Prostaglandin E$_2$ Promotes UV Radiation–Induced Immune Suppression through DNA Hypermethylation[1]

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Abstract

Exposure of mice to UV radiation results in suppression of the contact hypersensitivity (CHS) response. Here, we report that the UV-induced suppression of CHS is associated with increases in the levels of cyclooxygenase-2 (COX-2), prostaglandin E$_2$ (PGE$_2$), and PGE$_2$ receptors in the exposed skin. UV radiation–induced suppression of CHS was inhibited by topical treatment of the skin with celecoxib or indomethacin (inhibitors of COX-2) or AH6809 (an EP2 antagonist). Moreover, mice deficient in COX-2 were found to be resistant to UV-induced suppression of CHS. The exposure of wild-type mice to UVB radiation resulted in DNA hypermethylation, increased DNA methyltransferase (Dnmt) activity, and elevated levels of Dnmt1, Dnmt3a, and Dnmt3b proteins in the skin, and these responses were downregulated on topical treatment of the site of exposure after irradiation with indomethacin or EP2 antagonist. Topical treatment of UVB-exposed COX-2–deficient mice with PGE$_2$ enhanced the UVB-induced suppression of CHS as well as global DNA methylation and elevated the levels of Dnmt activity and Dnmt proteins in the skin. Intra-peritoneal injection of 5-aza-2′-deoxycytidine (5-Aza-dc), a DNA demethylating agent, restored the CHS response to 2,4-dinitrofluorobenzene in UVB-exposed skin and this was associated with the reduction in global DNA methylation and Dnmt activity and reduced levels of Dnmt proteins. Furthermore, treatment with 5-Aza-dc reversed the effect of PGE$_2$ on UV-induced suppression of CHS in COX-2–deficient mice. These findings reveal a previously unrecognized role for PGE$_2$ in the promotion of UVB-induced immunosuppression and indicate that it is mediated through PGE$_2$ regulation of DNA methylation.

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Introduction

It has been shown that UV radiation in the UVB (290–320 nm) range induces immunosuppression in laboratory animals and that xenografted tumor cells are more readily established and grow more rapidly in UV-irradiated mice than mice that have not been UV irradiated [1]. The exposure of the skin to UV radiation triggers the release of prostaglandins (PGs), such as PGD2, PGF$_{2\alpha}$, and PGE$_2$, which are produced from arachidonic acid by the action of cyclo-oxygenases (COX) [2,3]. The PGs have been implicated in UV radiation–induced immunosuppression in several studies that show that nonsteroidal anti-inflammatory drugs (NSAIDs), including indomethacin, that exert their effects through COX-2 inhibition can reverse the immunosuppressive effect of UV radiation [4,5]. Among the PG metabolites, PGE$_2$, which is produced abundantly by keratinocytes

in UV-exposed skin [6,7], is the major and most effective metabolite generated by COX-2 activity and is considered to be a potent mediator of inflammatory responses. Collectively, these data suggest that PGE$_2$ plays a key role in UV radiation–induced immunosuppression. The mechanisms by which PGE$_2$ induces or promotes UV-induced immunosuppression and the relationship of these mechanisms to those of other proposed mediators of UV-induced immunosuppression have not been elucidated fully, however.

These mechanisms are of considerable clinical interest as UV-induced immunosuppression has been implicated as a risk factor for melanoma and nonmelanoma skin cancers. Epidemiologic and clinical studies suggest a link between inflammation and skin cancer [8] and also suggest that the use of NSAIDs reduces the relative risk for developing skin cancer [9]. NSAIDs exert their anti-inflammatory and anti-tumor promoting effects, in part, by targeting PGs. It is known that UV radiation induces the expression of COX-2 and production of PGE$_2$ in mouse skin. We have demonstrated previously that there is a distinct pattern of DNA hypermethylation in UVB-exposed mouse skin and UVB-induced skin tumors in mice that is associated with elevated expression and activity of the DNA methyltransferase (Dnmt) 1, Dnmt3a, and Dnmt3b [10]. Together, these data suggested the possibility that PGE$_2$ may be involved in the promotion of UVB-induced immunosuppression and that the PGE$_2$ may act to promote the immunosuppression by enhancing the levels of DNA methylation.

To explore these possibilities, we used a combination of genetic and pharmacological approaches to assess the role of PGE$_2$ in standard contact hypersensitivity (CHS) model of UVB-induced immunosuppression. We found that UV-induced up-regulation of COX-2/PGE$_2$ is involved in suppression of the CHS response in UV-exposed mice. The role of PGE$_2$ is supported by the evidence that COX-2–deficient mice are resistant to UVB-induced suppression of CHS. We also show that administration of PGE$_2$ to the mice increases the levels of global DNA methylation in UV-exposed skin and that this increase in DNA methylation is associated with increased expression and activity of Dnmts.

Materials and Methods

Animals

Female C3H/HeN mice (6–7 weeks old) were purchased from Charles River Laboratory (Wilmington, MA). The COX-2–deficient male and female heterozygous mice (COX-2$^{+/-}$) were used in this study and were bred using heterozygous × heterozygous in our animal resource facility, as described [11]. The breeding pairs were kindly provided by Dr Langenbach of the National Institutes of Environmental Health Sciences (National Institutes of Health). In some experiments, wild-type littermates from this breeding strain were also used in this study. The survival of COX-2–deficient heterozygous mice is similar to that of wild-type mice and there are no gross phenotypical differences as compared with their wild-type counterparts. All mice were maintained under standard housing conditions of a 12-hour dark/12-hour light cycle, a temperature of $24 \pm 2°C$, and relative humidity of $50 \pm 10\%$. Food and water were provided *ad libitum*. The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Antibodies and Reagents

Antibodies were purchased as follows: 5-methylcytosine (5-mC) from Calbiochem, EMD Biosciences (New Jersey, NJ); Dnmt1,

Dnmt3a, and Dnmt3b from Imgenec Corporation (San Diego, CA); COX-2, EP2, EP3, and EP4 from Santa Cruz Biotechnology (Dallas, TX); EP1 from Abcam (Cambridge, MA), and DNA isolation kits were purchased from Qiagen Inc (Valencia, CA). The positive controls of EP1, EP2, EP3, and EP4 for Western blot analysis were obtained from Santa Cruz Biotechnology. The Methylamp Global DNA Methylation Quantification Kit and the EpiQuik DNA Methyltransferase Activity Assay Kit were purchased from Epigentek, Inc (New York, NY). The PGE$_2$ immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals of analytical grade were purchased from Sigma-Aldrich Chemical Co (St Louis, MO).

