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Carcinogen-Induced Inflammation and Immunosuppression Are Enhanced in Xeroderma Pigmentosum Group A Model Mice Associated with Hyperproduction of Prostaglandin E₂¹

Hiroko Miyauchi-Hashimoto,²*Kazue Kuwamoto,* Yoshihiro Urade, †Kiyoji Tanaka, ‡ and Takeshi Horio*¹

Xeroderma pigmentosum group A (XPA) gene-deficient mice easily develop skin cancers by the application of topical chemical carcinogens as well as by UV irradiation. As certain chemical carcinogens have been shown to be immunosuppressive, we examined the inflammatory and immunosuppressive effects of dimethylbenz(a)anthracene (DMBA) on XPA mice. Compared with wild-type mice, XPA mice showed greater ear swelling and reduction of epidermal Langerhans cells after DMBA application. Topical application of DMBA impaired the induction of contact hypersensitivity, initiated either locally or at distant sites. These DMBA-induced local and systemic immunosuppressions were more greatly enhanced in XPA mice than in wild-type mice. DMBA application induced increased production of PGE₂, IL-10, and TNF-α in the skin of XPA mice. Treatment with indomethacin, a potent inhibitor of PG biosynthesis, inhibited DMBA-induced inflammation and local immunosuppression. In XPA mice, increased serum IL-10 was detected after DMBA treatment. Excess production of PGE₂, TNF-α, and IL-10 after DMBA application may be involved in the enhanced local and systemic immunosuppression in DMBA-treated XPA mice. Susceptibility to DMBA-induced skin tumors in XPA mice may be due to easy impairment of the immune system by DMBA in addition to a defect in the repair of DMBA-DNA adduct. Enhanced immunosuppression by chemical carcinogens as well as the mutagenicity of these mutagens might be associated with the high incidence of internal malignancies seen in XP patients. Moreover, these results supported the hypothesis that persistent DNA damage is a trigger for the production of immunoregulatory cytokines. The Journal of Immunology, 2001, 166: 5782–5791.

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Abbreviations used in this paper: XP, xeroderma pigmentosum; XPA, XP group A; CHS, contact hypersensitivity; DMBA, 9,10-dimethyl-1,2-benz(a)anthracene; DNFB, 1-fluoro-2,4-dinitrobenzene; CPD, cyclobutane pyrimidine dimer; LC, Langerhans cell.

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As in the case of UVB radiation, certain chemical carcinogens, such as dimethylbenz(a)anthracene (DMBA), have been shown to be immunosuppressive. The following parameters have been reported to be affected in DMBA-exposed mice: proliferative response to T cell mitogens, unidirectional mixed lymphocyte response, generation of cytotoxic lymphocytes, and NK cell tumor cytotoxicity (14, 15). Application of DMBA to the skin depletes epidermal Langerhans cells (LCs) (16) and suppresses CHS locally, when a sensitizer is applied to DMBA-treated skin (17–19). Similar to UVB-induced immunosuppression, DMBA-induced local immunosuppression is reported to be associated with the development of Ag-specific suppressor T lymphocytes, which inhibit the induction of both cellular and humoral immune responses when adoptively transferred to naive syngeneic recipients (17, 18). In contrast to UVB, topically applied DMBA inhibits cutaneous immunological functions only at the site of application, because when DMBA is applied at one skin site, and animals are immunized to the hapten at another site, a normal CHS response is observed (18). Although these findings suggested that DMBA exerts immunosuppressive effects by reducing LC numbers and/or function, the precise mechanisms by which DMBA induces immunosuppression have not been clarified. XPA mice also showed a high incidence of skin tumors by topical application of DMBA as well as by UVB radiation (4). It is well established that the formation of DMBA adducts with DNA is a necessary precondition for the development of skin cancer and that the level of adducts correlates closely with tumorigenicity (20, 21). Because XPA protein is required to repair DMBA-DNA adducts, the high incidence of DMBA-induced skin cancer in XPA mice may be mainly due to a defect in the repair of DMBA-induced DNA lesions of cutaneous cells. We hypothesized that the immunosuppression by DMBA in addition to its mutagenicity may contribute to the development of skin tumors initiated by the application of DMBA to XPA mice. DNA damage caused by UVB radiation has been demonstrated to be one of the primary molecular mechanisms for initiation of UVB-induced immunosuppression. However, it is not known whether other forms of DNA damage, including the DMBA-DNA adduct, also cause immunosuppression, or whether the same immunosuppressive pathway is activated by chemical carcinogens and UVB radiation. We investigated the effects of DMBA on the immunologic function of XPA mice and the mechanisms of DMBA-induced immunosuppression.

