(−)-Epigallocatechin-3-Gallate Prevents Photocarcinogenesis in Mice through Interleukin-12–Dependent DNA Repair

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Abstract
We have shown previously that topical application of (−)-epigallocatechin-3-gallate (EGCG), the major polyphenol of green tea, prevents photocarcinogenesis in mice. EGCG prevents UVB-induced immunosuppression by inducing interleukin-12 (IL-12). As immunosuppression is a risk factor for photocarcinogenesis, we investigated the possibility that EGCG also prevents UVB-induced photocarcinogenesis through an IL-12–dependent DNA repair mechanism. To investigate this possibility, we determined the effects of EGCG on photocarcinogenesis in IL-12 knockout (KO) mice using the formation of cyclobutane pyrimidine dimers (CPD) as an indicator of the extent of UVB-induced DNA damage. Topical application of EGCG (1 mg/cm2 skin) prevented photocarcinogenesis in wild-type (C3H/HeN) mice in terms of tumor incidence and tumor multiplicity but did not prevent photocarcinogenesis in IL-12 KO mice. UVB-induced DNA damage, as determined by the formation of CPDs and the number of sunburn cells, was resolved more rapidly in the skin of wild-type mice treated with EGCG than untreated control mice. In contrast, the extent of UVB-induced DNA damage and the numbers of sunburn cells were not significantly different in the EGCG-treated IL-12 KO mice and untreated control mice. In addition, treatment of XPA-proficient human fibroblast cells with EGCG promoted repair of UVB-induced CPDs in a dose-dependent manner but not in an XPA-deficient cells, indicating that the nucleotide excision repair mechanism is involved in EGCG-mediated DNA repair. Taken together, these results indicate for the first time that EGCG can prevent photocarcinogenesis through an EGCG-induced IL-12–dependent DNA repair mechanism. (Cancer Res 2006; 66(10): 5512-20)

Introduction
Green tea polyphenols derived from the leaves of the Camellia sinensis, have a number of beneficial health effects, including the anticarcinogenic activity in various tumor models (1, 2). In previous studies, we and others have shown that either oral administration of green tea polyphenols (a mixture of several polyphenols) in drinking water or topical application of their major and most chemopreventive constituent, (−)-epigallocatechin-3-gallate (EGCG), inhibits solar UV radiation–induced skin carcinogenesis in mice in terms of tumor incidence, tumor multiplicity, and tumor growth (3–5). UVB radiation–induced immunosuppression also is inhibited by topical application of EGCG in a hydrophilic cream-based formulation in mice (6, 7). As suppression of the immune system by solar UV radiation, in particular the UVB spectrum (290-320 nm) of solar light, contributes to the induction of skin cancer (8, 9), the ability of EGCG to inhibit the UV-induced immunosuppression most likely contributes to its ability to inhibit UV radiation–induced skin carcinogenesis in mice. In addition to its immunosuppressive effects, solar UV light also contributes to premature aging of the skin and exacerbation of infectious diseases (10, 11). UV-induced DNA damage, predominantly in terms of the formation of cyclobutane pyrimidine dimers (CPD), has been recognized as an important molecular trigger for the initiation of UVB carcinogenesis (12–14). Reduction of CPDs through application of DNA repair enzymes considerably reduces the risk of UV-induced skin cancer in mice and in humans (14, 15).

Interleukin-12 (IL-12) is an immunoregulatory cytokine and composed of two disulfide bonded protein chains p35 and p40 (16). Colombo et al. have shown that the presence of IL-12 at the tumor site is critical for tumor regression (17). IL-12 has been shown to both augment immune responses and exhibit antitumor activity (17–19). Based on the immunoregulatory properties of IL-12, we previously investigated its role in the prevention of UVB-induced immunosuppression by EGCG and established that the ability of EGCG to induce the production of IL-12 contributed to its ability to prevent UVB-induced immunosuppression (7). In addition to its immunomodulatory effects, IL-12 also exhibits the capacity to remove or repair UV-induced DNA damage (20) with the repair of UV-induced CPDs by IL-12 being mediated through the induction of nucleotide excision repair (NER) enzymes (21). The highly conserved NER system that mediates DNA repair can accomplish CPD repair and prevent apoptosis. Depending on the severity of the DNA damage following UVB exposure of the skin, keratinocytes in the skin can undergo either DNA repair or apoptosis (22–24). If the DNA damage is irreparable, the keratinocyte cell cycle is arrested, and the keratinocyte is transformed into a sunburn cell, an initial morphologic indicator of epidermal cell apoptosis. Alternatively, the cell cycle is suspended in the keratinocyte, and DNA repair is initiated (24). We, therefore, investigated the possibility that the ability of EGCG to induce IL-12 results in stimulation of DNA repair, and that this contributes, at least in part, to the ability of EGCG to prevent UVB-induced skin cancer. We hypothesized that, if this is the case, treatment with EGCG would be unable to prevent UVB-induced skin carcinogenesis in IL-12–deficient mice or UVB-induced DNA damage in IL-12 knockout (KO) mice and NER-deficient cells.

