Interleukin-12-deficient mice are at greater risk of UV radiation–induced skin tumors and malignant transformation of papillomas to carcinomas

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Abstract

Solar UV radiation–induced immunosuppression is a risk factor for nonmelanoma skin cancer. Interleukin (IL)-12 has been shown to possess antitumor activity and inhibit the immunosuppressive effects of UV radiation in mice. In this study, we generated IL-12 knockout (KO) mice on a C3H/HeN background to characterize the role of IL-12 in photocarcinogenesis. After exposure of the mice to UVB (180 mJ/cm^2) radiation thrice a week for 35 weeks, the development of UV-induced tumors was more rapid and the tumor multiplicity and tumor size were significantly higher in IL-12 KO mice than their wild-type (WT) counterparts ($P < 0.05\text{-}0.001$). Moreover, the malignant transformation of UVB-induced papillomas to carcinomas was higher in IL-12 KO mice in terms of carcinoma incidence (55%, $P < 0.001$), carcinoma multiplicity (77%, $P < 0.001$), and carcinoma size (81%, $P < 0.001$). As IL-12 has the ability to repair UV-induced DNA damage, we determined this effect in our *in vivo* IL-12 KO mouse model. We found that UVB-induced DNA damage in the form of cyclobutane pyrimidine dimers was removed or repaired more rapidly in WT mice than IL-12 KO mice. Similarly, the UVB-induced sunburn cell formation is primarily a consequence of DNA damage. It was observed that UVB-induced sunburn cells were repaired rapidly in WT mice compared with IL-12 KO mice. The rapid removal or repair of UV-induced cyclobutane pyrimidine dimers or sunburn cells will result in reduced risk of photocarcinogenesis. Taken together, our data show that IL-12 deficiency is associated with the greater risk of photocarcinogenesis in mice, and this may be due to reduction in damaged DNA repair ability. [Mol Cancer Ther 2006;5(4):825–32]

Introduction

Solar UV radiation, particularly UVB (290–320 nm) radiation, is primarily responsible for the high incidence of melanoma and nonmelanoma skin cancers (1, 2). UV radiation has been shown to induce immunosuppression and the UV-induced immunosuppression is considered to be a risk factor for cancer induction (3, 4). Exposure of murine skin to UV radiation suppresses the development of allergic contact hypersensitivity, a prototypic T cell–mediated immune response (5). We have shown that treatment of mice with either a topical application of green tea polyphenols or dietary administration of grape seed proanthocyanidins prevents UV-induced skin carcinogenesis (6, 7) and that, in both cases, the prevention of skin carcinogenesis was associated with the prevention of UVB-induced suppression of the contact hypersensitivity response to a contact sensitizer, 2,4-dinitrofluorobenzene (8–10). Notably, the prevention of the UVB-induced suppression of the contact hypersensitivity response was associated with enhancement of the levels of interleukin (IL)-12 in treated mice compared with untreated mice on exposure to UV, suggesting that IL-12 plays a key role in the effects of these natural products in the prevention of UVB-induced suppression of contact hypersensitivity and UV-induced skin cancer (6, 7, 9, 10).

IL-12 has been previously shown to possess potent antitumor activity in a wide variety of murine tumor models (11–15) of various histologies, including carcinomas from the colon, kidney, and lung (11, 16–19), with the presence of IL-12 at the tumor site being critical for tumor regression (11). Conversely, it has also been shown that the tumor-bearing state is often characterized by a diminished capacity to produce IL-12 and an increase in IL-10 (20). The demonstration of significant antitumor activity in several preclinical animal tumor models has stimulated interest in the therapeutic use of IL-12 (21–23) but the mechanisms underlying the antitumor activity of IL-12 are only incompletely understood.

Solar UV radiation has both the immunosuppressive effect on the immune system as well as carcinogenic effects on the skin (3, 4, 24). UV-induced DNA damage, predominantly in the form of cyclobutane pyrimidine dimers (CPD), has been recognized as an important

molecular trigger for the suppression of immune responses (25) and initiation of UV carcinogenesis (25–27). Reduction of CPDs through application of DNA repair enzymes prevents the risk of UV-induced skin cancer (27). It has been observed that IL-12 exhibits the capacity to remove or repair UV-induced DNA damage (28). Depending on the severity of the DNA damage following UV exposure of the skin, keratinocytes in the skin could progress to either apoptosis or DNA repair (29, 30) pathways. If the DNA damage is irreparable, the keratinocyte cell cycle is arrested and it is transformed into a sunburn cell, an initial morphologic indicator of epidermal cell apoptosis. Based on the information that IL-12 possesses an antitumor activity and augment immune responses, we generated IL-12 knockout (KO) mice on a C3H/HeN background to characterize the role of IL-12 in UV radiation–induced skin carcinogenesis. By using IL-12 KO mice and their wild-type (WT) counterparts as a tool, we determined what role IL-12 has in UV-induced skin carcinogenesis.