Materials and Methods

Animals
XPA gene-deficient mice with CBA, C57BL/6, and CD-1 chimeric genetic background (4) were backcrossed with hairless albino mice of the inbred strains Hos/HR-1, which were supplied by Hoshino Experimental Animal Farm (Saitama, Japan), and the resultant hairless XPA Do/Do and XPA +/+ mice were used in this study. All mice were female and 8–10 wk of age at the beginning of each experiment, but within a single experiment, all mice were age-matched. Each experimental panel consisted of 5–14 mice.

Reagents
The [5,6,8,11,12,14,15-3H]PGE2 (7.4 × 106 Bq/mol) was obtained from New England Nuclear (Boston, MA), DMBA and indomethacin were purchased from Sigma (St. Louis, MO), and dinitrofluorobenzene (DNFB) was obtained from WAKO (Osaka, Japan).

Ear swelling response to DMBA application
The ears of mice were treated with 20 μl of different concentrations of DMBA in acetone. Ear thickness was measured with a dial thickness gauge (Peacock, Tokyo, Japan) immediately before and 1, 2, 3, 4, and 5 days after application. An ear swelling response was represented by an increment in thickness above the value before application.

ADPase staining
The epidermal sheets were prepared from skin samples using EDTA and were stained with ADP according to the method of Chaker et al. (22). Stained LCs were counted in 30 randomly selected interfollicular fields per sheet with the aid of a calibrated ocular grid.

Sensitization and elicitation of CHS
The mice were sensitized using an epicutaneous application of 25 μl of 1% DNFB solution in acetone/olive oil (4/1) on abdominal or ventral skin. CHS was elicited after using an application of 20 μl of a 0.2% DNFB solution on the surface of each left ear 6 days after sensitization. Ear thickness was measured before and 24 h after application of the challenge dose, and the difference between the two readings was recorded as ear swelling.

Assay for DMBA-induced immunosuppression of CHS
To assay the induction of local immunosuppression by DMBA, 60 μl of a 0.5 or 0.1% DMBA in acetone was applied on the dorsal area of the mice on day 0 and sensitization with DNFB was performed at the DMBA-applied site on day 4. Elicitation at the left ear was performed on day 10.

To assay the induction of systemic immunosuppression by DMBA, 100 μl of 0.05 or 0.1% DMBA was applied on the dorsal area of the mice on day 0 and sensitization with DNFB was performed at another site at the same time. CHS was elicited on day 4. Elicitation at the left ear was performed on day 10.

To determine whether any tolerance was induced, we sensitized all mice again by applying 25 μl of 1% DNFB on the nontreated dorsal skin immediately after measurement of ear swelling. The second challenge test was performed on the right ear 6 days after the second sensitization. Suppression of the ear swelling response was calculated as follows: [100 − (ear swelling of test mice/ear swelling of DMBA-nontreated control mice)] × 100%.

Determination of PGE2 in mouse skin
The amount of PGE2 in mouse ears at 0, 1, 2, 3, and 4 days after application of 20 μl of 0.5% DMBA was determined by enzyme immunoassay, as described previously (23). In brief, both ears of each mouse were cut off and immediately dropped into liquid nitrogen. Four frozen ears were combined into one sample, and then weighed and homogenized with a Polytron homogenizer (Brinkmann, Westbury, NY) in 10 ml of ethanol containing 0.1 ml of 5 N HCl, which was precooled at −20°C. [3H]PGE2 (10,000 dpm each) was added to the homogenates as a tracer for estimation of the recovery. After centrifugation, PGE2 in the ethanol extract was applied to Sep-Pak C18 cartridges (Waters Associates, Milford, MA) and purified by HPLC. The amount of PGE2 was measured in duplicate using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Immunohistochemical analysis for IL-10 and TNF-α
IL-10 and TNF-α in the skin were examined by an immunoperoxidase method using monoclonal rat anti-mouse IL-10 (BD PharMingen, San Diego, CA) and monoclonal rat anti-mouse TNF-α (BD PharMingen). DMBA-treated mouse skin was removed 2 days after treatment, embedded in an OTC compound, and frozen in liquid nitrogen; 4-μm cryostat sections were fixed with 2% paraformaldehyde. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide; slides were incubated with a protein blocking solution (10% normal horse serum and 1% normal goat serum) for 20 min, followed by treatment with rat anti-mouse IL-10 or TNF-α mAb overnight at 4°C. The slides were washed with PBS and incubated for 30 min with peroxidase-labeled streptavidin and for 20 min with diaminobenzidine. An isotype control, isotype rat IgG1 (BD PharMingen) was used instead of mAbs.