Materials and Methods
Animals. C3H/HeN mice (6-7 weeks old) were purchased from Charles River Laboratory (Wilmington, MA). The IL-12 KO mice were generated and
bred in our Animal Resource Facility at the University of Alabama at Birmingham, as described previously (25). Briefly, male IL-12−/− knockout mice on a C57BL/6j background and female C3H/HeN mice (6–7 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Male IL-12−/− and female C3H/HeN mice were mated to obtain male and female IL-12−/− mice. We then mated male IL-12−/− mice with IL-12−/− female or IL-12−/− male mice with IL-12−/− females and genotyped the progeny using a new rapid method of backcrossing for the generation of IL-12 KO mice onto a C3H/HeN mouse background (26). The IL-12−/− mice were constructed to carry a germ line disruption or mutation of the gene.

Photocarcinogenesis experiment. One week after the last UVB exposure of the mice, the UVB-irradiated back of the mice were examined on a weekly basis to check for the growth of papillomas or tumors. Growths larger than 5 mm in diameter were recorded at the termination of the experiment. Tumor volumes were calculated using the hemispherical model formula: tumor volume = 0.5 (4π/3) × (l/2) × (w/2) × h, where l = length, w = width and h = height, as described earlier (5). The mice were sacrificed and the supernatants were used to determine the IL-12 contents using ELISA kits (BioSource International, Camarillo, CA) following the manufacturer’s protocol.

Experimental design and photocarcinogenesis protocol. C3H/HeN mice were acclimatized for at least 1 week after their arrival in the Animal Resource Facility. The IL-12 KO mice on C3H/HeN background and their wild-type (WT) C3H/HeN counterparts were divided into three treatment groups with 10 mice in each group. These groups of mice included a control group, a group that was exposed to UVB in the absence of treatment with EGCG, and a group treated with EGCG before UVB irradiation. EGCG was applied topically (1.0 mg/cm² skin area) on the clipper-shaved backs of the mice in a hydrophilic cream-based formulation 25 to 30 minutes before each UVB exposure during the entire photocarcinogenesis experiment. The photocarcinogenesis protocol employed has been described previously (4, 5). Briefly, the shaved backs of the mice were irradiated every day with UVB (180 mJ/cm²) for a total of 10 days to stimulate tumor initiation. One week after the last UVB exposure of the tumor initiation stage, the mice were again irradiated with the same dose of UVB thrice a week for a total period of 35 weeks. The backs of the mice were shaved using clippers if hairs grown on the skin during the photocarcinogenesis experiment.

UVB irradiation of mice. Mice were UVB-irradiated as described previously (4, 5). Briefly, the clipper-shaved dorsal skin was exposed to UV radiation from a band of four FS20 UVB lamps (Dauvlin, UVA/UVB Research Irradiation Unit, Bryan, OH) equipped with an electronic controller to regulate UV dosage. The UV lamps emit UVB (315–340 nm), ~80% of total energy and UVA (320–375 nm, ~20% of total energy) with UVB emission being significantly less (~1%). The majority of the resulting wavelengths of UV radiation are in the UVB (290–320 nm) range with peak emission at 314 nm (4, 5). The UVB emission was monitored regularly with an IL-1700 photocarcinotherapy radiometer (International Light, Newburyport, MA).

Evaluation of tumor growth. During the photocarcinogenesis experiment, the UVB-irradiated back of the mice were examined on a weekly basis to check for the growth of papillomas or tumors. Growths larger than 1 mm in diameter that persisted for at least 2 weeks were defined as tumors and recorded. Tumor data on each mouse were recorded until the yield and size of the tumors had stabilized. The dimensions of all the tumors on each mouse were recorded at the termination of the experiment. Tumor volumes were calculated using the hemispherical model formula: tumor volume = 0.5 (4π/3) × (l/2) × (w/2) × h, where l = length, w = width and h = height, as described earlier (5). The mice were sacrificed, and the supernatants were used to determine the IL-12 contents using ELISA kits (BioSource International, Camarillo, CA) following the manufacturer’s protocol.