Materials and Methods

Animals

IL-12 KO mice were generated and bred in our Animal Resource Facility at the University of Alabama at Birmingham. Briefly, male IL-12 (−/−) KO mice on a C57BL/6J genetic background and female C3H/HeN mice (6–7 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Male IL-12 (−/−) and female C3H/HeN mice were mated to obtain IL-12 (+/−) mice. We then mated male IL-12 (−/−) mice with IL-12 (+/−) female or IL-12 (+/−) males with IL-12 (+/−) females and the progeny was genotyped using a new rapid method of backcrossing for the generation of IL-12 KO mice onto a C3H/HeN mice background. The Transgenic Animal Core Facility of the University of Alabama at Birmingham provided marker-assisted mouse genome scanning, genetic monitoring of the mouse strains, and the accelerated development of congenic mice (31). The IL-12 (−/−) mice carry a germline disruption of the gene. This mutation removes p35 chain of IL-12 protein molecule and therefore completely eliminates the synthesis of IL-12 protein. Mice that are homozygous for this IL-12 deletion mutation are viable but their viability is reduced compared with their WT counterparts (C3H/HeN).

Determination of IL-12 Genotype

The IL-12 genotype of the mice, including confirmation of the genotype of the mice used in the experiments, was determined using PCR analysis of chromosomal DNA extractions from the tail tissues using a standard protocol (32). The PCR protocol was used from The Jackson Laboratory with two pairs of primers in the same reaction. The DNA fragment (280 bp) from the bacterial neomycin resistance gene insert was amplified with 5′-CTTGGGT-GGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGA-GATC-3′, whereas the DNA fragment (680 bp) from the *IL-12* gene was amplified with 5′-AGTGAACCT-CACCTGTGACACG-3′ and 5′-TCTTTGCACCAGCCAT-

GAGC-3′. The PCR product from the IL-12 (−/−) homozygote is 280 bp, the PCR product from the IL-12 (+/−) heterozygote is 680 and 280 bp, and the PCR product from the IL-12 (+/+) WT mouse (C3H/HeN) is 680 bp.

The purchased WT mice (C3H/HeN) were acclimatized for at least 1 week before the start of experiments. All mice were maintained in our Animal Resource Facility under the following conditions: 12 hours dark/12 hours light cycle, $24 \pm 2°C$ temperature, and $50 \pm 10\%$ relative humidity. The mice were fed Purina Taklad diet (Harlan Teklad, Madison, WI) with water *ad libitum*. The experimental animal protocols were approved by Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

UVB Exposure of Mice

At least 48 hours before UVB exposure, the backs of the mice were shaved with electric clippers and treated with Nair depilatory lotion. The groups of mice that were not exposed to UVB were also shaved and depilatory lotion was applied to maintain a similar treatment protocol. UVB irradiation was done as described earlier (6, 7). Briefly, the clipper-shaved and chemically depilated 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 very little UVC emission (<1%). The majority of the resulting wavelengths of UV radiation are in the UVB range (290–320 nm) with peak emission at 314 nm as monitored (6). The UVB emission was also monitored with an IL-1700 phototherapy radiometer (International Light, Newburyport, MA).

Photocarcinogenesis Protocol

The female mice, 6 to 8 weeks old, were divided into the different treatment groups with 20 mice in each group. The control groups of mice were age- and sex-matched to the experimental group. A long-term photocarcinogenesis protocol was used as described previously (6, 7) in which mice were irradiated daily with UVB (180 mJ/cm^2) as described above for a total of 10 days to stimulate tumor initiation (the tumor initiation stage). One week after the last UV exposure of this tumor-initiation stage, the mice were again irradiated with the same dose of UVB thrice weekly (tumor promotion stage) until the end of the protocol. The UVB-irradiated backs of the mice were examined on a weekly basis for papillomas or tumor development. Growths >1 mm in diameter that persisted for at least for 2 weeks were defined as tumors and were recorded. Tumor data for each mouse were recorded until the yield and size stabilized.

Malignant Conversion of Papillomas to Carcinomas

To determine whether the IL-12 KO mice are susceptible to malignant progression of papillomas to carcinomas compared with their WT counterparts, the mice were observed for 35 weeks following the photocarcinogenesis protocol. Carcinoma development was identified grossly as the number of downward invading lesions per mouse per

week and each lesion was verified histopathologically at the time of termination of the experiment at 35 weeks (33, 34) or on sacrifice of mice that became moribund before the termination of the experiment. At the termination of the experiment or on sacrifice of moribund mice, the dimensions of all the tumors or carcinomas on each mouse were recorded, and all tumors and suspected carcinomas were harvested for histologic verification. The percentage malignant conversion of benign skin papillomas into squamous cell carcinomas was calculated by dividing the total number of carcinomas by the total number of papillomas and multiplied by 100 (35). Tumor or carcinoma volumes were calculated using the hemiellipsoid model formula: tumor volume = $1/2$ $(4\pi/3)$ $(l/2)$ $(w/2)$ h, where l is length, w is width, and h is height, as described earlier (7).

