(−)-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/cm^2 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

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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 genetic 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$^{+/-}$ males 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 mice background (26). The IL-12$^{-/-}$ mice were constructed to carry a germ line disruption or mutation of the gene. This mutation removes p35 chain of IL-12 protein molecule and therefore completely eliminates the synthesis of IL-12 protein.

All the mice were housed in the Animal Resource Facility of the University of Alabama at Birmingham and were maintained under the following conditions: 12-hour dark/12-hour light cycle, 24 ± 2°C temperature, and 50 ± 10% relative humidity. The mice were given control Purina chow diet and drinking water *ad libitum* throughout the experiment. A group of mice also was maintained that were given a control diet and water *ad libitum* but were not treated with EGCG or UVB irradiated. The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

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 20 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/cm^2 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 protocol. 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/cm^2) 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 (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH) equipped with an electronic controller to regulate UV dosage. The UV lamps emit UVB (280-320 nm, ≈80% of total energy) and UVA (320-375 nm, ≈20% of total energy), with UVC 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 phototherapy 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 hemiellipsoid model formula: tumor volume = 0.5 $(4\pi/3) \times (l/2) \times (w/2) \times h$, where l = length, w = width and h = height, as described earlier (5). The mice were sacrificed, and the tumors were then harvested and either frozen in liquid nitrogen or embedded and frozen in optimum cutting temperature medium for further examination.

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% H_2O_2. 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 diaminobenzidine substrate solution and counterstained with methyl green.

Tumor or epidermal (from control mice) tissues were homogenized in ice-cold lysis buffer as described previously (4), the homogenates were centrifuged, 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 CPDs. Immunohistochemical analysis was done to detect CPD$^+$ cells in the skin samples using a procedure described previously (27). Briefly, 5-μm-thick frozen skin sections were thawed and kept in 70 mmol/L NaOH in 70% ethanol for 2 minutes to denature nuclear DNA followed by neutralization for 1 minute in 100 mmol/L Tris-HCl (pH 7.5) in 70% ethanol. The sections were washed with PBS buffer and incubated with 10% goat serum in PBS to prevent nonspecific binding before incubation with a monoclonal antibody specific for CPDs (Kamiya Biomedical Co., Seattle, WA) or its isotype control (IgG1). Bound anti-CPD antibody was detected by incubation with biotinylated goat anti-mouse IgG1 followed by peroxidase-labeled streptavidin. After washing, sections were incubated with diaminobenzidine plus peroxidase substrate and counterstained with H&E.

Southwestern dot blot analysis. Genomic DNA from the epidermal skin samples or *XPA* cells was isolated following the standard procedures (28). Genomic DNA (500 ng) was transferred to a positively charged nitrocellulose membrane by vacuum dot blotting (Bio-Dot Apparatus, Bio-Rad, Hercules, CA) and fixed by baking the membrane for 30 minutes at 80C. After blocking the nonspecific binding sites in blocking buffer [5% nonfat dry milk, 1% Tween 20 in 20 mmol/L TBS (pH 7.6)], the membrane was incubated with the antibody specific to CPDs for 1 hour at room temperature. After washing, the membrane was incubated with horseradish peroxidase–conjugated secondary antibody. The circular bands of CPD dimers were detected by chemiluminescence using enhanced chemiluminescence detection system. The genomic DNA was used and tested from at least five mice in each group independently in each set of experiment.

***In vivo* treatment with IL-12.** To verify the role of EGCG-induced IL-12 on EGCG-mediated removal or repair of CPDs in UVB-exposed skin, recombinant murine IL-12 (100 ng/100 μL PBS; eBioscience, San Diego, CA) or an equal volume of PBS was injected s.c. 3 hours before UVB irradiation. The mice were sacrificed 24 hours later, and skin samples were collected for immunohistochemical detection of CPDs.