RT-PCR for IL-10
Whether the application of DMBA induced IL-10 mRNA signals was determined according to Enk and Katz (24) with some modification. At various times after application of DMBA, the mice were killed, and ears were excised, split, and floated dermal side down in 10,000 U of Dispase (Godo Shusei, Tokyo, Japan) in 10 ml of PBS for 2 h at 37°C. Epidermal cell suspensions were prepared, and total epidermal RNA was extracted by the method of Chomczynski and Sacchi (25).

The cDNA was synthesized from 1 μg of mRNA in a 20-μl volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl2, 10 mM DTT, 1 mM dNTPs, 0.5 μg of oligo(dT), and 2.5 U of SuperScript II RT (Life Technologies Oriental, Tokyo, Japan). The mixture was incubated at 42°C for 50 min and heated at 70°C for 10 min to inactivate the enzyme. The mixture was incubated with 2 U of Escherichia coli RNase H at 37°C for 20 min.
PCR amplification was performed according to Saiki et al. (26). The cycling conditions chosen were 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C, and 30 cycles were used. Primers for IL-10 were purchased from Clontech (Palo Alto, CA). Primer sequences for G3PDH were ACCA CAGTCCATGCGATC (5’ primer) and TCCACCCCGCTTTGCT GTA (3’ primer). After the final cycle, the temperature was maintained at 72°C for 10 min. PCR products were then electrophoresed though 1% agarose gels in Tris/acetate/EDTA buffer containing ethidium bromide, and the gels were photographed under UV light.

Measurement of TNF-α protein

Samples of ear tissue extract for ELISA were prepared as described by Ferguson et al. (27) with some modification. Briefly, at various times after application of DMBA, ears were excised and immediately homogenized with a 5-fold volume of 0.1% Tween 20 in PBS. Samples were then quickly frozen in liquid nitrogen, thawed in a 37°C water bath, sonicated for 15 s, and centrifuged for 5 min at 13,000 × g, and supernatants were used for ELISA (Genzyme, Minneapolis, MN).

Determination of serum IL-10 by ELISA

Peripheral blood was obtained by venipuncture 2 days after the epicutaneous application of 100 μl of 1% DMBA and was anticoagulated with heparin. Following centrifugation, serum IL-10 was measured in duplicate using an ELISA kit (BD PharMingen), according to the manufacturer’s procedures.

Statistical analysis

Student’s t test was used to determine the statistical difference between means, with p < 0.05 considered significant.

Results

XPA mice, but not wild-type mice, developed significant ear swelling response by DMBA application

A single application of 20 μl of 0.5% DMBA resulted in significant ear swelling in XPA mice 2 days after application, and edema was still increasing on day 5 (Fig. 1). XPA mice developed lesser ear swelling using a 0.1% DMBA application than with 0.5% DMBA, reaching a plateau on day 3. The application of 0.05% DMBA produced no significant ear swelling in XPA mice. In contrast, the wild-type mice did not develop any significant ear swelling even with a 2.5% DMBA application.

Next, we examined the susceptibility of the mice to other primary irritating reagents and/or carcinogens. Croton oil is known to be a tumor promoter and includes 12-O-tetradecanoylphorbol-13-acetate, which increases the amount of intracellular oxidative damage. Application of 0.2 and 1% croton oil produced a similar degree of ear swelling in XPA mice and wild-type mice (data not shown). Application of 10 and 20% phenol, which is a primary irritating reagent, also produced ear swelling in XPA mice as well as in wild-type mice (data not shown). These results indicate that XPA mice are highly susceptible to DMBA, but are no more susceptible to these other agents than are normal mice.

XPA mice developed stronger damage to ADPase+ LCs after DMBA application

In nontreated skin, approximately the same numbers of ADPase+ LCs were found in the epidermal sheet of XPA and wild-type mice (614.4 ± 104.32 and 577.6 ± 57.76/mm², respectively). The time course of change in the number of ADPase+ LCs after application of 0.5% DMBA was similar in both types of mice (Fig. 2). The number of ADPase+ LCs significantly decreased 2 days after application of 0.5% DMBA, reached a minimum on day 4 and then recovered, but was still significantly lower than the pretreated level on day 9. By day 16, the density of ADPase+ LCs in both types of mice recovered and reached a higher level than that before treatment. During the period from days 2–9, the number of ADPase+ LCs in XPA mice was smaller than that in wild-type mice. The DMBA application of a lower concentration (0.1%) significantly reduced ADPase+ LCs in XPA mice 2 and 4 days after application (p < 0.0001 compared with the pretreated level, on days 2 and 4), but not in the wild-type mice. Morphologically, dendritic cells in untreated XPA mice skin were indistinguishable from those in wild-type mice; both showed intense staining and multiple branched dendrites. The ADPase+ LCs remaining after the 0.1% DMBA application were significantly reduced in XPA mice but not in wild-type mice. The number of ADPase+ LCs in XPA mice was significantly lower than that in wild-type mice (p < 0.0001).
DMBA application were rounded and lacked dendrites in the XPA mice, but those in the wild-type mice had multiple branched dendrites.