Histologic Evaluation and Analysis of Tumor Type

The tumors, carcinomas, or suspected carcinomas were processed for histologic evaluation following preparation of conventional paraffin sections (5 μm thick) and H&E staining. The specimens were classified as being actinic keratoses, keratoacanthomas, squamous cell papillomas, or squamous cell carcinomas in a blinded manner by certified pathologists.

Immunohistochemical Detection of CPDs

To detect UVB-induced CPD$^+$ cells in the skin samples, immunostaining was done using a procedure described previously (36). Briefly, frozen skin or lymph node sections (5 μm thick) 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. Slides were washed with PBS and then incubated for 30 minutes with 10% goat serum in PBS. Sections were then incubated with thymine dimer–specific monoclonal antibody (Kamiya Biomedical Company, Seattle, WA) or an isotype control antibody (IgG1). Bound anti-CPD antibody was detected by incubation with biotinylated goat anti-mouse IgG1 followed by peroxidase-labeled streptavidin. Sections were then incubated with diaminobenzidine plus peroxidase substrate for 5 minutes. The sections were rinsed with distilled water and counterstained with H&E.

Dot-Blot Analysis

Genomic DNA from the epidermis was isolated following the standard procedures (32). 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 80°C. 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 then incubated with the antibody specific to CPDs for 1 hour at room temperature. After washing in washing buffer, the membrane was again incubated with horseradish peroxidase–conjugated secondary antibody. The CPDs containing DNA was detected by chemiluminescence using an enhanced chemiluminescence detection system. The experiments were repeated thrice.

Detection of Sunburn Cells

IL-12 KO as well as WT mice were exposed to UVB (60 mJ/cm^2). Mice were sacrificed later at indicated times, and the skin samples at least 1 cm long were obtained, fixed in formaldehyde, and embedded in paraffin. Tissue sections (5 μm) were stained with H&E. Sunburn cells were identified and counted throughout the epidermis of the section using light microscopy. Identification of apoptotic sunburn cell was based morphologically on cell membrane shrinkage and nuclear condensation attributable to fragmentation of the cells (30, 37). The percentage of apoptotic sunburn cells in the epidermis was calculated from the number of these cells / 100 cells counted from the entire 1 cm length of the epidermis for each skin section ($n = 5$).

Statistical Analysis

The susceptibility of IL-12 KO mice to tumors and carcinomas caused by UVB radiation was compared with the susceptibility of WT, C3H/HeN, mice. The four outcome variables used in this comparison were tumor incidence, tumor multiplicity, carcinoma incidence, and carcinoma multiplicity. The data were collected in an aggregate form over a period of 35 weeks. A linear model was fit to each of the outcome variables. The independent variables in the model were group, time, and a group/time interaction term. The interaction term helps to test if the rate of change in the outcome variables over time is a function of the group. To compare the numbers of CPD$^+$ cells or sunburn cells in different treatment groups, at least five to six different fields from each section were selected. A simple ANOVA followed by an appropriate *post hoc* test was used to calculate statistical significance of the data obtained from tumor volume, immunostaining of CPD$^+$ cells, dot-blot analysis, or sunburn cells. $P < 0.05$ was considered significant.