Immunohistochemical detection of IL-12 in tumors and quantification by ELISA. Intracellular staining of IL-12 in tumors was done as described previously (7). Briefly, 5-μm-thick frozen sections were fixed in ice-cold paraformaldehyde (4%, w/v), and endogenous peroxidase was blocked with HBSS buffer containing 0.1% saponin, 0.2 mol/L sodium azide, and 0.5% H₂O₂. Sections were incubated with rat monoclonal anti-mouse IL-12 antibodies diluted in HBSS + 0.1% saponin. After washing, sections were incubated for 30 minutes in biotinylated rabbit anti-rat IgG (Vector, Burlingame, CA) in HBSS + 0.1% saponin and subsequently with Vectastain Elite avidin-biotin complex method (horseradish peroxidase). After washing in HBSS alone, sections were incubated with dianisobenzene substrate solution and counterstained with methyl green.
UVB radiation (15 mJ/cm²) through PBS with or without pretreatment with EGCG. After UVB irradiation, the cells were then reincubated with EGCG for 24 hours.

**Cytostaining of CPDs in cultured cells.** The UVB-induced CPD⁺ cells were detected using a protocol as described previously with some modification (27). Briefly, at the indicated time period after UVB irradiation (15 mJ/cm²), cells were harvested and cytopun (~1 × 10⁵ per slide). After washing with PBS, the cells were fixed with 45% ethanol for 5 minutes followed by 70% ethanol at −20°C for 10 minutes and permeabilized with 0.3% Triton X-100 for 30 minutes. The cells were then treated with 0.5 N HCl and 0.05% pepsin at 37°C for 30 minutes to denature the DNA before incubation with the CPD-specific antibody for 1 hour at room temperature. After washing, the cells were incubated with biotinylated goat anti-mouse IgG1 followed by peroxidase-labeled streptavidin and then incubated with diaminobenzidine plus peroxidase substrate and thereafter counterstained with methyl green. CPD⁺ cells were counted in five to six different fields of each section, and the data are presented as the mean ± SD of the percentage of CPD⁺ cells from three independent experiments.

**Statistical analysis.** Statistical analysis of tumor data was done at the termination of the experiment. Tumor incidence in the UVB and EGCG + UVB groups was compared using the χ² test. Tumor multiplicity data were analyzed using the Wilcoxon rank sum test, and tumor volume was statistically analyzed using ANOVA followed by Tukey’s test. A simple ANOVA followed by an appropriate post hoc test was used to calculate statistical significance of the data obtained from tumor volume and the dot blot analysis. To compare the numbers of CPD⁺ cells in different treatment groups, at least five to six different fields from each section were selected, and the numbers of CPD⁺ cells were counted. P < 0.05 was considered significant.

**Results**

EGCG treatment prevents photocarcinogenesis in WT but not IL-12 KO mice. Previously, we have shown that topical application of EGCG inhibits UVB-induced carcinogenesis in the skin of mice in terms of tumor incidence, tumor multiplicity, and tumor growth (5). As UVB-induced immunosuppression has been implicated as a risk factor for photocarcinogenesis, we investigated the effect of topical treatment with EGCG on UVB-induced suppression of contact hypersensitivity to dinitrofluorobenzene and found that EGCG inhibits this reaction. UVB-induced suppression of contact hypersensitivity has been associated with an increase in the immunosuppressive cytokine IL-10 and suppression of the immunostimulatory cytokine IL-12 (7). EGCG reverses this UVB-induced alteration in the balance of IL-10 and IL-12 (7). To test the hypothesis that the mechanism by which EGCG prevents photocarcinogenesis is associated with the ability of EGCG to induce IL-12, we examined the effect of EGCG on photocarcinogenesis in IL-12 KO mice. We confirmed that when WT mice were exposed to a complete photocarcinogenesis protocol (UVB-induced initiation and promotion), topical application of EGCG resulted in a significant reduction in UVB-induced
skin tumorigenesis in terms of tumor incidence and tumor multiplicity compared with non–EGCG-treated WT mice (Fig. 1, left). Treatment with EGCG resulted in a 40% (P < 0.01) lower tumor incidence (% of mice with tumors) at the termination of the experiment at 35 weeks compared with non–EGCG-treated mice. Only 35% of the EGCG-treated mice developed tumors compared with 75% of the non–EGCG-treated mice. Treatment with EGCG also increased the latency period of the tumors by 3 weeks under the experimental conditions used in these studies. A total of 42 tumors (2.75 ± 0.6 tumors per tumor-bearing animal) were recorded in the group of mice that were irradiated with UVB but did not receive EGCG (non–EGCG), whereas only 16 tumors (2 ± 0.5 tumors per tumor-bearing mouse) were recorded in the group of irradiated mice treated with EGCG (Fig. 1, left). Each group had 20 mice. Topical treatment with EGCG resulted in a significantly lower UVB-induced tumor multiplicity (62%, P < 0.001) compared with that observed in the control group of mice that were UVB-irradiated but not treated with EGCG. In terms of the number of tumors per tumor-bearing mouse, although the tumor multiplicity was lower (27%) in the EGCG-treated mice, the difference between the groups was not statistically significant (Fig. 1). In addition, both the rate of appearance of the UVB-induced tumors and their development in the EGCG-treated WT animals were significantly lower (P < 0.01, Fisher-Irwin exact test). As illustrated in Fig. 2, the growth of tumors was significantly lower (81%, P < 0.001) in the group of EGCG-treated WT mice than the non-EGCG treated mice when measured and expressed in terms of tumor volume per tumor at the termination of the experiment. Tumor formation was not observed in the skin of mice that were treated with either hydrophilic cream alone or EGCG in hydrophilic cream for 35 weeks but not UV irradiated (data not shown).

