cultured for 16 h. Then, HaCaT cells were cultured with different concentrations of EGCG (1, 10, 50 μM) for 24 h. Studies of Hoechst staining assay showed that 50 μM EGCG significantly induced apoptosis (untreated cells: $3.2 \pm 1.3\%$, 50 μM EGCG: $6.0 \pm 2.0\%$, $p = 0.0015$, $n = 10$) (Table 1). In parallel, the numbers of viable cells were also counted by the trypan blue exclusion test. The numbers in control and EGCG-treated viable cells were $1.51 \pm 0.11 \times 10^6$ and $0.97 \pm 0.02 \times 10^6$ cells/plate, respectively. Cell viabilities were $93.9 \pm 2.0\%$ and $92.5 \pm 0.5\%$ in control and EGCG-treated cells, respectively. Thus, our results suggest that EGCG inhibited cell growth, also inducing apoptosis in these cells, although the magnitudes of apoptosis were small.

Inhibition of irradiation apoptosis by ECGC in HaCaT cells

Irradiation induces apoptosis in various types of cells [21]. We studied the effects of irradiation-induced

Table 1
Effect of EGCG on apoptosis induced by irradiation

	EGCG		
	0	10 μM	50 μM
0 Gy	$3.2 \pm 1.3^{*}$	4.5 ± 1.8	$6.0 \pm 2.0^{*}$
20 Gy	$10.6 \pm 2.7^{**}$	10.8 ± 2.6	$6.9 \pm 2.1^{**}$
80 Gy	$33.0 \pm 11.7^{***,****}$	$21.7 \pm 3.2^{***}$	$17.7 \pm 3.9^{****}$

HaCaT cells were pretreated with 10 or 50 μM of EGCG for 16 h and then irradiated at 20 or 80 Gy in the presence or absence of EGCG. Then cells were cultured for 24 h, fixed, and stained with Hoechst 33258. Apoptotic cells were counted under a fluorescence microscope. Data are presented as means ± standard error of counts from 10 fields.

$^{*}p = 0.0015$.
$^{**}p = 0.0038$.
$^{***}p = 0.015$.
$^{****}p = 0.0024$.

apoptosis in human keratinocytes HaCaT. HaCaT cells were spread at an initial concentration of 3×10^5 cells/plate and cultured for 16 h. Then, these cells were irradiated at different doses of γ-rays in the absence or presence of either 10 or 50 μM EGCG. After 24 h of irradiation, cells were harvested and apoptosis was determined by Hoechst staining (Table 1). Irradiation was shown to induce apoptosis in a dose-dependent manner. In cells irradiated with 20 Gy, 50 μM EGCG significantly inhibited the apoptosis by irradiation. However, 10 μM EGCG did not affect the apoptosis in these cells. On the other hand, treatment of cells with 10 μM EGCG significantly inhibited apoptosis of 80 Gy-irradiated cells in a dose-dependent manner. We also studied DNA fragmentation by irradiation in these cells. As shown in Fig. 1A, 10 Gy of irradiation induced DNA fragmentation in HaCaT cells, and treatment of cells with 50 μM of EGCG inhibited the DNA fragmentation. Further study found that irradiation with 40 Gy induced DNA-laddering after 4 h and EGCG prevented the DNA fragmentation in these cells (Fig. 1B).

Mechanisms for inhibition of apoptosis by EGCG in irradiated HaCaT keratinocytes

To evaluate the nature of the anti-apoptotic effect mediated by EGCG, we analyzed the effect of EGCG on activation of caspase-3 and cleavage of PARP. Cells were cultured with 50 μM of EGCG for 16 h and then irradiated at 10 Gy in the presence of EGCG. Cells were harvested at the times indicated in Fig. 2. Western blot analysis showed that treatment of cells with EGCG alone did not affect cleavage of these proteins at detectable levels. On the other hand, irradiation of these cells with 10 Gy cleaved caspase-3 from 32 kDa to its cleaved form, a 17 kDa fragment (Fig. 2). A cleaved form of caspase-3 was detected at 18 h after irradiation and these levels increased in a time-dependent manner. Furthermore, cleavage of PARP was observed in almost the same manner as that of caspase-3. Treatment of cells