UVB Irradiation of Mice

The dorsal hairs of mice were trimmed or shaved with electric clippers at least 24 hours before UVB exposure. The shaved backs of the mice were UVB irradiated as described earlier [12] using 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 insignificant. The peak emission of UV radiation is at 314 nm. This UV unit enables us to enter the UV dose in millijoules and variations in energy output are compensated automatically so that the desired UV dose is delivered.

UVB-induced CHS Model and Assay

The shaved backs of the mice were exposed to UVB radiation (150 mJ/cm^2) on four consecutive days. Twenty-four hours after the last UV exposure, the mice were sensitized by painting 25 μl of 0.5% 2,4-dinitrofluorobenzene (DNFB) in acetone/olive oil (4:1, vol/vol) on the UVB-irradiated skin site. The CHS response was elicited 5 days later by painting both surfaces of both ears of each mouse with 20 μl of 0.2% DNFB in acetone/olive oil (4:1, vol/vol). The ear skin thickness was measured 24 hours after the challenge using an engineer's micrometer (Mitutoyo, Tokyo, Japan) and was compared with the ear thickness just before the challenge, as detailed previously [13,14]. Mice that were not UV irradiated but were sensitized and challenged as described served as a positive control. Mice that were not UV irradiated and received only ear challenge without sensitization with DNFB served as a negative control. During the UV exposure of the mice, the ears of the mice were protected from the UV irradiation. The UV-induced suppression of CHS was determined as described previously [13,14]. Each group consisted of four to five mice.

UVB Irradiation of Mice for the Biochemical Analysis Including Epigenetic Regulators

The shaved backs of the mice were exposed to UVB radiation (150 mJ/cm^2) on four consecutive days with or without the treatment with PGE$_2$, COX-2 inhibitors, or EP2 antagonist. Twenty-four hours after the last UV exposure, mice were sacrificed and dorsal skin samples were collected for preparing the samples for biochemical analysis.

Treatment of Mice with PGE$_2$, Indomethacin, Celecoxib, and 5-Aza-2′-deoxycytidine

To assess the effect of PGE$_2$ or COX-2 inhibitors (indomethacin or celecoxib) on UVB-induced suppression of the CHS response, the mice were treated topically with celecoxib (500 μg in 0.2 ml acetone) [15,16], indomethacin (50 μg in 0.2 ml of acetone) [16,17], or AH6809 (25 μg in 0.2 ml of acetone) [16,18], which were applied

to the shaved dorsal skin 30 minutes before UVB exposure. PGE$_2$ (50 μg in 0.2 ml of acetone) was applied to the COX-2–deficient mouse skin after UVB exposure [16,17]. Control animals were treated topically with acetone (0.2 ml of acetone). Some mice were administered 5-aza-2′-deoxycytidine (5-Aza-dc; 1 mg/kg body weight) by intraperitoneal (i.p.) injection after exposure to UVB radiation [19]. Two doses of 5-Aza-dc were administered: first dose of 5-Aza-dc was injected i.p. after first UVB exposure of mice and second dose of 5-Aza-dc was administered i.p. after third exposure of UVB irradiation of mice.

Dot-blot Analysis of 5-mC for DNA Methylation

Genomic DNA from skin samples was isolated, and dot-blot analysis was performed as detailed previously [12]. Briefly, genomic DNA (50 ng) was transferred to a positively charged nitrocellulose membrane by vacuum dot-blot analysis (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, the membrane was incubated with the antibodies specific for 5-mC at room temperature for 1 hour. After washing, the membrane was incubated with HRP-conjugated secondary antibody. The enhanced chemiluminescence reagents were used to detect the circular bands of 5-mC.

Analysis of Global DNA Methylation

Total genomic DNA was extracted from the mouse skin samples using the Qiagen amp DNA Mini Kit (Qiagen Sciences, Valencia, CA) following the manufacturer's protocol. The levels of global DNA methylation were determined using the Methylamp Global DNA Methylation Quantification Kit (Epigentek, Inc) following the manufacturer's instructions.

Assay of Dnmt Activity

Skin samples from different treatment groups were used to prepare nuclear extracts. Nuclear extracts were prepared using the EpiQuik Nuclear Extraction Kit (Epigentek, Inc), and Dnmt activity was determined using the EpiQuik DNA Methyltransferase Activity Assay Kit (Epigentek, Inc) according to the instructions provided by the manufacturer.

Western Blot Analysis

The epidermis was separated from the whole skin as described earlier [20] and pooled from two or three mice in each group (n = 4–5 per group). Lysates of the epidermis were prepared for Western blot analysis as described previously [21]. Proteins (25–60 μg) were resolved on 8% to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were incubated in blocking buffer for 1 hour at room temperature and then incubated with the primary antibodies in blocking buffer overnight at 4°C. The membrane was then washed with phosphate-buffered saline and incubated with HRP-conjugated secondary antibody. Protein bands were visualized using the enhanced chemiluminescence detection reagents. Equal loading of proteins on the gel was verified by reprobing the membrane with anti–β-actin or anti-histone H3 antibody.

Immunoassay Analysis of PGE$_2$

Skin samples were homogenized in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 10 μM indomethacin using a polytron homogenizer (PT3100; Fisher Scientific, Pittsburg, PA). The supernatants were collected for analysis of the levels of PGE$_2$ using the Cayman PGE$_2$ Enzyme Immunoassay Kit (Cayman Chemical) following the manufacturer's protocol.

Statistical Analysis

The statistical significance of difference between the values of control and treatment groups was determined using analysis of variance. $P < .05$ was considered as statistically significant.

Results

UV-induced Suppression of CHS Is Associated with an Increase in the Levels of COX-2, PGE$_2$, and PGE$_2$ Receptors

It is well recognized that exposure of mice to UV radiation induces suppression of the immune system or CHS response. We first verified that exposure of C3H/HeN mouse skin to UV radiation on four consecutive days resulted in suppression of the CHS response to DNFB, a skin contact sensitizer (Figure 1A). To determine the mechanism of UV-induced suppression of CHS, we investigated whether UV-induced suppression of CHS is associated with enhancement of the levels of mediators of inflammation, such as COX-2. For this purpose, mice were UVB irradiated for four consecutive days and sacrificed 24 hours after the last UVB irradiation. Skin samples from UVB-irradiated and non–UVB-irradiated mice were collected, and skin lysates were subjected to Western blot analysis. As shown in Figure 1, Western blot analysis revealed that the expression of COX-2 was approximately 2.5-fold higher in UVB-exposed skin than in non–UVB-exposed mouse skin (Figure 1B). The production of PGE$_2$ metabolite and the expression levels of PGE$_2$ receptors also were higher in the UVB-exposed skin than non–UVB-exposed mouse skin (Figure 1, C and D). Positive controls of EP receptors were used for proper recognition of receptors during gel electrophoresis.