Treatment with EGCG did not protect against photocarcinogenesis in IL-12 KO mice (Fig. 1, right). The IL-12 KO mice seemed to be at greater risk of UVB radiation-induced skin tumor development than their WT counterparts, as is evident from the data concerning tumor incidence and tumor multiplicity (Fig. 1, right). In the group of irradiated IL-12 KO mice, 90% developed tumors, whereas only 75% of their WT counterparts developed tumors. A total of 72 tumors were recorded in the group of IL-12 KO mice compared with only 42 tumors in the group of WT mice (Fig. 1). Similarly, both the number of tumors per tumor-bearing mouse and the size of the tumor per tumor were higher in the IL-12 KO mice than their WT counterparts. Histopathologic analysis of the UVB-induced tumors from both WT and IL-12 KO mice was done using routinely used procedure of H&E staining by pathologists in a blinded manner, which revealed that majority of the tumors were squamous cell papillomas and of epidermal origin (30). Taken together, these tumor data indicate that mice deficient in IL-12 are at greater risk of UVB-induced carcinogenesis and further suggest that the chemopreventive effect of EGCG against photocarcinogenesis is mediated through the induction of IL-12.

**EGCG treatment induces IL-12 in UVB-induced tumors of WT mice.** We next determined whether EGCG prevents photocarcinogenesis through the induction of IL-12 in skin tumors. As shown in Fig. 3A, immunohistochemical examination revealed that the number of IL-12-producing cells (IL-12+ cells shown by arrows) was greater in the tumors of the UVB-exposed mice that were treated with EGCG than in the tumors in the non–EGCG-treated mice. IL-12+ cells were not detectable in the skin of mice that were not UVB irradiated (data not shown). Analysis of epidermal skin homogenates (control mice) and tumor homogenates using ELISA revealed detectable levels of IL-12 (Fig. 3B). The levels of IL-12 in UVB-induced skin tumors were relatively higher than the non–UVB-exposed epidermal skin samples, indicating the induction of IL-12 by UVB radiation. The levels of IL-12 in the tumors of the mice treated with EGCG were 2-fold higher (P < 0.001) than those of the mice treated with UVB alone. EGCG treatment enhances the levels of IL-12 in UVB-induced skin tumors of WT mice. A, EGCG treatment increases the numbers of IL-12–producing cells in tumors. Intracellular staining was done to detect IL-12–positive cells, as described in Materials and Methods. Arrows indicate presence of IL-12–producing cells. Magnification, ×20. B, levels of IL-12 were determined in epidermal skin homogenates and tumor homogenates using ELISA kit. Levels of IL-12 in each treatment group are expressed in terms of ng/mg protein. Columns, mean (n = 5); bars, SD. *, P < 0.001, significant induction versus UVB alone.

**Figure 2.** EGCG treatment inhibits the growth of UVB-induced tumors greatly in WT mice than in IL-12 KO mice. The tumor volume in each treatment group was recorded at the termination of the experiment. *, P < 0.001; ¶, P < 0.05. Significant decreases in tumor size versus UV alone.
compared with the tumors obtained from non-EGCG-treated mice. The presence of enhanced level of IL-12 in the skin tumors of EGCG-treated mice may be critical in the inhibition or regression of UVB-induced tumor growth. As expected, IL-12 was not detectable in the skin or the tumors of the UVB-irradiated IL-12 KO mice.