**DMBA-induced local immunosuppression was enhanced in XPA mice**

The immune status of mice sensitized to DNFB through DMBA-treated skin was investigated. Both XPA and wild-type mice developed a strong CHS response when nontreated skin was sensitized with DNFB (Fig. 3a). Sensitization with DNFB on skin that had received a 0.1 or 0.5% DMBA application resulted in almost complete suppression of the CHS response in XPA mice (96 and 94% suppression, respectively). In contrast, less suppression was induced in the wild-type mice with pretreatment using 0.1 or 0.5% DMBA-treated skin was significantly suppressed in XPA mice. In these mice, the induction of tolerance was confirmed by the second challenge test. In XPA and wild-type mice that had received the same concentration of DMBA treatment ($p < 0.0001$ at 0.1% DMBA, $p < 0.005$ at 0.5% DMBA).

**DMBA-induced systemic immunosuppression was enhanced in XPA mice**

Application of 0.1% DMBA on the back 4 days before sensitization with DNFB on the abdominal skin induced a stronger suppression of CHS in the XPA mice than in wild-type mice (82 vs 45% suppression; Fig. 4a). Although 0.05% DMBA induced only slight suppression in wild-type mice (32% suppression), it produced pronounced suppression in XPA mice (59% suppression). In these mice, the induction of tolerance was also confirmed by the second challenge tests. Because the intensity of the second challenge tests was almost the same as that of the first challenge (Fig. 4b), it was thought that systemic suppression induced by DMBA application may be an active tolerance, but not a transient unresponsiveness.

**Production of PGE$_2$ in DMBA-treated skin increased in XPA mice but not in wild-type mice**

PGE$_2$ is known to be not only an inflammatory mediator, but also an immunomodulator. To investigate the molecular mechanisms of enhanced DMBA-induced inflammation and immunosuppression in XPA mice, the amounts of PGE$_2$ in mouse ears were measured by enzyme immunoassay at various times after 0.5% DMBA application (Table I). In nontreated skin, approximately the same amounts of PGE$_2$ were detected in XPA (0.97 ± 0.02 ng/g tissue) and wild-type mice (2.3 ± 0.88 ng/g tissue). Although the amount of PGE$_2$ in the skin of the wild-type mice had not changed by 4 days after application of 0.5% DMBA, the amount in the skin of XPA mice had increased at 2 days after application (15.9 ± 6.1 ng/g tissue) and was still increasing on day 4 (53.9 ± 2.2 ng/g tissue).

**Treatment of indomethacin after DMBA application inhibited DMBA-induced ear swelling response in XPA mice**

To determine whether the DMBA-induced edema was mediated by PGE$_2$, the effect of indomethacin (a potent inhibitor of PG biosynthesis) on the DMBA-induced ear swelling response was examined. Twenty microliters of 1% indomethacin was applied to the ears of XPA mice immediately after the application of 0.1% DMBA, and the thickness of the ears was measured (Fig. 5). A slight ear swelling response developed 1 day after application in the mice that only received DMBA (3.67 ± 0.47 × 10$^{-3}$ cm), but there was no observable swelling in the mice that also received indomethacin after DMBA application (0.58 ± 0.76 × 10$^{-3}$ cm;}

![FIGURE 3.](http://www.jimmunol.org/)

Enhanced local immunosuppression was induced by DMBA application in XPA mice. Groups of mice received applications of 60 μl of DMBA at concentrations of 0.1 and 0.5%. Sensitization was attempted 4 days after DMBA treatment by applying 25 μl of 1% DNFB to the site that had received DMBA treatment. Six days after sensitization the mice were challenged at the left ears epicutaneously with 20 μl of 0.2% DNFB. To determine whether tolerance was induced, all mice were sensitized again by applying 25 μl of 1% DNFB to the nontreated skin immediately after the first challenge. The second challenge test was performed on the right ear 6 days after the second sensitization. Data are expressed as the mean intensity of ear swelling (± SD) of 5–10 mice/group 24 h after the first challenge test (a) and after the second challenge tests (b). a, Sensitization with DNFB through 0.1 and 0.5% DMBA-treated skin was significantly suppressed in XPA mice. b, In these mice, the induction of tolerance was confirmed by the second challenge test. *$p < 0.001$ compared with the positive control group (animals sensitized without DMBA application). Statistically significant differences in the intensity of suppression were present between XPA and wild-type mice that had received the same concentration of DMBA treatment ($p < 0.0001$ at 0.1% DMBA, $p < 0.005$ at 0.5% DMBA).
To determine whether PGE$_2$ is involved in the DMBA-induced local immunosuppression in XPA mice, treatment with indomethacin after DMBA application inhibited swelling by 40–70% at all of the time points studied. After application, indomethacin inhibited DMBA-induced ear swelling, reaching maximum swelling 3–4 days after treatment. In these mice, the induction of tolerance was confirmed by the second challenge test. $p < 0.0005$. Although the ear swelling in both groups of mice increased after treatment, reaching maximum swelling 3–4 days after application, indomethacin inhibited DMBA-induced ear swelling by 40–70% at all of the time points studied.