Detection of apoptotic sunburn cells. IL-12 KO as well as WT mice were exposed to UVB (60 mJ/cm^2) with or without pretreatment with EGCG. The mice were sacrificed at the indicated time points, and skin samples (at least 1 cm in length) were obtained, fixed in formaldehyde, and embedded in paraffin. Tissue sections (5 μm) were obtained and stained with H&E. Sunburn cells were identified and counted throughout the epidermis in each section using light microscopy. The identification of apoptotic sunburn cells was based on morphologic characteristics, including cell membrane shrinkage and nuclear condensation attributable to fragmentation of the cells (23, 29). The percentages of sunburn cells in the specimens of epidermis were calculated as the number of the apoptotic sunburn cell cells identified per 100 cells counted in the entire 1-cm length of the epidermis in each skin section.

Cells, culture conditions, and UVB irradiation. Xeroderma pigmentosum complementation group A (*XPA*)–deficient and *XPA*-proficient human fibroblasts were obtained from the Coriell Institute for Medical Research (Camden, NJ). The *XPA*-deficient cells had been collected from a patient suffering from xeroderma pigmentosum, whereas the *XPA*-proficient cells had been obtained from a healthy human individual. The cells were cultured in Modified Eagle Medium with Earle's salts supplemented with 2 mmol/L L-glutamine, 10% fetal bovine serum (Hyclone, Logan, UT) and maintained in a incubator at 37C in a humidified atmosphere of 5% CO_2. The UV source was the same as that employed for irradiation of the mice as described above. The cells were exposed to the

UVB radiation (15 mJ/cm^2) 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/cm^2), cells were harvested and cytospun ($\approx 1 \times 10^5$ 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 χ^2 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