Treatment of Mice with COX-2 Inhibitors Prevents UVB-Induced Suppression of CHS in Mice

To determine whether the induction of COX-2 in UVB-exposed skin plays a role in UVB-induced suppression of immune reactions, the effect of UVB radiation on CHS was determined in mice that had been administered COX-2 inhibitors. As shown in Figure 2A, topical administration of indomethacin (sixth bar) or celecoxib (seventh bar) before each exposure to UVB resulted in inhibition of UVB-induced suppression of the CHS response in the mice. Importantly, treatment of mice with indomethacin or celecoxib did not affect the CHS response to DNFB in non–UV-irradiated mice (third or fourth bar). We also found that the production of PGE$_2$ was significantly lower ($P < .001$) in indomethacin- or celecoxib-treated UVB-exposed mouse skin compared to UV-exposed skin that was not treated with these COX-2 inhibitors (Figure 2B). As this suggested a role for PGE$_2$ in UVB-induced immunosuppression, we examined the effect of the EP2 antagonist, AH6809, on UVB-induced suppression of CHS in mice. Topical treatment of the skin site with AH6809 before each exposure of UVB enhanced sensitization to DNFB in UVB-exposed mice (Figure 2A, eighth bar) compared to the UVB-exposed mice that were not treated with the AH6809 (fifth bar).

Effect of Indomethacin and EP2 Antagonist on DNA Methylation in UVB-Irradiated Mouse Skin

We previously have shown that exposure of the mouse skin to UV radiation results in aberrant DNA hypermethylation in the UVB-exposed
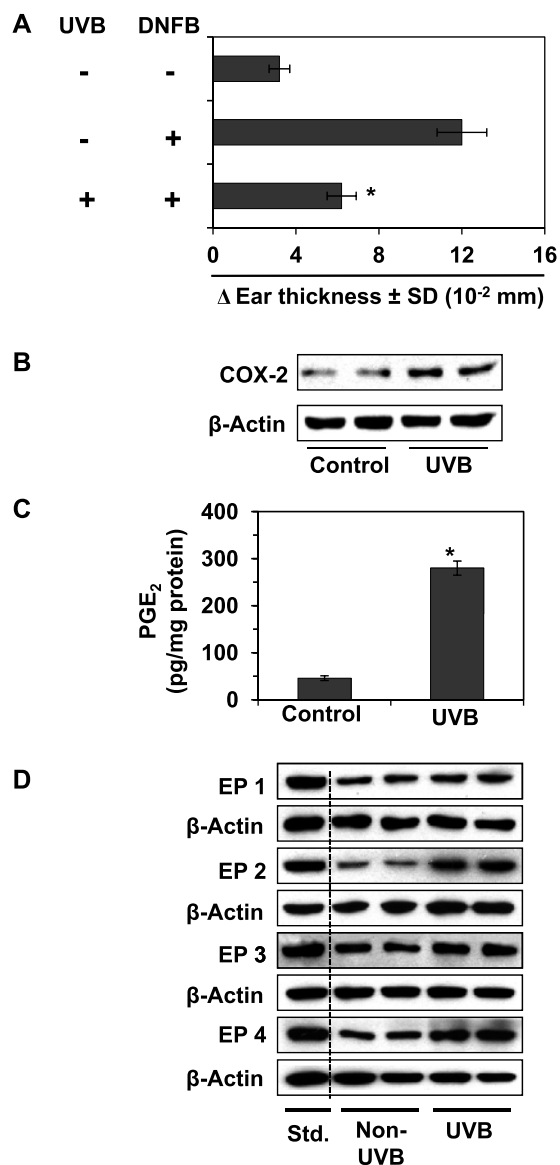
Figure 1. Effect of UVB radiation on CHS response and the levels of inflammatory mediators. The shaved dorsal skin of female C3H/HeN mice was exposed to UVB radiation (150 mJ/cm^2) on four consecutive days as described in Materials and Methods section. (A) The mice were sensitized to DNFB, and the CHS response to application of DNFB on ear skin (challenge) was assessed by measurement of the ear swelling response 24 hours later as described in Materials and Methods section. The change in ear skin thickness is reported in millimeter ($\times 10^{-2}$) as the mean $\pm$ SD, with n = 4 to 5 per group. (B–D) The mice were exposed to UVB (150 mJ/cm^2) on four consecutive days and then sacrificed 24 hours after the last UVB exposure. Dorsal skin samples from UVB-exposed and non–UVB-exposed mice were collected for analysis. (B) The levels of COX-2 were determined in lysates of the skin using Western blot analysis. (C) The concentration of PGE$_2$ was determined in homogenates of the epidermis using an ELISA kit. PGE$_2$ concentration is expressed in terms of pg/mg protein as the mean $\pm$ SD. (D) The levels of expression of the PGE$_2$ receptors (EP1, EP2, EP3, and EP4) were determined by Western blot analysis. To recognize and verify the exact band of EP receptors, a positive control of individual EP receptor was run on the same gel. For B to D, data are shown in duplicates, and each sample was prepared by pooling the skin biopsies from at least two mice in each group. Std., standards or positive controls of EP receptors.
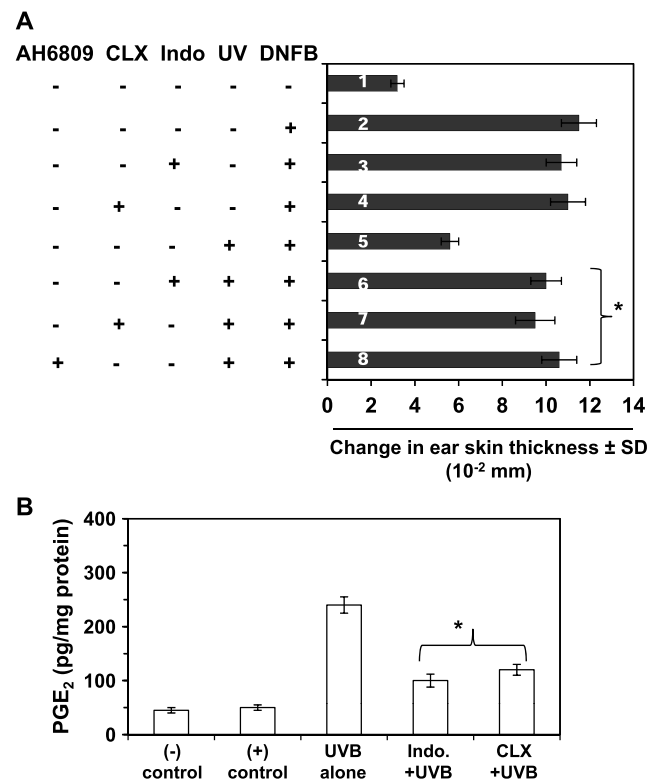