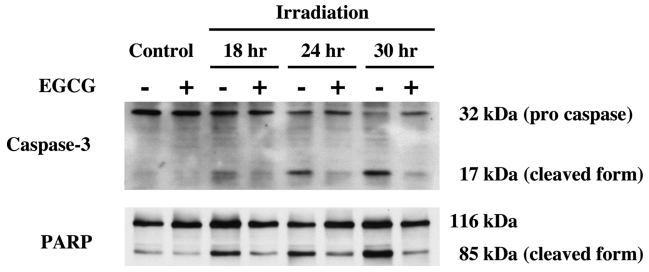

Fig. 2. Effects of EGCG on activation of caspase-3 and PARP cleavage by irradiation in HaCaT cells. Cells were irradiated at 10 Gy after culturing with or without 50 μM EGCG for 16 h. At the indicated times, protein (50 μg/lane) from total cell lysates was subjected to SDS–PAGE and Western blot analysis was performed using either caspase-3 or PARP antibody.

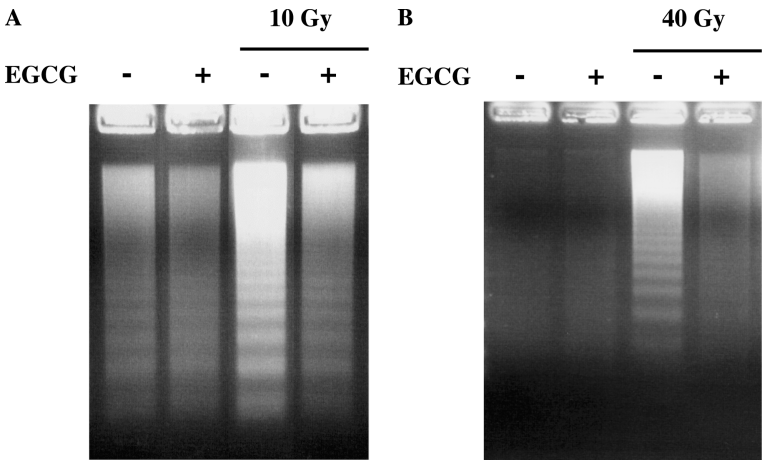

Fig. 1. Inhibition of irradiation-induced DNA fragmentation by EGCG in human HaCaT keratinocytes. HaCaT cells were pretreated with 50 μM EGCG for 16 h and then irradiated at 10 (A) or 40 Gy (B) in the presence or absence of EGCG. Cells were then cultured for 4 (B) or 24 h (A). Cellular DNA was extracted as described in Materials and methods, and analyzed by agarose gel electrophoresis, followed by ethidium bromide staining.

with EGCG almost completely suppressed the irradiation-induced cleavage of the executioner caspase-3 and its substrate PARP. Thus, activation of caspase-3 and cleavage of PARP during irradiation-mediated were blocked by EGCG.

Discussion

Green tea and its constituent polyphenols have been shown to have anti-tumor properties in a wide variety of experimental systems including animal models [22–24]. Furthermore, human clinical trials have also indicated some positive link between the drinking of green tea and the decrease of cancer incidence [25]. Recently, the anti-tumor effects of these green tea polyphenols have been studied at the cell biological level; the major cellular phenomena induced by the catechins were found to be apoptosis and cell cycle arrest [26]. EGCG is the most abundant compound among the polyphenols in green tea and is the most potent in terms of bioactivity. Recent studies have shown that EGCG has anti-inflammatory and anti-oxidant activities [7,8,27]. However, the effects of EGCG on irradiated cells or tissues remain to be elucidated. In the present study, we investigated the effects of EGCG on apoptosis induced by irradiation using a human keratinocyte line, HaCaT, and analyzed the intracellular apoptotic signal transduction pathway linked to the activation of caspase.