Treatement with indomethacin after DMBA application inhibited DMBA-induced local immunosuppression in XPA mice

To determine whether PGE$_2$ is involved in the DMBA-induced immunosuppression, we studied the effect of indomethacin on DMBA-induced local immunosuppression. Immediately after the application of 40 $\mu$l of 0.1 or 0.05% DMBA, 40 $\mu$l of 1% indomethacin was applied to the DMBA-treated area, and sensitization with DNFB was performed on the DMBA- and indomethacin-treated skin 4 days later. Epicutaneous application of indomethacin on DMBA-treated skin abrogated the suppressive effect of DMBA in both XPA and wild-type mice (Fig. 6). In XPA mice, sensitization with DNFB on skin that had been exposed to 0.1 or 0.05% DMBA resulted in a significantly decreased CHS response (98 and 75.3% suppression, respectively). However, application of 1% indomethacin immediately after exposure to 0.1 or 0.05% DMBA inhibited the degree of suppression (43.2 and 30% suppression, respectively). Similarly, the degree of suppression decreased after treatment with indomethacin in wild-type mice, although less suppression was induced using DMBA in wild-type mice than in XPA mice.

DMBA-induced IL-10 and TNF-\(\alpha\) production in the epidermis was enhanced in XPA mice

IL-10 and TNF-\(\alpha\) have been implicated as participants in the immunosuppressive cascade. The ability of DMBA to induce IL-10 and TNF-\(\alpha\) production in mouse skin in vivo was analyzed by mAb and immunoperoxidase staining of skin sections. Constitutive expression of IL-10 was not detected in the skin of XPA (Fig. 7A) and wild-type mice (data not shown). IL-10 could be detected in the epidermis 2 days after 0.1% DMBA application in XPA mice (Fig. 7B), but not in wild-type mice (Fig. 7C). There was no detectable staining using isotype control rat IgG1 as a negative control in epidermis of XPA mice treated with 0.1% DMBA (Fig. 7D). Similarly, application of 0.1% DMBA induced detectable TNF-\(\alpha\)-a production in the epidermis of XPA mice, but not in that of wild-type mice (data not shown).

To confirm that DMBA application induces IL-10 production more easily in XPA mice than in wild-type mice, the effect of DMBA application on IL-10 gene expression in epidermis was examined. We studied mRNA expression for IL-10 at various time points after DMBA application in both types of mice. There was no demonstrable mRNA expression for IL-10 in nontreated skin of XPA mice. Although IL-10 could be detected one day after 1% DMBA application, mRNA expression for IL-10 was not detected in the skin of XPA (Fig. 7A) and wild-type mice (data not shown). There was no statistically significant difference in the intensity of suppression between wild-type mice and XPA mice that had received the same concentration of DMBA treatment ($p < 0.0005$ at 0.05% DMBA, $p < 0.01$ at 0.1% DMBA).

### Table I. Amount of PGE$_2$ in skin treated with 0.5% DMBA

<table>
<thead>
<tr>
<th>Day</th>
<th>Wild-type mice (ng/g tissue)</th>
<th>XPA mice (ng/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.3 ± 0.88</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>1</td>
<td>1.0 ± 0.72</td>
<td>0.80$^b$</td>
</tr>
<tr>
<td>2</td>
<td>0.16$^b$</td>
<td>15.9 ± 6.1</td>
</tr>
<tr>
<td>3</td>
<td>2.9 ± 2.7</td>
<td>40.4 ± 7.3</td>
</tr>
<tr>
<td>4</td>
<td>0.45 ± 0.25</td>
<td>53.9 ± 2.2</td>
</tr>
</tbody>
</table>

$^a$ Experimental details are described in Materials and Methods. Data are expressed as mean ± SD for two samples. One sample consists of four ears that received treatment.