Figure 2. COX-2 inhibitors (indomethacin and celecoxib) and an EP2 antagonist (AH6809) inhibit UVB-induced suppression of the CHS response. Mice were exposed to UVB radiation, and the CHS response was measured as described in Figure 1A, except that some mice were treated by topical administration of the indicated agents to the skin 30 minutes before each UVB irradiation as described in Materials and Methods section. The change in ear skin thickness is reported in millimeter ($\times 10^{-2}$) as the mean $\pm$ SD, with n = 4 to 5 per group. Mice in treatment group 1 were not sensitized with DNFB but were challenged with DNFB on the ear skin. Mice were treated with indomethacin (sixth bar), celecoxib (seventh bar), or EP2 antagonist (eighth bar). Significant CHS response *versus* UVB alone–exposed group of mice (fifth bar), *P < .001. Indo, indomethacin; CLX, celecoxib. (B) The levels of PGE$_2$ in the dorsal skin samples were determined using a PGE$_2$ immunoassay kit. Significant inhibition *versus* UVB alone group, *P < .001. Data are shown as the means $\pm$ SD.

skin and UVB-induced skin tumors in mice [10]. PGE$_2$ regulates many fundamental cellular functions and has been implicated in regulation of DNA methylation in fibroblasts [22]. As most of the biologic functions of PGE$_2$ are mediated through the PGE$_2$ receptor EP2, we tested the effects of PGE$_2$ on global DNA methylation by using an EP2 antagonist to block the action of PGE$_2$ in UV-exposed skin. In these experiments, the mice were UVB irradiated on four consecutive days with or without topical application of indomethacin or the EP2 antagonist. The mice were sacrificed 24 hours after the last UVB exposure and skin samples were processed for analysis. Quantitative analysis of the global DNA methylation levels showed that the DNA methylation in the UVB-exposed skin was more than 2.5-fold higher than in the non–UVB-exposed mouse skin (Figure 3A). Topical application of indomethacin or EP2 antagonist before each exposure to UVB resulted in significantly lower levels of global DNA methylation (P < .01) in the mouse skin compared to the levels in mouse skin that was exposed to UVB radiation in the absence of treatment with indomethacin or EP2 antagonist. This
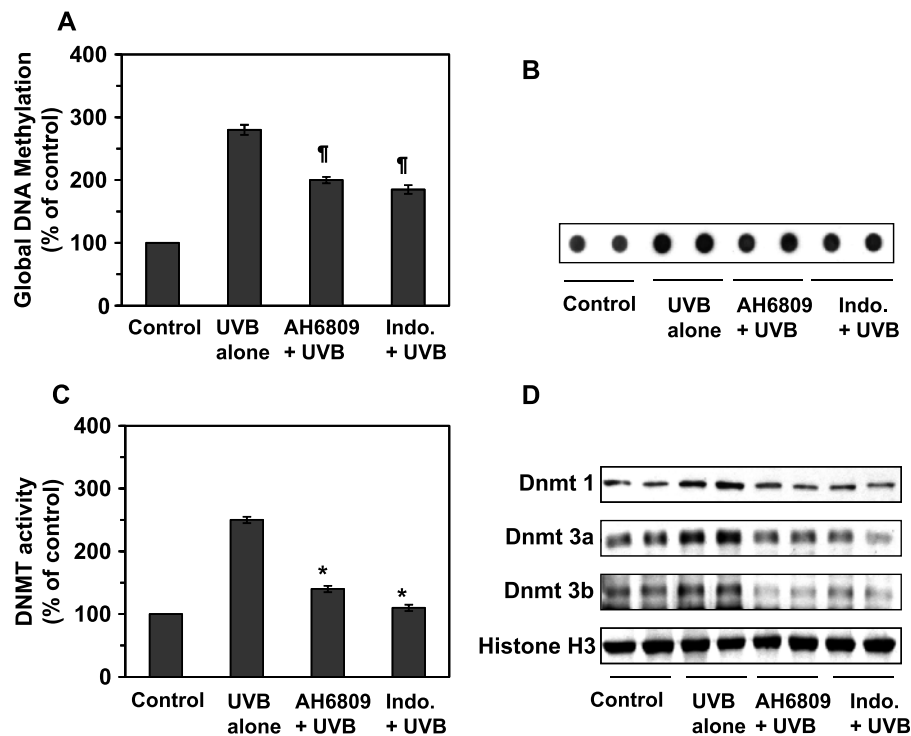
Figure 3. Indomethacin and EP2 antagonist (AH6809) affect UVB-induced epigenetic regulators in UVB-irradiated mouse skin. Mice were UVB exposed in the presence and absence of topical treatment of the mouse skin with indomethacin or EP2 antagonist on four consecutive days. Samples of the dorsal skin were collected 24 hour later. (A, B) Total DNA was extracted from the epidermis. (A) The levels of global DNA methylation were determined in skin samples using a Global DNA Methylation Kit. (B) The levels of 5-mC in the DNA were estimated by dot-blot analysis. (C) Nuclear extracts were prepared and total Dnmt activity was determined using the Dnmt Activity Assay Kit. Data are presented in terms of percentage *versus* non–UVB-irradiated control skin. (D) The skin samples from the mice described in A were analyzed for the presence of Dnmt1, Dnmt3a, and Dnmt3b proteins using Western blot analysis. Significant inhibition *versus* the group exposed to UVB but not treated with indomethacin or PGE$_2$ antagonist, *$P < .001$, ¶$P < .01$.

observation was further verified by dot-blot analysis in which the levels of DNA methylation were determined using an antibody specific for 5-mC (Figure 3B).