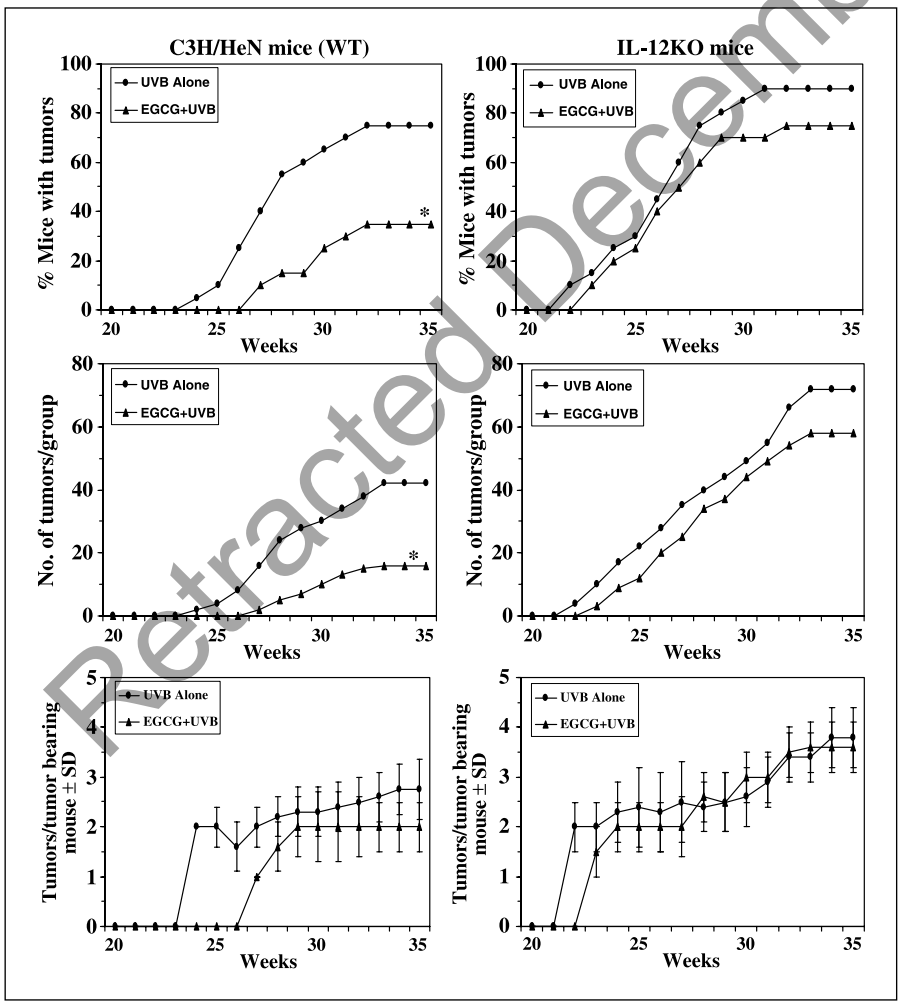

Figure 1. EGCG treatment significantly inhibits UVB-induced skin tumorigenesis in WT (C3H/HeN) mice but does not inhibit it in IL-12 KO mice. The details of experimental protocol are provided in Materials and Methods. The percentage of mice with tumors, total number of tumors per group, and number of tumors per tumor-bearing mouse are plotted as a function of the number of weeks on treatment. *Points,* mean; *bars,* SD. Tumor data in WT mice (*left*) and tumor data in IL-12 KO mice (*right*). Each treatment group had 20 mice. *, $P < 0.001$; significant reduction in tumors versus UVB alone at the termination of the experiment.

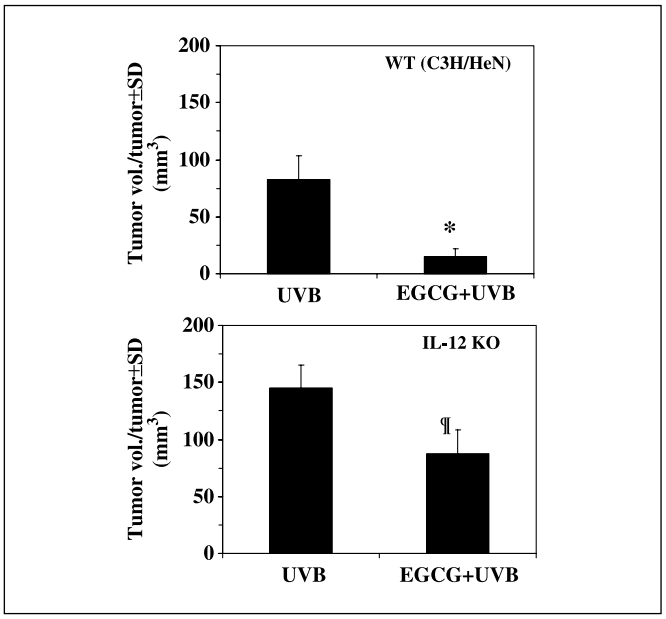

Figure 2. EGCG treatment inhibits the growth of UVB-induced tumors greatly in WT mice than in IL-12 KO mice. The tumor volume in each treatment group was recorded at the termination of the experiment. *, $P < 0.001$; ¶, $P < 0.05$, significant decreases in tumor size versus UV alone.

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. 3*A*, 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. 3*B*). 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 ($P < 0.001$)