$^b$ Data was obtained from one sample.
XPA and wild-type mice (Fig. 8A, lane 1, and Fig. 8B, lane 1, respectively). In XPA mice, IL-10 mRNA expression was observed at 9 h after 0.1% DMBA application (Fig. 8A, lane 3), peaked at 12 h (Fig. 8A, lane 5), and disappeared at 15 h (Fig. 8A, lane 7). Furthermore, strong expression of IL-10 mRNA was induced in XPA mouse skin treated with 0.5% DMBA at 12 h (Fig. 8A, lane 4), the signal was decreased at 15 h (Fig. 8A, lane 6), and disappeared at 24 h (Fig. 8A, lane 8). In contrast, IL-10 gene expression was undetectable in wild-type mice treated with 0.1 and 0.5% DMBA throughout the observation period (Fig. 8B).

Next, to confirm that DMBA application induces TNF-α production more easily in XPA mice than wild-type mice, production of TNF-α at protein level in the DMBA-treated skin of both types of mice. There was no detection of TNF-α protein in nontreated skin of XPA and wild-type mice. In the skin of XPA mice, TNF-α was detected at 15 h after DMBA application in XPA mice. The levels of mRNA for IL-10 and G3PDH on the ears of DMBA-treated XPA mice (A) and wild-type mice (B) determined by RT-PCR. RT-PCR was performed as described in Materials and Methods. PCR products were separated on 1% agarose gels and stained with ethidium bromide.
production at the protein level was detected 24 h after the application of 0.1% DMBA and increased at 48 h (Table II). The level of TNF-α production was enhanced by 0.5% DMBA application in XPA mice. However, production of TNF-α protein was undetectable in wild-type mice treated with 0.1 and 0.5% DMBA throughout the observation period.

**IL-10 release in serum was detectable only in DMBA-treated XPA mice**

IL-10 has been reported to play a role in the induction of systemic immunosuppression by alteration of the Ag-presenting property of APC in draining lymph nodes. The release of IL-10 in peripheral blood was examined after DMBA application. IL-10 was not detected in serum constitutively in either XPA or wild-type mice. However, production of TNF-α protein was undetectable in wild-type mice treated with 0.1 and 0.5% DMBA throughout the observation period.

**Table II. Amount of TNF-α in skin treated with 0.1% and 0.5% DMBA**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0.1% DMBA</th>
<th>0.5% DMBA</th>
<th>0.1% DMBA</th>
<th>0.5% DMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>18</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>27</td>
</tr>
<tr>
<td>24</td>
<td>Not detected</td>
<td>Not detected</td>
<td>27.5</td>
<td>54</td>
</tr>
<tr>
<td>48</td>
<td>Not detected</td>
<td>Not detected</td>
<td>56</td>
<td>75</td>
</tr>
</tbody>
</table>

* Experimental details are described in Materials and Methods. Data were obtained from one sample (four ears).

**Discussion**

Immunological factors play an extremely important role in controlling the growth and metastasis of tumors (28). As well as UVB radiation, certain chemical carcinogens, including polyaromatic hydrocarbons, have been shown to be immunosuppressive. Although much is known about how UVB radiation modulates immunologic responses, relatively little is known about interactions between the immune system and the carcinogenesis pathway caused by chemical carcinogens. Moreover, a complete picture of the molecular steps leading to UV-induced immune suppression is not yet available. Kripke and colleagues proposed UVB-induced DNA damage as a primary molecular target for initiation of immune suppression. We previously demonstrated that UVB-induced local and systemic immunosuppression was enhanced in XPA mice (5). Our results supported the hypothesis proposed by Kripke and colleagues, because XPA mice are different from wild-type mice only in a defect in the repair of UVB-induced DNA lesion. However, it is unclear whether cutaneous DNA damage caused by other than UVB radiation might also induce immunosuppression. DMBA, one of the prototype carcinogenic polyaromatic hydrocarbons, is a chemical carcinogen and causes DNA adduct, and XPA protein is needed for its repair. We have reported that XPA mice showed a high incidence of skin tumors initiated by topical application of DMBA as well as by UVB radiation (4). Based on this background, we investigated the effects of DMBA on inflammation and the immune system in XPA mice.

A single cutaneous application of 0.1 or 0.5% DMBA induced significant ear swelling in XPA mice, but not in wild-type mice. In contrast, the intensities of ear swelling responses after treatment with croton oil or phenol were almost the same in both types of mouse. Croton oil and phenol do not form DNA adduct, or XPA protein is not required to repair DNA damage, if any, caused by these chemicals. Thus, a defect in the repair of DMBA-DNA adduct in XPA mice might be involved in the enhancement of DMBA-induced inflammation. In XPA mice, LCs are impaired at a lower DMBA concentration than in wild-type mice. Moreover, DMBA-induced local immunosuppression was enhanced in XPA mice, but not in wild-type mice. In XPA mice, enhanced reduction of LC number after application of DMBA might be involved in the enhancement of DMBA-induced local immunosuppression.