Effect of Indomethacin and EP2 Antagonist on Dnmt Activity and Dnmt Proteins in UVB-Irradiated Mouse Skin

As Dnmts play a key role in DNA methylation, we determined the effect of indomethacin on Dnmt activity in UVB-exposed skin. Mice were exposed to UVB radiation on four consecutive days with and without topical application of indomethacin or AH6809. They were then sacrificed 24 hours after the last UV exposure, and skin samples were prepared for the analysis of Dnmt activity and Dnmt proteins. As shown in Figure 3C, Dnmt activity was significantly higher in the UVB-exposed mouse skin ($P < .001$) than the non–UVB-exposed mouse skin. Furthermore, the Dnmt activity was significantly lower ($P < .001$) in indomethacin-treated UVB-exposed skin than UVB alone–exposed mouse skin. To determine whether the indomethacin-induced reduction in Dnmt activity was associated with reduced expression of Dnmt proteins, the levels of Dnmt proteins were determined in the skin lysates using Western blot analysis. As shown in Figure 3D, there was a reduction in the levels of Dnmt1, Dnmt3a, and Dnmt3b proteins in the indomethacin-treated UVB-exposed skin as compared with non–indomethacin-treated UVB-exposed skin.

To test directly whether PGE$_2$ promotes UVB-induced suppression of immune reactivity through DNA methylation, we treated mice with EP2 antagonist before each exposure to UVB irradiation and analyzed

Dnmt activity and the levels of Dnmt proteins in skin samples. As shown in Figure 3C, the levels of Dnmt activity were significantly lower in the AH6809-treated UVB-exposed mouse skin ($P < .001$) than in non–AH6809-treated UVB-exposed skin. Similarly, the levels of Dnmt1, Dnmt3a, and Dnmt3b proteins, as determined by Western blot analysis, were reduced in the AH6809-treated UVB-exposed mouse skin (Figure 3D).

COX-2–deficient Mice Are Resistant to UVB-Induced Suppression of CHS

If PGE$_2$ promotes UVB-induced immunosuppression in mice, then PGE$_2$ deficiency in mice should prevent UVB-induced suppression of CHS. Thus, to confirm the role of PGE$_2$ in UVB-induced suppression of CHS, we used COX-2–deficient mice that are unable to produce sufficient PGE$_2$ to adequately suppress the CHS response. The COX-2–deficient mice were subjected to the CHS assay protocol as described in Materials and Methods section. As shown in Figure 4, the sensitization reactions after DNFB challenge (third bar) of the COX-2–deficient mice after UVB exposure on four consecutive days were almost equal to those of mice in the positive control group (second bar), indicating that the COX-2–deficient mice are indeed resistant to UVB-induced suppression of the CHS response. We have also examined the effect of UVB irradiation on the CHS response in wild-type littermates of COX-2–deficient mice. Following CHS protocol, it was observed that UVB irradiation of wild-type littermates resulted in suppression of CHS response to DNFB, as was observed in C3H/HeN

mice (data not shown). To further verify the role of PGE$_2$ in UVB-induced immunosuppression, another group of COX-2–deficient mice was exposed to UVB and treated with PGE$_2$ as described in Materials and Methods section. As shown in Figure 4, the COX-2–deficient mice that were UVB irradiated and treated with PGE$_2$ showed a significant suppression of the CHS response (fourth bar) compared with the mice that were not treated with PGE$_2$ but exposed to UVB.

Treatment of Mice with the DNA Demethylating Agent, 5-Aza-dc, Abrogates PGE$_2$-Induced Suppression of CHS in UVB-Exposed COX-2–Deficient Mice

To test whether PGE$_2$ promotes UVB-induced suppression of CHS in mice by promoting DNA methylation, we treated another group of COX-2–deficient mice with PGE$_2$ and then with the DNA demethylating agent 5-Aza-dc following the protocol described in Materials and Methods section. As shown in Figure 4, treatment of COX-2–deficient mice with PGE$_2$ and subsequently with 5-Aza-dc resulted in a full sensitization reaction after treatment of the mice with DNFB (fifth bar), and this CHS response was comparable to that observed in the positive control group (second bar). These data suggest that PGE$_2$-induced DNA methylation might be blocked or that demethylation may occur in the mice treated with 5-Aza-dc resulting in full sensitization and restoration of the CHS response.

Effect of PGE$_2$ on DNA Methylation, Dnmt Activity, and Dnmt1, Dnmt3a, and Dnmt3b Proteins in UVB-Exposed Skin of COX-2–Deficient Mice

The results of the current study show that inhibition of PGE$_2$ in UVB-exposed skin by topical administration of indomethacin resulted
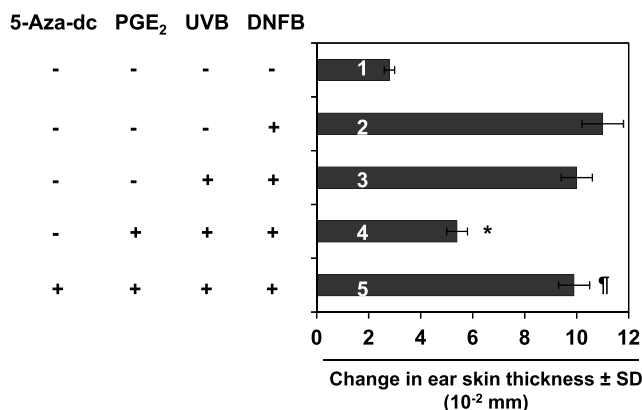

Figure 4. Effect of UVB irradiation on the CHS response in COX-2–deficient mice. Mice were UVB irradiated on four consecutive days with or without topical application of PGE$_2$ after each UVB exposure and the CHS response assessed 24 hours later, as described in Figure 1 and in Materials and Methods section. The COX-2–deficient mice were resistant to UVB-induced suppression of the CHS response. Topical application of PGE$_2$ after each UVB exposure promoted suppression of CHS in UV-exposed mice (fourth bar) and treatment of the mice with a DNA demethylating agent 5-Aza-dc (1 mg/kg body weight) by i.p. injection after exposure to UVB radiation restored the CHS response in the PGE$_2$-treated COX-2–deficient mice. Significant suppression of CHS *versus* positive control group (fourth bar), *$P <$ .001. Significant restoration of CHS *versus* PGE$_2$-induced suppression of CHS, ¶$P <$ .01.