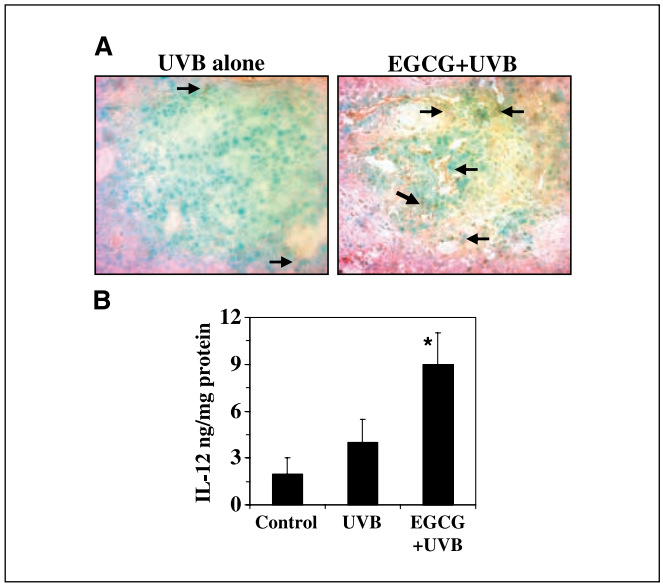

Figure 3. EGCG treatment enhances the levels of IL-12 in UVB-induced skin tumors of WT mice. *A,* EGCG treatment increases the numbers of IL-12–producing cells in tumors. Intracellular staining was done to detect IL-12–positive cells, as described in Materials and Methods. Arrows indicate presence of IL-12–producing cells. Magnification, ×20. *B,* levels of IL-12 were determined in epidermal skin homogenates and tumor homogenates using ELISA kit. Level of IL-12 in each treatment group is expressed in terms of ng/mg protein. *Columns,* mean ($n = 5$); *bars,* SD. *, $P < 0.001$, significant induction versus UVB 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/cm^2) 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. 4*A* 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