Results

IL-12-Deficient (KO) Mice Are Susceptible to UVB-Induced Skin Carcinogenesis

As IL-12 has been shown to possess antitumor activity (11, 12, 16), we determined the effect of IL-12 deficiency on susceptibility to UVB-induced skin carcinogenesis. On exposure to a standard photocarcinogenesis protocol, we found that UVB-induced tumor development occurred 14 days earlier in IL-12 KO mice than their WT counterparts (Fig. 1). Throughout the photocarcinogenesis protocol, the percentage of IL-12 KO mice with tumors was higher than the percentage of WT mice with tumors. This effect persisted through the termination of the experiment but did not attain statistical significance under the conditions used in this study (Fig. 1A). In terms of tumor multiplicity, the total number of tumors in the group of IL-12 KO mice remained higher than the total number in the group of WT mice throughout the experimental protocol (Fig. 1B). At the termination of the experiment, the total number of tumors was significantly higher in the group of IL-12 KO mice than the group of WT mice (72 versus 42, $P < 0.01$). Similarly, in terms of tumors per tumor-bearing mouse, a higher
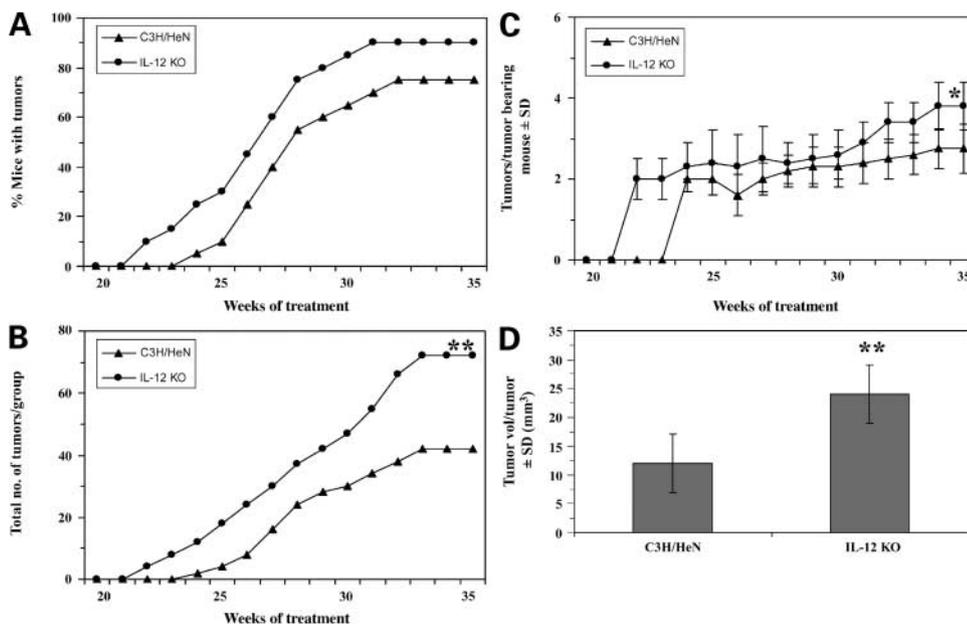
Figure 1. IL-12-deficient (IL-12 KO) mice are at higher risk to UVB radiation–induced skin tumor development compared with WT, C3H/HeN, mice. The percentage of mice with tumors (**A**), total number of tumors per group (**B**), and the number of tumors per tumor-bearing mouse (**C**) are plotted as a function of the weeks of treatment ($n = 20$). *Points*, mean number of tumors per tumor-bearing mouse (**C**); *columns*, mean tumor volume per tumor (**D**); *bars*, SD. IL-12 KO, $n = 18$; WT group, $n = 15$. **, $P < 0.001$; *, $P < 0.05$, significant difference from WT mice.

number of tumors per tumor-bearing mouse was observed throughout the experiment in the group of IL-12 KO mice than in the group of WT mice (Fig. 1C) and was significantly higher at the termination of the experiment ($P < 0.05$). At the termination of the experiment, the tumor volume of each mouse in each group was measured (Fig. 1D), and it was observed that tumor volume per tumor was significantly higher in the group of IL-12 KO mice compared with their WTs ($P < 0.001$). Taken together, these data concerning incidence and size of the UV-induced tumors clearly indicate that IL-12-deficient mice are extremely susceptible to UVB-induced carcinogenesis.

Tumor Type

Histopathologic examinations of the tumors revealed that there were 40 squamous cell carcinomas, 30 squamous cell papillomas, and 2 keratoacanthomas in the group of UVB-exposed IL-12 KO mice that were exposed to the photo-carcinogenesis protocol. In contrast, there were 9 squamous cell carcinomas, 23 squamous cell papillomas, 6 keratoacanthomas, and 4 actinic keratoses in the group of WT mice, as shown in Table 1. This difference in the type of tumors suggested that the IL-12-deficient mice are at a greater risk of UVB-induced skin cancer and malignant transformation of papillomas to carcinomas.

IL-12-Deficient Mice Are Susceptible to the Malignant Progression of UV-Induced Papillomas to Carcinomas

It was intriguing to note that some of the skin tumors that occurred in IL-12 KO mice transformed rapidly into squamous cell carcinomas. Moreover, ulceration was noted in most of the squamous cell carcinomas. Compared with the WT mice (25%), a significantly higher percentage (80%) of the IL-12 KO mice had carcinoma at the termination of the experiment at 35 weeks ($P < 0.001$; Fig. 2A). The kinetics of malignant conversion of papillomas into carcinoma in IL-12 KO mice was also significantly higher compared with

WT mice (Fisher-Irwin exact test; $P < 0.01$; Fig. 2B). The total number of carcinomas at the termination of the experiment in IL-12 KO group was 40, whereas only 9 carcinomas were recorded in the WT group (Fig. 2B; Table 1); thus, 55% papillomas were converted into carcinomas in the group of IL-12 KO mice, whereas 21% papillomas were converted into carcinomas in WTs. Thus, the risk of malignant progression of papilloma into carcinoma in IL-12 KO mice was higher compared with their WT counterparts ($P < 0.01$). The number of carcinomas per carcinoma-bearing mouse also remained higher throughout the experimental protocol in IL-12 KO (Fig. 2C) and was significantly higher ($P < 0.05$) at the termination of the experiment. The size of the individual carcinomas in IL-12 KO mice was significantly larger ($\sim$2-fold; $P < 0.01$; Fig. 2D) than in WT mice. Interestingly, we noted that the carcinomas that developed in the IL-12 KO mice tended to occur in clusters of three to four and that this clustering