in reduced expression of global DNA methylation and Dnmt activity and lowered the levels of Dnmt proteins (Figure 3). To further verify that PGE$_2$ affects epigenetic regulators on UVB exposure, we used the COX-2–deficient mice. In these experiments, COX-2–deficient mice were exposed to UVB radiation on four consecutive days with or without topical treatment with PGE$_2$, as detailed in Materials and Methods section. Mice were sacrificed 24 hours after the last UVB exposure and skin samples were collected for the analysis of epigenetic biomarkers. As shown in Figure 5A, exposure of COX-2–deficient mice to UVB did not significantly influence DNA methylation. In contrast, the levels of global DNA methylation were significantly higher in the UVB-exposed COX-2–deficient mice that were treated with PGE$_2$ than in the UVB-exposed COX-2–deficient mice that were not treated with PGE$_2$. This effect of PGE$_2$ on DNA methylation was further verified by dot-blot analysis using antibody specific to 5-mC (Figure 5B). We also determined the effects of PGE$_2$ on Dnmt activity and Dnmt proteins (Dnmt1, Dnmt3a, and Dnmt3b) in the skin samples from these treatment groups. We found that the levels of Dnmt activity were not significantly higher in the COX-2–deficient mice that were exposed to UVB compared to the levels in the COX-2–deficient mice that were not exposed to UVB. However, treatment of COX-2–deficient mice with PGE$_2$ resulted in significantly higher ($P <$ .001) levels of Dnmt activity in the UVB-exposed skin of the COX-2–deficient mice compared to the levels of activity in the non–PGE$_2$-treated UVB-exposed COX-2–deficient mice (Figure 5C). Similarly, treatment with PGE$_2$ enhanced the levels of Dnmt1, Dnmt3a, and Dnmt3b proteins in the UVB-exposed COX-2–deficient mice (Figure 5D). Interestingly, the levels of the Dnmt3a protein in the UVB-exposed PGE$_2$-treated COX-2–deficient mouse skin were greater than the levels of the Dnmt1 and Dnmt3b proteins. To further verify that PGE$_2$ is responsible for enhanced DNA methylation, the wild-type littermates of COX-2–deficient mice were treated with PGE$_2$ for four consecutive days, then sacrificed 24 hours after the last treatment of PGE$_2$, and skin samples were collected for the analysis of global DNA methylation levels. It was found that PGE$_2$ treatment significantly enhanced ($P <$ .01) the levels of global DNA methylation in PGE$_2$-treated mouse skin compared to non–PGE$_2$-treated control skin (Figure 5E).

PGE$_2$ Increases Dnmt3a Expression through Increased Expression of Sp1 and Sp3

The expression of Dnmt3a can be regulated by the transcription factors Sp1 and Sp3 [23]. To determine whether the marked PGE$_2$-induced increase in the levels of Dnmt3a protein is associated with up-regulation of Sp1 and/or Sp3, we determined the expression of these transcription factors in the skin of COX-2–deficient mice that had been exposed to UVB with and without treatment with PGE$_2$. Western blot analysis revealed that UVB irradiation did not affect the expression of Sp1 and Sp3 transcription factors in the COX-2–deficient mice. Treatment of UVB-exposed COX-2–deficient mice with PGE$_2$ markedly increased the expression of both transcription factors compared to the expression in either UVB-exposed COX-2–deficient mice that were not treated with PGE$_2$ or non–UVB-irradiated mouse skin, as shown in Figure 5F. In contrast, the expression levels of Sp1 and Sp3 were increased in UVB-irradiated skin of wild-type littermates of COX-2–deficient mice compared to non–UVB-irradiated control skin (Figure 5G). These data suggest that PGE$_2$-mediated increase in Dnmt3a is due, at least in part, to increased expression of the Sp1 and Sp3 transcription factors.
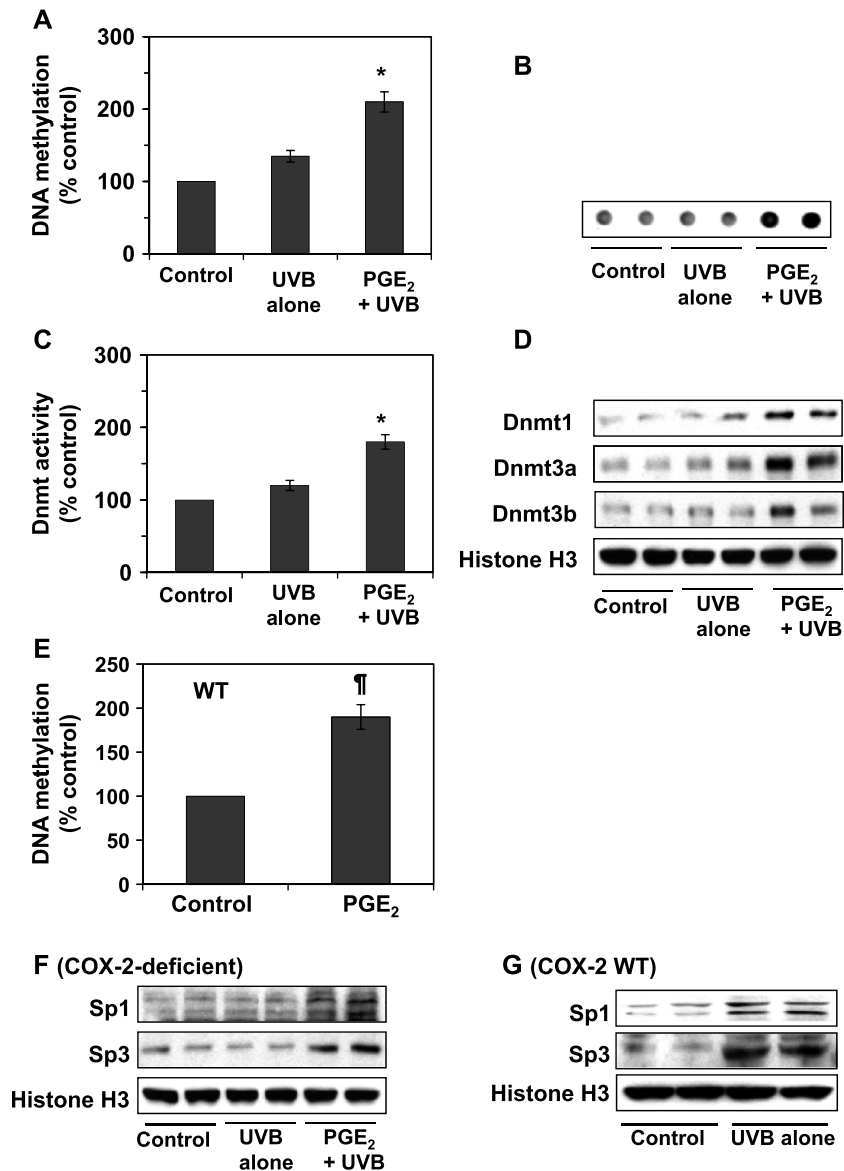
Figure 5. Effect of UVB irradiation on epigenetic regulators in COX-2–deficient mice. Mice were UVB irradiated on four consecutive days with or without topical application of PGE$_2$ and samples of the dorsal skin were taken 24 hours later, as described in Materials and Methods section ($n = 5$). (A) The levels of global DNA methylation in the mouse skin were determined using a Global DNA Methylation Assay Kit. In these mice, the UVB irradiation did not significantly affect the levels of global DNA methylation. Topical treatment of the UVB-exposed mouse skin with PGE$_2$ enhanced the levels of global DNA methylation. (B) The levels of 5-mC in the extracted DNA were estimated by dot-blot analysis. (C) Total Dnmt activity in nuclear extracts of skin samples was determined using the Dnmt Activity Assay Kit. Topical treatment of the UVB-exposed mouse skin with PGE$_2$ increased Dnmt activity in the skin. Data are presented in terms of percentage *versus* non–UVB-irradiated control group. Significantly higher activity *versus* mice exposed to UVB without topical application of PGE$_2$, *$P < .001$. (D) The levels of Dnmt1, Dnmt3a, and Dnmt3b proteins in the skin samples from the mice described in A were determined using Western blot analysis. (E) The effect of topical treatment of PGE$_2$ on DNA methylation levels in the wild-type littermates was determined. Significant increase *versus* non–PGE$_2$-treated mouse skin, ¶$P < .01$. (F, G) The expression of the transcription factors, Sp1 and Sp3, were determined using Western blot analysis. (F) COX-2–deficient mice were resistant to UVB-induced increases in expression of Sp1 and Sp3 and topical application of PGE$_2$ in UVB-exposed COX-2–deficient mice resulted in overexpression of Sp1 and Sp3 transcription factors. (G) UVB irradiation increased the expression of Sp1 and Sp3 in the skin of wild-type littermates of COX-2–deficient mice. Equal nuclear protein loading on gels was verified using anti-histone H3 antibody.