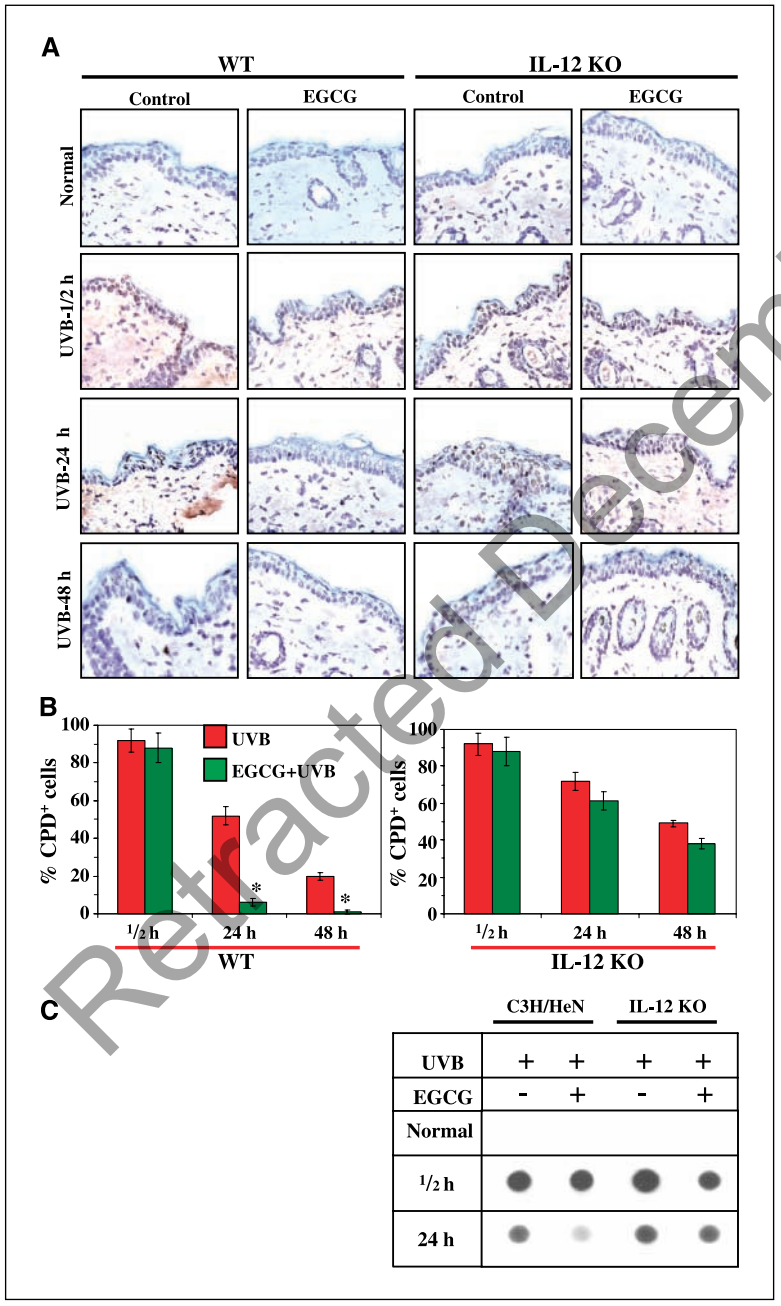

Figure 4. EGCG removes or repairs UVB-induced CPDs more rapidly in WT mice than in IL-12 KO mice. *A,* WT or IL-12 KO mice were treated topically with EGCG then exposed to UV (60 mJ/cm^2) 30 minutes later as described in the Materials and Methods. Mice were sacrificed at 30 minutes (immediate), 24, or 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/cm^2), 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 reduced from that in the IL-12 KO mice that had not been treated with EGCG (Fig. 4*A* 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. 4*A* 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. 4*C*, 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 pattern 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 blots of IL-12 KO mice whether or not they had been treated with EGCG (Fig. 4*C*).

***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/cm^2) with one group of mice receiving endotoxin-free recombinant IL-12 s.c. 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 ($61 \pm 7\%$) were similar to the numbers observed in the group that did not receive EGCG ($70 \pm 8\%$), as shown in Fig. 5*A* 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 $20 \pm 4\%$, 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.