Table 1. Distribution pattern of tumor/carcinoma types in IL-12 KO mice and in their WT counterparts at the termination of the photocarcinogenesis protocol

Mouse strain	Tumor types			
	AK	Papillomas	Keratoacanthomas	SCC
IL-12 KO ($n = 18$)	—	30	2	40
WT ($n = 15$)	4	23	6	9

NOTE: Histologic examination of tumor type was done at the termination of the photocarcinogenesis protocol at 35 weeks. The tumors or suspected carcinomas were obtained and processed for histologic evaluation following conventional H&E staining.
Abbreviations: AK, actinic keratosis; SCC, squamous cell carcinoma; n, number of tumor-bearing mice in each group.

tendency was not apparent in the WT mice. These data clearly indicate that not only are IL-12-deficient mice extremely susceptible to UV-induced carcinogenesis, but they are extremely susceptible to progression of papillomas to carcinomas.

UVB-Induced DNA Damage Removes or Repairs More Rapidly in WT Mice than in IL-12 KO Mice

UVB-induced DNA damage in the form of CPDs play an important role in initiation of photocarcinogenesis and that IL-12 has the ability to repair CPDs; therefore, we determined whether UVB-induced CPDs were removed or repaired rapidly in WT mice than in IL-12 KO mice. To do this, the shaved backs of IL-12 KO and WT mice were exposed to UVB (60 mJ/cm^2). One set of mice was sacrificed immediately or within 30 minutes after UV exposure and a second set of mice was sacrificed 24 hours later, skin samples were obtained, frozen in OCT, and subjected to immunohistochemical detection of CPD^{+} cells using an antibody directed against CPD (Kamiya Biomedical Co., Seattle, WA). In skin samples obtained immediately after UV exposure, no differences in the staining pattern of CPDs were observed between IL-12 KO and WT mice. However, in samples obtained 24 hours after UVB exposure, the numbers of CPD^{+} cells were significantly lower in the WT mice compared with the number of CPD^{+} cells obtained immediately after UV exposure in WT ($P < 0.01$). As anticipated, the number of CPD^{+} cells in IL-12 KO mice 24 hours after UVB exposure decreased; however, it had not significantly decreased compared with the number of CPD^{+} cells detected immediately after UV exposure. Moreover, the spontaneous DNA repair in WT mice was greater than IL-12 KO mice (Fig. 3A and B). This suggests that endogenous DNA repair mechanisms, including both IL-12-dependent and IL-12-independent mechanisms, may contribute to the repair of UV-induced DNA damage, and that the difference in DNA repair in between WT and IL-12 KO may be due to the absence of IL-12 in IL-12 KO mice. The skin samples obtained from the groups of mice that were not exposed to UV (normal skin) were devoid of any CPD^{+} cells.

Further, the effect of UVB-induced DNA damage in the form of CPDs was checked and confirmed using Southwestern dot-blot analysis. Epidermal genomic DNA from the skin samples of different treatment groups was isolated and subjected to Southwestern dot-blot analysis (Fig. 4). As shown in Fig. 4, in skin samples obtained immediately (within 30 minutes) after UV exposure, no significant differences in the dot-blot pattern was observed between IL-12 KO and WT mice. However, in samples obtained 24 hours after UVB exposure, the intensity of dot-blot or the presence of genomic DNA containing CPDs in WT mice was significantly lower compared with the dot-blot obtained immediately after UV exposure. In contrast, the intensity of dot-blot in IL-12 KO mice 24 hours after UV exposure was not significantly less compared with the dot-blot obtained immediately after UV exposure (Fig. 4). These data indicated that WT mice were able to rapidly remove or repair UV-induced DNA damage in the form of CPDs compared with IL-12 KO mice, and this difference may be due to the presence of IL-12 in WT mice or absence of IL-12 in IL-12 KO mice. The genomic DNA obtained from the groups of mice that were not exposed to UVB (normal skin) was devoid of CPDs or dot-blots.