**Table III. Amount of IL-10 in serum from mice treated with 1% DMBA**

<table>
<thead>
<tr>
<th>Day</th>
<th>Wild-type mice</th>
<th>XPA mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>2</td>
<td>Not detected</td>
<td>55.2 ± 17.5</td>
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* Experimental details are described in Materials and Methods. Data are expressed as mean ± SD for five samples.
responses when adoptively transferred to naive syngeneic recipients (17, 18). However, the mechanisms by which DMBA induces suppressor T cells have not been clarified. We assumed that, similar to UVB radiation, DMBA may exert its immunosuppressive effects by altering APC function in skin. In our experiments although the number of epidermal ADPase + LCs was significantly decreased in both XPA and wild-type mice 4 days after a 0.5% DMBA application (Fig. 2), local immunosuppression was greatly enhanced in XPA mice when DNB sensitization was performed 4 days after 0.5% DMBA application (Fig. 3). This result might indicate that LC depletion alone cannot explain DMBA-induced inhibition of the CHS. It is possible that mechanisms similar to UVB-induced immunosuppression might be involved in DMBA-induced local immunosuppression. Exposure to DMBA was demonstrated to suppress the IL-2 production of Th cells in vivo (29) and in vitro (30). These observations suggest the dysfunction of Th1 cells in establishing DMBA-induced immunosuppression. It has been demonstrated that UV-treated LCs are unable to stimulate Th1 cells, whereas their ability to stimulate Th2 cells is unaffected. UVB radiation induces the production and secretion of a variety of immunomodulatory factors in the skin. Among them, PGE2 is known to be an inflammatory mediator as well as an immunosuppressive factor. We previously reported that the amount of PGE2 in skin significantly increased after UVB irradiation in XPA mice, which might account for the enhanced inflammation and local immunosuppression induced by UVB radiation in XPA mice (13). In the present study, we demonstrated that the application of DMBA also induced the production of a pronounced amount of PGE2 in the skin of XPA mice. Moreover, in XPA mice, the DMBA-induced ear swelling response and local immunosuppression were partially inhibited by treatment with indomethacin, a potent inhibitor of PG biosynthesis. Therefore, enhanced production of PGE2 after DMBA application, similar to that after UVB radiation, might cause enhancement of inflammation and local immunosuppression in XPA mice. Unrepaired DMBA-induced DNA lesions might cause an excess production of PGE2 in XPA mice. In addition to PGE2, IL-10 and TNF-α production in the epidermis were detected by 0.1% DMBA application in XPA mice, but not in wild-type mice. Previously, Nishigori et al. provided direct evidence that UVB-induced DNA damage initiates the production of IL-10 in keratinocytes (31). They showed that treatment with liposomal T4 endonuclease V, an excision repair enzyme specific for CPD in DNA, reduced UVB-induced production of IL-10 in mice in vivo and in vitro. Similarly, it is possible that unrepaired DMBA-DNA adduct in XPA mice stimulates keratinocytes to produce IL-10 and TNF-α, thereby modifying critical steps in the immunological pathway and ultimately leading to the suppression of the immune response and generation of Ag-specific suppressor T cells.

In this study, DMBA-induced systemic immunosuppression was enhanced in XPA mice. These mice became tolerant against DNB, because they could not be re sensitized against the same hapten. Therefore, DMBA-induced systemic immunosuppression might be mediated by the development of Ag-specific suppressor T cells, similar to DMBA-induced local immunosuppression. A key element in UVB-induced systemic immunosuppression is the production of epidermal cytokines, PGs, and cis-urocanic acid, which mediate the transfer of signals from UV-irradiated skin to lymphoid cells. Shreedhar et al. suggested that the cytokine cascade, including PGE2, IL-4, and IL-10, is responsible for UV-induced systemic immunosuppression (12), because PGE2 can induce the secretion of IL-10 from PBMC (32) or from keratinocytes via IL-4 (12). We demonstrated that DMBA treatment induced a detectable release of serum IL-10 in XPA mice. Thus, it is possible that PGE2 derived from DMBA-treated skin induces the secretion of IL-10 from keratinocytes and/or PBMC. Consequently, the APC function in the lymph nodes is altered by PGE2 and/or IL-10 to develop systemic immunosuppression. Although systemic suppression was induced by application of 0.05 and 0.1% DMBA in XPA mice, there was detectable serum level of IL-10 by ELISA only in 1% DMBA-treated XPA mice. There was also detectable serum IL-10 in two of five XPA mice treated with 0.1% DMBA, but not in 0.05% DMBA-treated XPA mice. These findings might be due to the limitation of the detectability of this assay. Similarly, elevation of serum IL-10 was detectable only after high dose UVB radiation (33). Therefore, even an undetectable amount of IL-10 in serum seems to play a crucial role in the induction of systemic immunosuppression.