**EGCG removes or repairs UVB-induced CPD* cells more rapidly in WT mice than in IL-12 KO mice.** We next determined whether induction of IL-12 by EGCG contributes to the ability of EGCG to prevent photocarcinogenesis in mice by inducing DNA repair. To do this, the shaved backs of IL-12 KO and WT mice were exposed to UVB (60 mJ/cm²) with and without pretreatment of EGCG and the time kinetics of the presence of CPD* cells after UVB irradiation was determined by immunohistochemical analysis of skin samples. Mice were sacrificed either immediately (within 30 minutes), 1, 2, 5, or 7 days after UVB exposure. UVB-induced CPDs were detectable immediately after UVB exposure. In skin samples obtained immediately after UVB exposure, no differences in the staining pattern of CPDs were observed between the samples obtained from IL-12 KO and WT mice whether or not the mice had been treated with EGCG (Fig. 4A and B). Thus, topical application of EGCG does not prevent UVB-induced CPDs immediately after UVB irradiation, and this indicates that EGCG does not have a significant filtering effect on the UVB radiation. In skin samples obtained at 24 and 48 hours after UVB exposure, the numbers of CPD* cells were significantly declined time dependently in the EGCG-treated WT mice than the control group that were not treated with EGCG but were exposed to UVB (P < 0.001). Further

**Figure 4.** EGCG removes or repairs UVB-induced CPDs more rapidly in WT mice than in IL-12 KO mice. A. WT or IL-12 KO mice were treated topically with EGCG then exposed to UV (60 mJ/cm²) 30 minutes later as described in the Materials and Methods. Mice were sacrificed at 30 minutes (immediate), 24, or 48 hours after UVB exposure, and skin samples were collected and frozen in optimum cutting temperature medium. Frozen sections (5 μm thick) were subject to immunoperoxidase staining to detect CPD* cells that are dark brown. CPD* cells were not detected in non–UVB-exposed skin whether treated or not treated with EGCG. B. numbers of CPD* cells were counted in five to six different areas of the sections, and the numbers reported represent the percentage of CPD* cells in epidermis. Columns, mean (n = 5); bars, SD. *, P < 0.001, significant reduction versus non–EGCG-treated UVB-exposed mice. C. EGCG removes or repairs UVB-induced CPDs rapidly in WT mice than in IL-12 KO mice. CPDs were estimated using Southwestern dot blot analysis. The treatment protocol was similar to (A). Mice were sacrificed 0.5 or 24 hours after UVB irradiation with or without EGCG treatment (1 mg/cm²), and skin samples were collected. Genomic DNA was extracted from the epidermal skin samples and subjected to Southwestern dot blot analysis using an antibody against CPD. The mice that were not exposed to UVB did not show the presence of CPDs in the dot blot analysis. Experiments were conducted and repeated separately in five animals in each group with identical results.
kinetics of removal of UVB-induced CPDs indicated that the epidermis of WT mice was clear from CPDs after 5 days of UVB irradiation, whereas the epidermis of EGCG + UVB-treated mice was completely cleared from CPD+ cells in <72 hours of UVB irradiation (data not shown). In contrast, the numbers of UVB-induced CPD+ cells in the IL-12 KO mice that had been treated with EGCG did not significantly reduce from that in the IL-12 KO mice that had not been treated with EGCG (Fig. 4A and B). Furthermore, the rate of repair or removal of UVB-induced CPDs in IL-12 KO mice whether treated or not treated with EGCG was slower compared with WT mice. The immunostaining of IL-12–positive cells in IL-12 KO mice skin samples indicated that it took about 7 days to clear the epidermis from UVB-induced CPD+ cells. The CPD+ cells were not detectable in those skin samples, which were obtained from the groups of mice that were not exposed to UVB, whether or not they were treated with EGCG. Interestingly, we found some reduction in the number of CPD+ cells in the skin samples of WT and IL-12 KO mice that were not treated with EGCG at 24 and 48 hours after UVB exposure. This observation suggests that an endogenous defense mechanism may play a role in the repair of UVB-induced CPDs; however, it seems to be much less efficacious than the EGCG-mediated mechanism. Although this spontaneous DNA repair or removal of CPD+ cells occurred in IL-12 KO mice, it was more significant in the WT mice (Fig. 4A and B), suggesting that both IL-12-dependent and IL-12-independent endogenous mechanisms may contribute to the repair of UVB-induced DNA damage, and that the difference in CPDs repair or removal between WT and IL-12 KO mice may be due to the presence of IL-12 in WT mice and/or the absence of IL-12 in IL-12 KO mice.