UVB-Induced Sunburn Cells Repair More Rapidly in WT Mice than in IL-12 KO Mice

The UVB-induced sunburn cell formation is primarily a consequence of DNA damage. Sunburn cells are keratinocytes undergoing apoptosis after they have received a

Figure 2. IL-12-deficient mice are at higher risk of UVB radiation–induced malignant transformation of papillomas to carcinomas compared with their WT mice. The percentage of mice with carcinoma (**A**), the total number of carcinoma per group (**B**), and the number of carcinomas per carcinoma-bearing mouse are plotted as a function of the weeks of treatment ($n = 20$). *Points,* mean number of carcinoma per carcinoma-bearing mouse (**C**); *columns,* mean carcinoma volume per carcinoma (**D**); *bars,* SD. IL-12-deficient group, $n = 16$; WT group, $n = 5$. **, $P < 0.001$; *, $P < 0.05$, significant difference from WT mice.
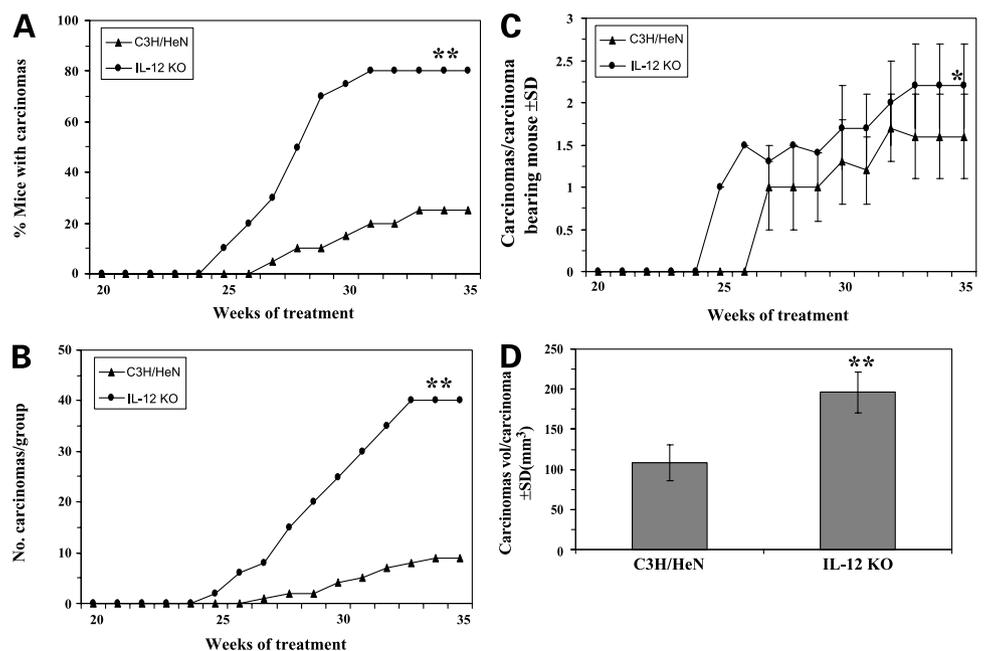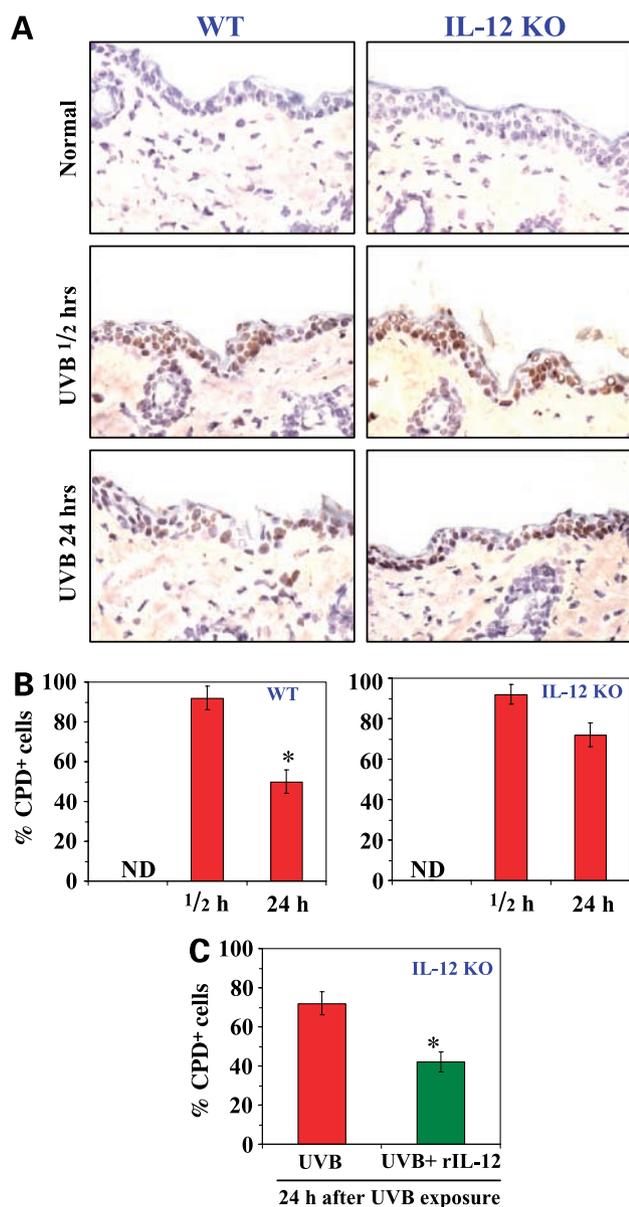
Figure 3. UVB-induced CPD removes or repairs more rapidly in WT mice than in IL-12 KO mice. **A,** WT or IL-12 KO mice were exposed to UVB (60 mJ/cm²). Mice were sacrificed at 30 min (immediate) or 24 h later and skin samples were collected and frozen in optimum cutting temperature medium. Frozen sections (5 μm thick) were subject to immunoperoxidase staining to detect CPD⁺ cells. *Dark brown,* CPD⁺ cells. CPD⁺ cells were not detected in non–UV-exposed skin. **B,** numbers of CPD⁺ cells were counted in five to six different areas of the sections under microscope. *Columns,* percentage of CPD⁺ cells in epidermis ($n = 5$); *bars,* SD. *, $P < 0.005$, significant reduction at 24 h versus immediately after UVB exposure. **C,** *in vivo* treatment of IL-12 to IL-12 KO mice reduces UVB-induced CPDs. IL-12 KO mice were exposed to UVB (60 mJ/cm²), as described above. In the indicated group, mice were injected with 1,000 ng/mouse of recombinant IL-12 s.c. to the UVB-irradiated skin site 3 h before UVB irradiation. Mice were sacrificed 24 h after UVB irradiation and skin samples were collected. Frozen skin sections were subjected to immunoperoxidase staining to detect CPDs. The 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. *, $P < 0.005$, significant difference versus UVB-alone group.