The results presented here suggest that DMBA-DNA adduct formation is a primary molecular event for the initiation of immunosuppression induced by DMBA, because XPA mice, having a defect in the repair of DMBA-DNA adduct, showed enhanced local and systemic immunosuppression in response to DMBA. Regarding UVB-induced immunosuppression, DNA damage caused by UVB has been demonstrated to be one of the primary molecular mechanisms for initiation of immunosuppression. Kripke and colleagues reported that reducing the number of CPDs in UV-irradiated skin abrogated the systemic suppression of CHS and delayed-type hypersensitivity (34, 35). The action spectra for UV-induced suppression of the mixed lymphocyte and mixed epidermal cell-lymphocyte reactions were shown to closely resemble those for the induction of CPD and 6-4 photoproducts (36). Action spectra for local and systemic suppression of CHS are also consistent with DNA damage as initiators of these effects (37, 38). Our previous study, in which XPA mice showed increased local and systemic immunosuppression induced by UVB radiation, supported this hypothesis (5). Moreover, Kripke and colleagues indicated that DNA damage other than CPD also causes immune suppression. They reported that the liposomal HinIII restriction enzyme, which causes double-strand breaks at the 5′-AAGCTT-3′ base sequence in DNA, induced local and systemic immunosuppression (39, 40). However, treatment of HindIII failed to induce suppressor T cells, suggesting that the mechanisms inducing immunosuppression were different for UVB and HindIII treatment. Our results support the possibility that DNA damage by other than UVB can also induce immune suppression and trigger the production of immunomodulatory cytokines. Recently, Yarosh et al. reported that UV-induced DNA damage associated with an increase in and activation of FKB12-rapamycin-associated protein kinase, resulted in gene expression of TNF-α (41). Moreover, it was demonstrated that TNF-α induced cyclooxygenase-2 expression, leading to the production of PGE2 in human lung epithelial cells (42). In our model it is speculated that DMBA-induced DNA damage might lead to the production of TNF-α mediated by DNA protein kinase, resulting in the expression of cyclooxygenase-2 and production of PGE2. Further studies are necessary to elucidate the precise molecular mechanisms by which DNA damage in cells induces production of immunomodulately cytokines and PGE2.

Clinically, XP patients show increased rates of neoplasms in organs that are not exposed to UV radiation. Kraemer and colleagues reported that XP patients had a 10- to 20-fold increase in the occurrence of neoplasms in sites not exposed to UV radiation compared with the general population (43). We have previously shown that NK function is easily suppressed by UVB radiation in XPA mice, suggesting that impairment of NK function by UVB radiation might contribute to an increased internal malignancy in
XP patients (44). Exposure to environmental carcinogens, endogenous mutagens, and/or oxygen-free radicals (45) may be important for the development of these internal neoplasms in XP patients.

XP cells are hypersensitive to mutagenic tryptophan pyrolys products in charbroiled meat and fish (46, 47) and are also hypersensitive to killing and are hypermutable to DNA-damaging chemical carcinogens, such as benzo[a]pyrene, found in cigarette smoke (48, 49). Moreover, oral treatment of XPA mice with benzo[a]pyrene, acetylaminofluorene, and aminomethylphenylidazopyridine resulted in lymphomas, liver and bladder tumors, and intestinal adenomas and lymphomas, respectively (50). DMB is a prototype carcinogenic polyaromatic hydrocarbon. Polyaromatic hydrocarbons are ubiquitous environmental contaminants generated as byproducts of the incomplete combustion of fossil fuels, wood, and other organic materials (51). The high incidence of internal cancers in XP patients may be due to a defect in the repair of such chemical carcinogen-damaged DNA. Moreover, the present study suggested that easy impairment of the immune surveillance system by chemical carcinogens demonstrated in XPA mice could also be involved in the high incidence of malignancy in XP patients. The role of cyclooxygenase and PGs in tumorigenesis is well established (52, 53), and cyclo-oxygenase inhibitors are effective in tumor patients in both prophylactic and therapeutic settings (52, 54). Overexpression of PGE2 induced by UBV and chemical carcinogens may also be responsible for the increased incidence of neoplasms in XP patients. If this is true, cyclooxygenase inhibitors may be beneficial for XP patients.

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References