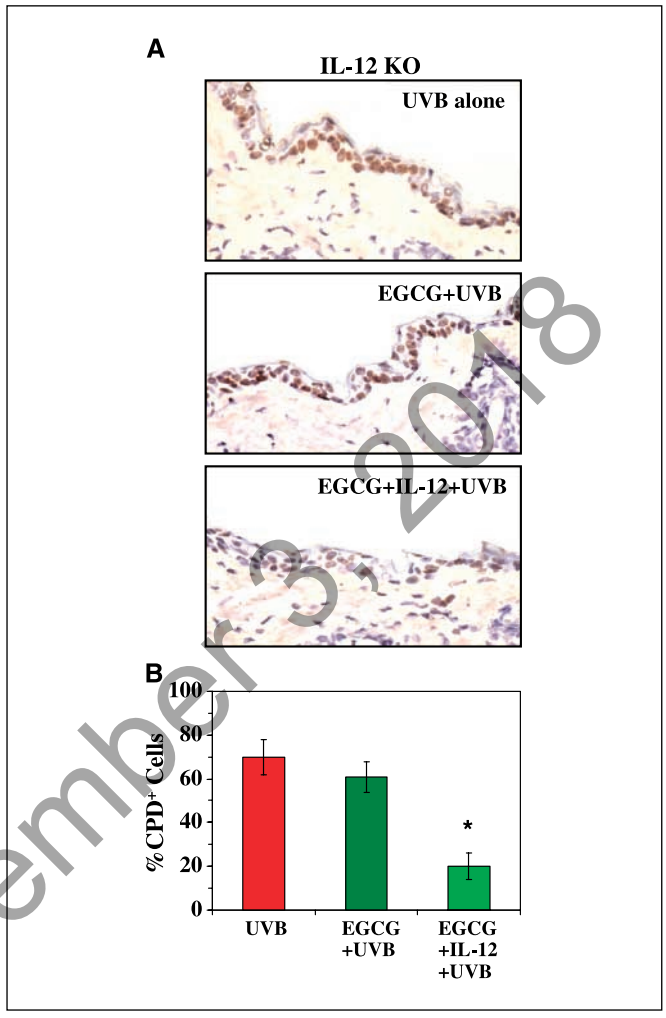

Figure 5. *In vivo* treatment with IL-12 of EGCG-treated IL-12 KO mice reduces UVB-induced CPDs. IL-12 KO mice with or without treatment of EGCG exposed to UVB (60 mJ/cm^2), as described in Fig. 4. In the indicated group, mice were injected with 1,000 ng IL-12 s.c. 3 hours before UVB irradiation. Mice were sacrificed 24 hours after UV irradiation, and skin samples were collected and frozen in optimum cutting temperature. *A,* frozen skin sections were subject to immunoperoxidase staining to detect CPDs. *B,* numbers of CPD^+ cells were counted in five to six different areas of the sections. *Columns,* mean percentage of CPD^+ cells in the epidermis ($n = 5$); *bars,* SD. Magnification, ×40. *, $P < 0.001$, significant difference versus UVB alone and/or EGCG + UVB groups.

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 UVB 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/cm^2 of UVB radiation, and mice were sacrificed at different time points, and skin samples were

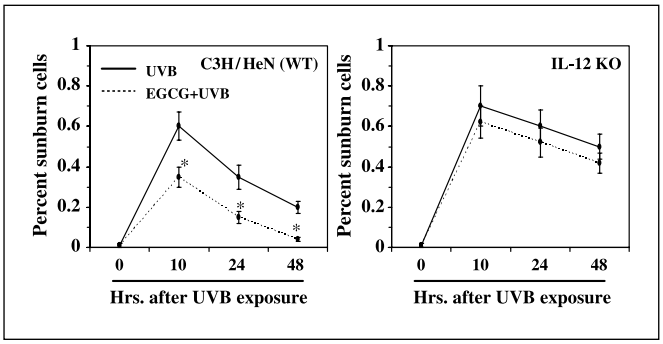

Figure 6. EGCG reduces UVB-induced increases in apoptotic sunburn cells in the epidermis of WT mice more rapidly than in IL-12 KO mice. The WT or IL-12 KO mice were treated with vehicle or EGCG and exposed to UVB (60 mJ/cm^2) 30 minutes later on the shaved back. The animals were sacrificed at the indicated 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.

collected and processed for routine H&E staining. As shown in Fig. 6, in WT mice, the number of sunburn cells were maximum (0.6% of total keratinocytes) 10 hours after UVB exposure and decreased thereafter. Treatment with EGCG resulted in a significant reduction in the numbers of sunburn cells at each time point after UVB irradiation. In IL-12 KO mice, the numbers of sunburn cells observed 10 hours after UV exposure were slightly more (0.7%) than those observed in WT mice; however, in these mice, the spontaneous reduction in sunburn cells was slow and significantly less than in WT mice. Also in contrast to WT mice, EGCG treatment of IL-12 KO mice did not result in the rapid removal of sunburn cells after UVB exposure (Fig. 6). This difference in the repair kinetics of sunburn cells in the IL-12 KO and WT mice may be attributed due to the EGCG induction of IL-12 in the WT mice.

EGCG induces repair of UVB-induced CPDs in *XPA*-proficient cells but does not reduce the numbers of UVB-induced CPDs in *XPA*-deficient cells. IL-12 has the ability to repair UVB-induced CPDs in healthy cells but not in cells from patients with xeroderma pigmentosum A (20). The *XPA* gene is an essential component of the NER; thus, cells with a mutated *XPA* gene completely lack a functional NER. To determine whether the NER activity is required for the EGCG-induced IL-12–mediated repair of UVB-induced CPDs, NER-deficient fibroblasts from an *XPA* patient and NER-proficient fibroblasts from a healthy person were exposed to UVB with or without prior treatment with EGCG (20 μg/mL). CPD$^+$ cells were not detectable in non–UVB-irradiated cells whether or not they were treated with EGCG (data not shown). When the cells were analyzed for CPDs immediately after UVB exposure, no differences were observed in the cells treated with or without EGCG in terms of the number of CPD$^+$ cells (data not shown). This finding implies that EGCG does not prevent immediate formation of CPDs after UVB exposure and excludes a UVB radiation filtering effect. However, when the cells were analyzed 24 hours after UVB irradiation, the numbers of CPD$^+$ cells were significantly reduced (65%, $P < 0.001$) in the *XPA*-proficient cells (Fig. 7*A* and *B, left column*), 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 *XPA* gene is an essential component of the NER; thus, cells with a mutated *XPA* gene completely lack a functional NER. As shown in Fig. 7*A* and *B* (*right columns*), treatment with EGCG did not significantly remove or repair the UVB-induced CPDs in *XPA*-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 *XPA*-proficient and *XPA*-deficient cells using Southwestern dot blot analysis. As indicated in Fig. 7*C*, treatment of *XPA*-proficient cells with EGCG (0, 10, 20 and 30 μg/mL) resulted in a dose-dependent