physiologic UV dose that irreversibly and severely damaged their DNA or other chromophores. IL-12 has been shown to have the ability to repair UVB-induced DNA damage and thus inhibits sunburn cell formation in the mouse epidermis (28). The inhibition of UV-induced apoptosis or sunburn cell formation by IL-12 may be due to a reduction in UV-induced DNA damage, and that may lead to the reduction in photocarcinogenesis. Using IL-12 KO mice and their WT counterparts as a tool, we determined the repair kinetics of UVB-induced sunburn cell in these mice. For this purpose, IL-12 KO mice and their WT (C3H/HeN) mice were exposed to 60 mJ/cm² of UVB radiation. Mice were sacrificed at different time points (Fig. 5), and skin samples were collected and processed for conventional paraffin sections (5 μm thick) and H&E staining for the detection of sunburn cell microscopically. As shown in Fig. 5, the numbers of sunburn cell were maximum in WT mice (0.6% of total epidermal cells) at 10 hours after UV irradiation and thereafter decreased. The number of sunburn cell in WT mice were significantly decreased at 24 and 48 hours after UV irradiation compared with 10 hours after UV irradiation (Fig. 5). A little higher number of sunburn cell was recorded in IL-12 KO mice (0.7% of total epidermal cells) at 10 hours after UV irradiation compared with WT mice. However, the spontaneous reduction in sunburn cell in IL-12 mice was slow and significantly less compared with WT mice at each time point studied. Further, the difference in decreasing kinetics of sunburn cell in between WT and IL-12 mice may be due to the endogenous presence and absence of IL-12, respectively, in WT and IL-12 KO mice. In additional study, recombinant IL-12 was administered s.c. to IL-12 KO mice 3 hours before to UVB-irradiated skin site. In this treatment group, the percentage of sunburn cell was significantly less than the percentage of sunburn cell in IL-12 KO mice, which were not treated with recombinant IL-12 ($P < 0.01$). These data further provide evidence that the presence of IL-12 may have a role in reduction of UVB-induced photocarcinogenesis.

Discussion

The photocarcinogenesis experiments conducted in IL-12 KO and their WT mice (C3H/HeN) indicated that IL-12 KO mice are at a greater risk of UVB radiation–induced skin carcinogenesis in terms of tumor incidence, tumor multiplicity, and the growth of the developing tumors than their WT counterparts (Fig. 1). Additionally, the present study also establishes that mice that are deficient in IL-12 are at greater risk of malignant progression of papillomas to carcinomas (Fig. 2). These data clearly provide an indication that IL-12 plays a preventive role in UV-induced skin carcinogenesis, and are in concordance with the results of other studies in which IL-12 has been shown to cure or improve the survival of tumor-bearing mice in several other tumor models (11, 16–19).

It has been recognized that UV-induced immunosuppression is a risk factor in the development of UV

radiation–induced skin carcinogenesis (3, 4), and that IL-12 has the capability to reverse the UVB-induced suppression of immune responses, at least in mice (38–40). Therefore, the data obtained from our genetically modified mouse model, where IL-12 KO mice and their WTs were used, clearly indicates that immunoregulatory cytokine IL-12 may have a role in prevention of UVB-induced skin carcinogenesis.