The effect of EGCG on UVB-induced DNA damage was further tested using Southwestern dot blot analysis of genomic DNA isolated from the skin samples from UVB-exposed skin at 30 minutes and 24 hours after UVB irradiation. Genomic DNA obtained from skin samples from non–UVB-exposed mice served as a control. As shown in Fig. 4C, the results confirmed those obtained using immunohistochemical analysis. The genomic DNA samples obtained from the groups of mice that were not exposed to UV (normal skin), including those that were or were not treated with EGCG were negative in the dot blot assay. No significant differences in the dot blot patterns of CPDs were observed between skin samples obtained immediately after UVB exposure from IL-12 KO and WT mice whether or not they were treated with EGCG. In samples obtained 24 hours after UVB exposure, the intensity of the dot blot was significantly lower in the EGCG-treated WT mice than the control WT mice that were not treated with EGCG. In contrast, there was no significant difference in the dot blot pattern of IL-12 KO mice whether or not they had been treated with EGCG (Fig. 4C).

In vivo treatment of EGCG-treated IL-12 KO mice with recombinant IL-12 removes or repairs UVB-induced CPD+ cells. To further confirm the role of IL-12 in the EGCG-mediated repair of UVB-induced DNA damage, the shaved backs of EGCG-treated IL-12 KO mice were exposed to UVB (60 mJ/cm²) with one group of mice receiving endotoxin-free recombinant IL-12 before 3 hours before UVB exposure. Mice were sacrificed 24 hours later, skin samples were obtained, and the presence of CPDs assessed using immunohistochemical analysis. In the UVB-irradiated IL-12 KO mice, the numbers of CPD+ cells in the EGCG-treated group (61 ± 7%) were similar to the numbers observed in the group that did not receive EGCG (70 ± 8%), as shown in Fig. 5A and B. In contrast, the number of CPD+ cells in the skin samples of IL-12 KO mice that had been treated with EGCG and had received IL-12 before UVB exposure was only 20 ± 4%, indicating a significant reduction of DNA damage 24 hours after UVB exposure (P < 0.001). These data further support that EGCG promotes the removal or repair of damaged DNA in UVB-exposed skin through a mechanism that requires IL-12 activity.

EGCG removes UVB-induced sunburn cells more rapidly in WT mice than in IL-12 KO mice. The formation of sunburn cells after UVB irradiation is primarily a consequence of DNA damage. Sunburn cells are keratinocytes undergoing apoptosis after they have received a physiologic UV dose that has severely and irreversibly damaged their DNA or other chromophores. Using IL-12 KO and their WT counterparts as a tool, we determined whether treatment with EGCG inhibits UVB-induced sunburn cell formation through an IL-12–dependent mechanism. IL-12 KO and their WT mice were exposed to 60 mJ/cm² of UVB radiation, and mice were sacrificed at different time points, and skin samples were
analyzed 24 hours after UVB irradiation, the numbers of CPD+ cells were significantly reduced (65%, P < 0.001) in the XP-A-proficient cells (Fig. 7A and B, left columns), suggesting that EGCG might accelerate the repair of UVB-induced CPDs through an NER mechanism. Because NER is the major mechanism by which CPDs are removed in mammalian cells, the effect of EGCG on UVB-induced CPDs as a marker of DNA damage in NER-deficient cells also was determined. The XP-A gene is an essential component of the NER; thus, cells with a mutated XP-A gene completely lack a functional NER. As shown in Fig. 7A and B (right columns), treatment with EGCG did not significantly remove or repair the UVB-induced CPDs in XP-A-deficient cells. This observation indicates that EGCG-induced DNA repair in the form of CPDs is mediated through a functional NER mechanism.

We also determined the effect of EGCG on UVB-induced CPDs in XP-A-proficient and XP-A-deficient cells using Southwestern dot blot analysis. As indicated in Fig. 7C, treatment of XP-A-proficient cells with EGCG (0, 10, 20 and 30 μg/mL) resulted in a dose-dependent

Figure 6. EGCG reduces UVB-induced increases in apoptotic sunburn cells in the epidermis of WT mice more rapidly than in IL-12 KO mice. The WT or IL-12 KO mice were treated with vehicle or EGCG and exposed to UVB (60 mJ/cm²) 30 minutes later on the shaved back. The animals were sacrificed at the indicated times, and skin samples were collected. Morphologically distinct apoptotic sunburn cells were counted in H&E-stained sections. Points, mean percentage of sunburn cells from five independent experiments (n = 5); bars, SD. Statistically significant difference at individual times after UVB exposure were observed for the percentage of apoptotic sunburn cells between EGCG-treated and non–EGCG-treated WT and IL-12 KO mice. *, P < 0.01, significant reduction versus UVB alone.