Caspases are synthesized as inactive pro-enzymes, and their activation during apoptosis results in cleavage at specific aspartate cleavage sites [28]. The downstream signals during apoptosis are transmitted via caspases. Upon conversion from pro- to active forms, caspases mediate the cleavage of PARP, followed by DNA fragmentation. The DNase responsible for the fragmentation is reportedly activated directly by caspase-3 [18,19]. Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme PARP. Thus, PARP is known to be cleaved in the execution phase of apoptosis. Although cell death by apoptosis has been recognized as an important control mechanism in the maintenance of tissue homeostasis and in the elimination of cells with damaged DNA, information on the induction and characteristics of apoptosis in keratinocytes is rather scarce. In the present study, we showed that irradiation induced apoptosis, which was accompanied by the activation of the caspase cascade in human keratinocytes. Furthermore, EGCG inhibited the radiation-induced apoptosis and the activation of the caspase cascade in these cells. Catechins are water-soluble substances with limited ability to pass through the plasma membrane of cells. Therefore, EGCG may bind to certain surface receptors of cells, thereby provoking anti-apoptotic intracellular signals [29]. Further clarification of this issue will require additional studies.

Genotoxic stress such as irradiation induces different patterns of p53-dependent and p53-independent apoptosis in cells of various organs [30]. Recent studies have shown that green tea and EGCG selectively inhibit cell growth and induce apoptosis in cancer cells without adversely affecting normal cells [31]. Furthermore, EGCG has been reported to induce G1 phase arrest and apoptosis in human prostate carcinoma cells via $p21^{WAF1}$, irrespective of the p53 status, and that EGCG affects the expression of Bax, also a p53-dependent downstream target, and favors apoptosis [6,32]. In our study, the tumor-suppressor p53 was highly expressed in HaCaT cells. EGCG did not induce the phosphorylation of p53 or the expression of $p21^{WAF1}$ or Bax (data not shown), whereas EGCG inhibited cell growth and induced apoptosis. High levels of p53 generally reflect mutant protein, and it is known that both alleles of p53 are mutated in HaCaT cells [33]. We studied the effects of EGCG on the activation of p53 and the expression of $p21^{WAF1}$ and Bax by irradiation. Irradiation did not induce the phosphorylation of p53 at Ser15 or the expression of $p21^{WAF1}$ or Bax (data not shown). Treatment with EGCG did not affect these proteins in irradiated keratinocytes. Thus, our results suggest that EGCG inhibits apoptosis by irradiation through a pathway independent of p53.

The water-extractable fraction of green tea contains abundant polyphenols, of which EGCG is the major constituent. After a common brewing procedure, 30–42% (w/w) of green tea is dissolved in water [2]. Therefore, a single cup of green tea may contain as much as 300 μM as calculated from the fact that a single cup of green tea may contain 150–200 mg EGCG [34–36]. On the other hand, studies of [^{3}H]EGCG have shown that 10% of the initial dose of EGCG was detected 24 h after a single intra-gastric administration [37]. EGCG can also be absorbed rapidly through oral mucosa [38]. In the present study we used concentrations of EGCG up to 50 μM, and the 50-μM-concentration level is suggested to be within the range of EGCG concentration in normal green tea beverage. Our results suggest that the oral intake of EGCG may act as a radioprotector against radiation burn.

EGCG is well known as a scavenger of ROS in extracellular environments, although it induces the production of ROS [39,40]. Our results demonstrate that apoptosis was induced in keratinocytes in vitro by irradiation, and that EGCG exhibited a cytoprotective effect by inhibiting this irradiation-induced apoptosis in these cells. There are, however, also studies reporting that EGCG induces apoptosis, has a pro-apoptotic effect, or stimulates proliferation [6–8,41]. In our study, treatment of HaCaT keratinocytes with EGCG also induced apoptosis, but the degree was minor. It remains

uncertain how the different mechanisms function during the antioxidant (cytoprotective) versus oxidant (cytostatic/cytotoxic) actions of EGCG. Since keratinocytes are affected by numerous external and internal stimuli, they may have several protective mechanisms to prevent apoptosis and to ensure the structural integrity of the outermost barrier of the body. The growth inhibition by EGCG may be one of the mechanisms for the radioprotective effect.

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