It has been shown that UVB-induced DNA damage in the form of CPDs has a role in immunosuppression and initiation of photocarcinogenesis. IL-12 has the ability to remove or repair UVB-induced CPDs and can reverse the UVB-induced immunosuppression (28, 38, 40). Therefore, we determined whether there is any difference in repair kinetics of UVB-induced CPDs in between IL-12 KO and their WT mice. Our observations that WT mice after UV irradiation removes or repairs UV-induced CPDs faster than IL-12 KO mice indicates the susceptibility of IL-12 KO mice toward photocarcinogenesis compared with their WT counterparts. Moreover, the s.c. injection of recombinant IL-12 to UV-irradiated mouse skin in IL-12 KO mice enhanced the repair of UVB-induced CPDs compared with non–recombinant IL-12-treated, UVB-exposed IL-12 KO mice. This effect in IL-12 KO mice was similar to WT mice. These data further support that the deficiency of IL-12 in mice may stimulate the susceptibility for photocarcinogenesis.

Induction of apoptosis is an additional protective mechanism as it eliminates cells that were unable to repair UVB-induced DNA damage completely (41). Therefore, dysregulation of UV-induced apoptosis will enhance the risk of photocarcinogenesis. There are several ways to inhibit UVB-induced apoptosis, such as overexpression of heat-shock proteins (42), induction of antiapoptotic proteins (43), and disruption of p53 function (30), etc. As these events allow the survival of cells carrying DNA damage, they may give rise to mutations and skin cancer development. Here, we provide the evidence that UVB-induced
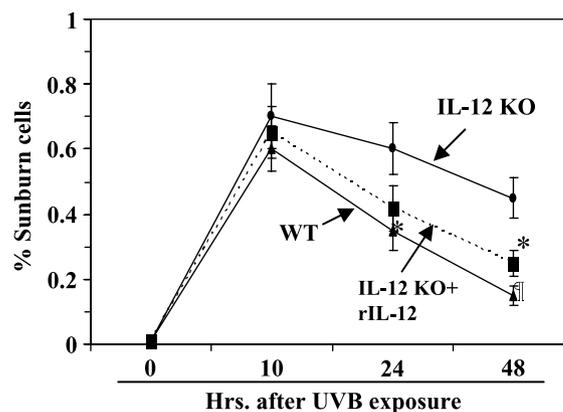

Figure 5. UVB-induced increases in apoptotic sunburn cells in the epidermis of WT mice removes more rapidly than in IL-12 KO mice. The WT or IL-12 KO mice were exposed to UVB (60 mJ/cm²) on the shaved back. In one group, IL-12 KO mice were injected with 1,000 ng/mouse of recombinant IL-12 s.c. to the UVB-irradiated skin site 3 h before UVB irradiation. The animals were sacrificed at the indicated time points after UV irradiation and skin samples were collected. Morphologically distinct apoptotic sunburn cells in the epidermis were counted in H&E-stained sections. *Points,* mean percentage of sunburn cells (n = 5); *bars,* SD. *, $P < 0.01$; ¶, $P < 0.005$, significant reduction versus IL-12 KO mice.

sunburn cells were repaired or removed rapidly in WT mice when determined at different time points after UV irradiation of the skin (Fig. 5). In contrast, this effect was not seen in IL-12 KO mice (Fig. 5). However, the treatment of IL-12 in *in vivo* IL-12 KO mice has resulted in a rapid removal or repair of UVB-induced sunburn cell compared with non–recombinant IL-12-treated, IL-12 KO mice, and this effect of recombinant IL-12 injection was similar to that found in WT mice. The fact that there was no significant difference in the amount of DNA damage immediately after UVB irradiation (Figs. 3–5) but a difference at a later time point can only be explained by enhanced removal or repair of damaged DNA lesions. These data confirms that the DNA repair mechanism in WT mice is faster than IL-12 KO mice and that may be mediated through the presence of IL-12.

The data obtained in this study clearly indicate that IL-12 deficiency is a risk factor for photocarcinogenesis in mice. It is tempting to suggest that IL-12 may have a role in prevention of photocarcinogenesis and therefore use of those natural products (whether through topical or dietary), which can induce the IL-12 induction *in vivo* or IL-12 therapy, may help to reduce the risk of UV radiation–induced skin cancers in humans.

Time (h)		$^1/_2$	24
UVB	-	+	+
WT			
IL-12 KO			

Figure 4. UVB-induced CPDs removes more rapidly in WT mice than in IL-12 KO mice. UVB-induced DNA damage in the form of CPDs was determined using Southwestern dot-blot analysis. Treatment protocol was similar to Fig. 3. Mice were sacrificed after half an hour or 24 h of UV irradiation and skin samples were collected. Epidermal genomic DNA was extracted from the epidermal skin samples and subjected to Southwestern dot-blot analysis using an antibody specific to CPD. The mice that were not exposed to UVB did not show the presence of CPDs in the dot-blot. Experiments were conducted and repeated separately in five animals in each group with identical results.

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