Figure 7. EGCG reduces UVB-induced DNA damage in XP-A-proficient cells but does not reduce UVB-induced DNA damage in XP-A-deficient cells. A, XP-A-proficient cells and XP-A-deficient cells were exposed to UVB (15 mJ/cm²) with or without EGCG treatment (20 μg/mL), and cells were harvested 24 hours later, cytoplasm, and subject to immunocyto staining to detect CPD⁺ cells. CPD-positive cells are dark brown. Magnification, >40. B, numbers of CPD⁺ cells in XP-A-proficient and XP-A-deficient experimental cell sets were counted in five to six areas of the slide. Columns, mean percentage of CPD⁺ cells from three independent experiments; bars, SD. *, P < 0.001, significant decrease versus non-EGCG (UVB alone) treatment in XP-A-proficient cells. C, dose-dependent effect of EGCG on UVB-induced DNA damage in the form of CPDs in XP-A-proficient and XP-A-deficient cells. XP-A-proficient and XP-A-deficient cells were exposed to UVB (15 mJ/cm²) with or without EGCG treatment (0, 10, 20, or 30 μg/mL), and cells were harvested 24 hours later. Genomic DNA was extracted from the cells and subjected to Southwestern dot blot analysis using an antibody against CPDs. The cells that were not exposed to UVB did not show the presence of CPDs in the dot blot analysis. Experiments were repeated thrice with identical results.
repair or reduction of UVB-induced CPDs after 24 hours, whereas this chemopreventive effect of EGCG was not evident in the XPA-deficient cells 24 hours after UVB irradiation. In the absence of UVB irradiation, the cells whether XPA-deficient or XPA-proficient and whether treated or not with EGCG did not show the presence of CPDs in the dot blot assay. Thus, the data from dot blot analysis further confirm our observation that EGCG-induced DNA repair in UVB-exposed cells is mediated through functional NER mechanism.

Discussion

In the present study, we show a new mechanism by which EGCG prevents photocarcinogenesis (i.e., the promotion of IL-12–dependent DNA repair). The significant chemopreventive effect of EGCG on photocarcinogenesis was not observed in IL-12 KO mice but observed in their wild-type mice, suggesting that the prevention of photocarcinogenesis by EGCG is mediated primarily through IL-12. The treatment of IL-12 KO mice with EGCG did result in inhibition, although not statistically significant, of UVB-induced tumor development and enhancement of repair of UVB-induced DNA damage. The slight or nonsignificant chemopreventive effect of EGCG against UVB-induced skin carcinogenesis in IL-12 KO mice may be due to some other pathways that are not dependent on IL-12 activity. As IL-12-induced antitumor activity has been shown against established cutaneous deposits and experimental metastases (17, 18, 31, 32), it is possible that the induction of this cytokine by EGCG may be involved in the anticarcinogenic activity of EGCG. IL-12 is a key component of numerous immune functions, including stimulation of T cells, and has been found to cure or improve the survival of tumor-bearing mice by enhancing in vivo antitumor immune responses and antitumor activity in a number of different tumor models (17, 18, 31, 32). The presence of IL-12 at the tumor site is critical for tumor regression (17). As we observed that treatment of WT mice with EGCG increased the levels of IL-12 in tumors in our system, it is possible that this enhancement of the levels of IL-12 is involved in the inhibition of the growth of tumors. In contrast, this observation was not found in IL-12 KO mice whether treated or not treated with EGCG. Thus, the comparison of available information from other tumor models with our present study conducted in IL-12 KO mouse model indicates that IL-12 has an inhibitory effect on UV-induced skin tumor development, and its deficiency renders the mice susceptible to UV carcinogenesis.