Effect of DNA Demethylating Agent (5-Aza-dc) on UVB-Induced Suppression of CHS in C3H/HeN Mice

To verify whether PGE$_2$-enhanced DNA methylation and Dnmt activity have a role in UVB-induced CHS in mice, additional experiments were conducted. Mice were exposed to UVB with and without

treatment with 5-Aza-dc and the CHS response to DNFB was evaluated, as detailed in Materials and Methods section. As shown in Figure 6A, exposure of the mice to UVB irradiation confirmed significant suppression ($P < .001$) of CHS (third bar) compared to the positive control group of mice (second bar). Treatment of mice with

5-Aza-dc by i.p. injection after each UVB exposure resulted in significant enhanced sensitization of mouse skin ($P < .001$) after challenge to DNFB (fourth bar) compared to the CHS response in UVB-exposed mice (third bar) that were not treated with 5-Aza-dc.
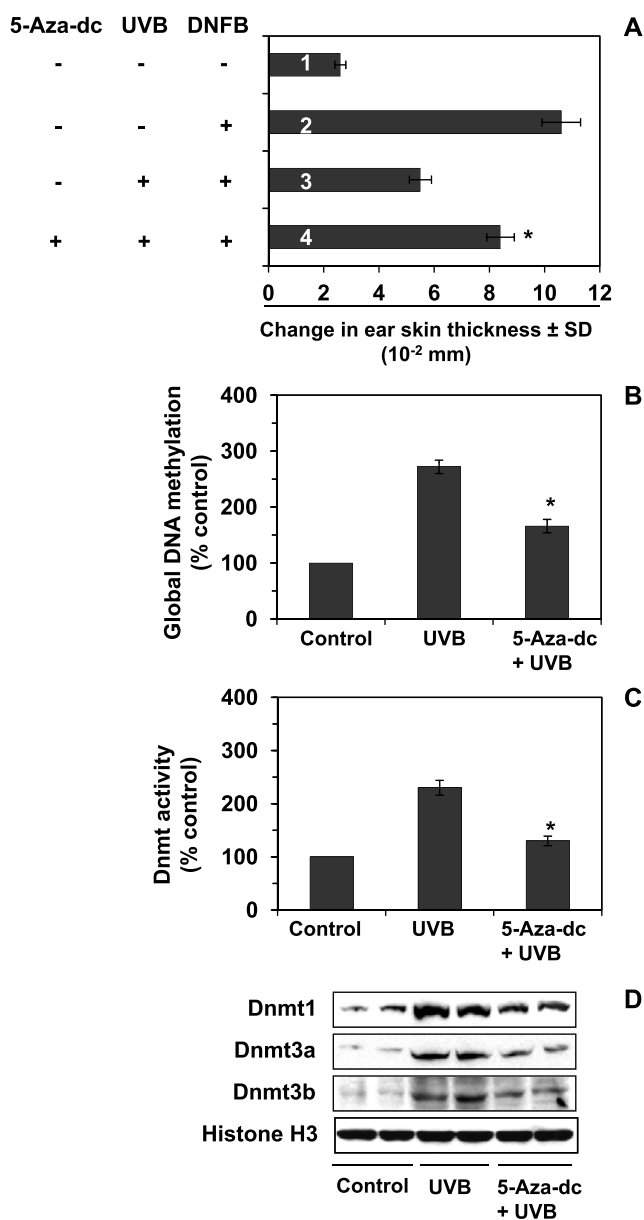

Figure 6. Effect of i.p. treatment of 5-Aza-dc on UVB-induced suppression of CHS and DNA methylation. Mice were UVB irradiated on four consecutive days with or without treatment with 5-Aza-dc. The CHS response was measured as described in Materials and Methods section ($n = 4-5$). (A) Injection of 5-Aza-dc after UVB irradiation reduced UVB-induced suppression of the CHS response or restored the CHS response in UVB-irradiated mice. For the analysis of epigenetic regulators, shaved mouse skin was UVB irradiated for four consecutive days with and without the treatment of 5-Aza-dc. Skin samples were collected 24 hours after the last UVB exposure. Treatment of mice with 5-Aza-dc reduced (B) the levels of UVB-induced global DNA methylation in UVB-irradiated mouse skin and (C) Dnmt activity in UVB-irradiated mouse skin. Significant difference *versus* UVB irradiation in the absence of application of 5-Aza-dc, *$P < .01$; and (D) the levels of Dnmt proteins, as determined by Western blot analysis in UVB-irradiated mouse skin.