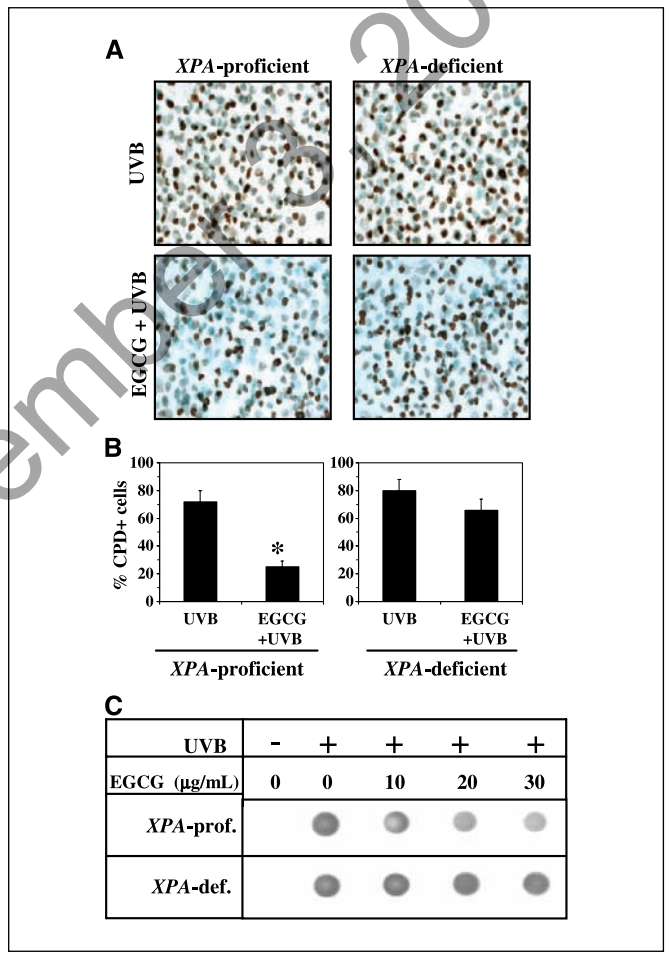

Figure 7. EGCG reduces UVB-induced DNA damage in *XPA*-proficient cells but does not reduce UVB-induced DNA damage in *XPA*-deficient cells. *A, XPA*-proficient cells and *XPA*-deficient cells were exposed to UVB (15 mJ/cm^2) with or without EGCG treatment (20 μg/mL), and cells were harvested 24 hours later, cytospun, and subject to immunocytostaining to detect CPD$^+$ cells. CPD-positive cells are dark brown. Magnification, ×40. *B,* numbers of CPD$^+$ cells in *XPA*-proficient and *XPA*-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 *XPA*-proficient cells. *C,* dose-dependent effect of EGCG on UVB-induced DNA damage in the form of CPDs in *XPA*-proficient and *XPA*-deficient cells. *XPA*-proficient and *XPA*-deficient cells were exposed to UVB (15 mJ/cm^2) 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 UVB-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 *in vivo* 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.

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