It has been recognized that UV-induced DNA damage in the form of CPDs initiates photocarcinogenesis and is involved in UV-induced immunosuppression, which has been implicated in UV carcinogenesis (8, 9). IL-12 has the ability to repair UVB-induced CPDs (19, 20, 33). Therefore, we examined the effect of EGCG on UVB-induced DNA damage in the form of CPDs, employing IL-12 KO mouse model as a tool. Our observation that treatment of EGCG removes or repairs UVB-induced CPDs rapidly in WT mice than in IL-12 KO mice indicates that prevention of photocarcinogenesis by EGCG may be associated with its ability to stimulate DNA repair through induction of IL-12. Further the s.c. injection of IL-12 at the site of UVB irradiation enhanced the repair of UVB-induced CPDs in EGCG-treated IL-12 KO mice. These data support the concept that EGCG stimulates the removal or repair of CPDs from the UVB-exposed skin through a mechanism that involves IL-12 activity. Using a similar approach, we also have shown that EGCG prevents UVB radiation–induced immunosuppression through IL-12–dependent DNA repair (25).

Induction of apoptosis is an additional protective mechanism that eliminates cells that are unable to repair UVB-induced DNA damage completely (34). Thus, dysregulation of UV-induced apoptosis enhances the risk of photocarcinogenesis. UVB-induced apoptosis can be disrupted by several events, including overexpression of heat shock proteins (35), induction of antiapoptotic proteins (36), and disruption of p53 function (23), among others. As these events allow the survival of cells carrying DNA damage, they may give rise to mutations and skin cancer. Here, we provide evidence that treatment with EGCG inhibits sunburn cell formation or repair of sunburn cells in WT mice but not in IL-12 KO mice (Fig. 6). As the formation of sunburn cells is primarily a consequence of DNA damage, and treatment with IL-12 of human keratinocytes in vitro and C3H/HeN mice or rat has been shown to reduce the numbers of sunburn cells (20), the inhibition or removal of UVB-induced sunburn cells by EGCG in WT mouse skin may be due to the induction of IL-12. The fact that there was no difference in the amount of DNA damage observed immediately after UVB irradiation (Figs. 4 and 5), but a difference was observed at a later time points, can only be explained by enhanced removal or repair of damaged DNA lesions. The involvement of NER in DNA repair was confirmed by using cultured XPA-proficient and XPA-deficient cells. EGCG was able to repair UVB-induced CPDs in XPA-proficient cells but not in XPA-deficient cells (Fig. 7), thus confirming that the DNA repair mechanism promoted by EGCG is mediated through NER mechanism. Although the detailed mechanism by which EGCG and especially IL-12 induce NER gene expression remains to be determined, induction of transcription seems to be essential. Other studies have reported that UVB-mediated DNA damage can be reduced by the application of exogenous DNA repair enzymes. The bacterial DNA repair enzyme T4 endonuclease V (T4N5) can be delivered into cells by liposomes (37). Topically applied T4N5 liposomes penetrate the skin, increase the removal of CPDs, and reduce the incidence of skin cancer (14, 15). In accordance with the accelerated removal of DNA lesions by T4N5, sunburn cell formation also was found to be reduced (38). In contrast to the external application of DNA repair enzymes, the present study indicates that EGCG affects the cell’s own NER system by inducing IL-12.

NER is the main mechanism of repair of UVB-induced DNA damage in mammalian cells. It is important to identify natural products or botanicals that can promote NER activity. We have determined that EGCG, an important constituent of green tea, has the ability to enhance the repair of UVB-induced DNA damage through induction of immunoregulatory cytokine IL-12 and ultimately to reduce the risk of photocarcinogenesis. Although it is possible that not all cells with UVB-induced DNA damage may be repaired or eliminated using this strategy, the reduction in UVB-induced DNA damage should be beneficial. Based on our data in the present study, it is tempting to suggest that supplementation of sunscreens or skin care lotions with EGCG might have potential as a chemopreventive agent for the prevention of melanoma and nonmelanoma skin cancers in humans.

Acknowledgments

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References

Retraction: (-)-Epigallocatechin-3-Gallate Prevents Photocarcinogenesis in Mice through Interleukin-12-Dependent DNA Repair

Syed M. Meeran, Sudheer K. Mantena, Craig A. Elmets, and Santosh K. Katiyar

This article (1) has been retracted at the request of the editors. Following a joint institutional investigation by the Birmingham VA Medical Center and the University of Alabama at Birmingham, the primary affiliations for several of the authors, it was determined that the same histologic image was used to represent results from different animals and treatment conditions in Fig. 4A. The original research records related to the figures were not available during the review. Therefore, the institutions were not able to determine which of the published images were correct and recommended retraction; upon internal review, the editors agreed with this recommendation.

A copy of this Retraction Notice was sent to the last known e-mail addresses for all four authors. Two authors (S.M. Meeran and S.K. Katiyar) did not agree to the retraction; the two remaining authors (S.K. Mantena and C.A. Elmets) did not respond.

Reference

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