Effect of 5-Aza-dc on UVB-Induced DNA Methylation and Dnmt Activity

To examine whether reversal of UVB-induced suppression of CHS by 5-Aza-dc is associated with suppression of DNA methylation and Dnmt activity, additional experiments were conducted. For this purpose, C3H/HeN mice were exposed to UVB on four consecutive days with and without treatment with 5-Aza-dc by i.p. injection, and 24 hours after the last UVB exposure, the mice were sacrificed and skin samples were collected for the analysis of DNA methylation and Dnmt activity. As shown in Figure 6, the results confirmed that in the untreated mice UVB irradiation significantly increased the levels of global DNA methylation (Figure 6B) and Dnmt activity (Figure 6C) in the UVB-exposed mice compared to non–UVB-exposed mouse skin. The levels of global DNA methylation and Dnmt activity were significantly lower ($P < .01$) in the UVB-exposed mice that were treated with 5-Aza-dc than in the UVB-exposed mice that were not treated with 5-Aza-dc. Western blot analysis of the levels of Dnmt proteins in the skin lysate samples from the same treatment groups revealed that the treatment of mice with 5-Aza-dc resulted in reduced expression of Dnmt1, Dnmt3a, and Dnmt3b proteins compared to the levels in the skin of the non–5-Aza-dc–treated UVB-exposed mice (Figure 6D).

Discussion

UV-induced PGE$_2$ overproduction has been implicated in various stages of tumor development, invasion, and metastasis of tumor cells [8,24]; however, it is not yet clearly understood how PGE$_2$ contributes to the UV-induced immunosuppression and how the action of PGE$_2$ is related to the other proposed mechanisms that have been implicated in this process. In the present study, using a well-established CHS protocol, we show that UV-induced suppression of CHS is associated with the overexpression of COX-2, PGE$_2$, and PGE$_2$ receptors in the mouse skin. Our study shows that treatment of mice with indomethacin, celecoxib, or antagonist of PGE$_2$ receptor EP2 reverses or blocks UVB radiation–induced immunosuppression in mice and thus supports the concept that inhibitors of PGE$_2$ or PGE$_2$ signaling play a crucial role in immunosuppression after UV irradiation. Under similar conditions, exposure of the skin to UVB enhanced expression of Dnmt1, Dnmt3a, and Dnmt3b proteins as well as Dnmt activity, which subsequently increased global DNA methylation. In the reverse situation, treatment of mice with indomethacin or EP2 antagonist (AH6809) decreased the levels of DNA methylation, Dnmt activity, and the protein expressions of Dnmt1, Dnmt3a, and Dnmt3b in UVB-exposed mouse skin. These data suggest that PGE$_2$-mediated DNA methylation may have a role in the suppression of the immune system in UVB-irradiated mice.

To verify the role of PGE$_2$ in UVB-induced immunosuppression, we used COX-2–deficient mice, which were unable to synthesize PGE$_2$ after UVB irradiation. It was observed that COX-2–deficient mice were resistant to UVB-induced suppression of CHS, whereas the treatment of UVB-exposed COX-2–deficient mice with PGE$_2$ resulted in suppression of CHS response, thus suggesting the role of PGE$_2$ in UVB-induced immunosuppression. To establish a link between PGE$_2$ and DNA hypermethylation in UVB-exposed skin, COX-2–deficient mice that were treated with PGE$_2$ were also treated with DNA demethylating agent (5-Aza-dc) after UVB irradiation. The treatment of mice with 5-Aza-dc resulted in full sensitization reaction after DNFB challenge in PGE$_2$-treated COX-2–deficient mice,

thus suggesting the role of PGE$_2$-mediated DNA hypermethylation in UVB-induced immunosuppression.

The link between overexpression of PGE$_2$ and DNA methylation on exposure of the skin to UVB irradiation was further verified using COX-2–deficient mice. A significant increase in DNA methylation and Dnmt activity was not observed when COX-2–deficient mice were irradiated multiple times to UVB radiation. However, the treatment of these UVB-irradiated mice with PGE$_2$ resulted in significant greater Dnmt activity, overexpression of various Dnmt proteins (Dnmt1, Dnmt3a, and Dnmt3b), and increased DNA methylation compared to the mice that were exposed to UVB but were not treated with PGE$_2$. Epigenetic alterations including DNA hypermethylation or hypomethylation have been implicated in tumor growth and progression including the skin tumors in mice and humans [10]. The role of inflammatory mediators such as PGE$_2$ in UV irradiation–induced immunosuppression has been indicated by studies showing that NSAIDs that exert their effect through COX-2 inhibition can reverse the immunosuppressive effect of UV radiation [4,5]. We therefore further tried to establish a link between DNA methylation and UVB-induced immunosuppression. For this purpose, C3H/HeN mice were exposed to UVB radiation with or without treatment with a DNA demethylating agent (5-Aza-dc) and CHS response was evaluated. The results demonstrate that treatment of mice with 5-Aza-dc reversed or inhibited UVB-induced suppression of CHS response to DNFB accompanied with lowering the levels of global DNA methylation, Dnmt activity, and the protein expressions of Dnmt1, Dnmt3a, and Dnmt3b compared to non–5-Aza-dc–treated UVB-exposed mice. It was interesting to note that the expression of Dnmt3a was greater in PGE$_2$-treated mice.

Several immunomodulatory mediators have been shown to play a role in the UV-induced immunosuppression [13,14,25–27]. Previously, we have shown that the UV-induced suppression of CHS is associated with an imbalance of interleukin-10 and interleukin-12 [13] and that the repair of UV-induced DNA damage in the form of cyclobutane pyrimidine dimers and nucleotide excision repair mechanisms play a role in this process [14]. The current study clearly implicates PGE$_2$, which is known to affect multiple immune and inflammatory factors. This suggests that exposure of skin to UVB radiation can induce immune suppression by different events that trigger widespread disruption of immune and inflammatory response. Elucidation of the relative roles of these events and their interactions in the context of dose responses and genetic predisposition requires further study.

The findings of the current studies have significant clinical relevance as PGE$_2$ generation is upregulated in inflammation, infection, and cancer. In general, the outcome of our findings allow us to speculate that changes in PGE$_2$ biosynthesis may contribute to changes in DNA methylation patterns associated with a wide variety of diseases including the various skin diseases associated with chronic exposure to sunlight and other environmental factors. Collectively, the results of our study demonstrate the role of PGE$_2$-mediated DNA methylation in UVB-induced immunosuppression. UV-induced immunosuppression has been considered as a risk factor in nonmelanoma skin cancers caused by excessive exposure to UV radiation [1,28], and the incidence of nonmelanoma skin cancer was reduced in subjects receiving a selective COX-2 inhibitor (celecoxib) compared to subjects receiving placebo [9]. Therefore, the outcome of our study not only provides substantial insight into the intricate roles of PGE$_2$ in UVB-induced immunosuppression and how DNA methylation is regulated by PGE$_2$ in UVB-exposed skin but also provide a rationale for considering the development of new strategies using the inhibitors of PGE$_2$ and demethylat-ing agents alone or in combination for the prevention or treatment of nonmelanoma skin cancers.

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