Lack of Expression of the EP2 but not EP3 Receptor for Prostaglandin E2 Results in Suppression of Skin Tumor Development

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Abstract

The EP2 receptor for prostaglandin E2 (PGE2) is a membrane receptor that mediates at least part of the action of PGE2. It has been shown that EP2 plays a critical role in tumorigenesis in mouse mammary gland and colon. However, the possibility that the EP2 receptor is involved in the development of skin tumors was unknown. The purpose of this study was to investigate the role of the EP2 receptor in mouse skin carcinogenesis. Unlike EP3 knockout mice, the EP2 knockout mice produced significantly fewer tumors and reduced tumor incidence compared with wild type (WT) mice in a 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) two-stage carcinogenesis protocol. EP2 knockout mice had significantly reduced cellular proliferation of mouse skin keratinocytes in vivo and in vitro compared with that in WT mice. In addition, the epidermis of EP2 knockout mice 48 hours after topical TPA treatment was significantly thinner compared with that of WT mice. The inflammatory response to TPA was reduced in EP2 knockout mice, based on a reduced number of macrophages in the dermis and a reduced level of interleukin-1α mRNA expression, compared with WT mice. EP2 knockout mice also had significantly reduced epidermal cyclic AMP levels after PGE2 treatment compared with WT mice. Tumors from WT mice produced more blood vessels and fewer apoptotic cells than those of EP2 knockout mice as determined by immunohistochemical staining. Our data suggest that the EP2 receptor plays a significant role in the protumorigenic action of PGE2 in skin tumor development.

Introduction

Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid into the intermediate PGH2, which is a rate-limiting step in prostaglandin E2 (PGE2) production. Two isoforms of COX have been identified. COX-1 is constitutively expressed in many tissues and has functions in tissue homeostasis. COX-2 is induced by mitogenic or inflammatory factors and is highly expressed in most epithelial cancer cells including skin (1). In epidermal keratinocytes, PGs produced by COX-1/2 are highly regulated and necessary for skin tumor formation and development (2). UV irradiation or application of various tumor promoters highly induce the expression of COX-2 and elevate PGE2 production in mouse skin (3, 4). Both genetic deletions of COX and nonspecific COX inhibitory nonsteroidal anti-inflammatory drugs (NSAID) or COX-2-specific inhibitors prevent skin tumor development following a 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA) protocol or UV irradiation (1, 3). PGE2 is the major prostaglandin produced in the skin (5) and is a comitogen for mechanical wounding (6) and for phorbol ester-induced hyperproliferation of epidermis (7). PGE2 binds to four receptors, designated EP1, EP2, EP3, and EP4 (8). These receptors are seven transmembrane G-protein-coupled receptors. EP1 receptor–mediated signaling increases intracellular calcium levels. EP2 and EP4 receptor–mediated signaling increases cyclic AMP (cAMP) levels via activation of adenylate cyclase, whereas EP3 receptor–mediated signaling decreases cAMP levels (8). All four PGE2 receptors were found to be present in normal human epidermis (8), and we have found that all four EP receptors are expressed in mouse epidermis. It has been difficult to determine whether the biological effects ascribed to PGE2 (i.e., proliferation, apoptosis, and angiogenesis) are EP2 dependent or not. To address this, a number of investigators used knockout mice to study the role of the EP2 receptor in several cancer tissues, such as mammary gland and colon tissues (8–10). EP2 is also implicated in the growth of intestinal polyps in vivo and plays an important role in PGE2-mediated signaling pathways in intestinal polyposis with COX-2 overexpression (10). In the APCΔT16 mice, a murine model of familial adenomatous polyposis, homozygous disruption of the EP2 receptor caused a decrease in the number and size of intestinal polyps as well as a decrease in COX-2 and vascular endothelial growth factor (VEGF) expression (10). This suggests that EP2 receptor activation contributes to induction of tumor-mediated angiogenesis, at least in this mouse model system (10). Similarly, in human pancreatic cancer cells, PGE2 synthesized by COX-2 elevates VEGF expression via the EP2 receptor (11). These studies therefore showed that the PGE2/EP2 receptor interaction may contribute to tumor development by promoting neovascularization and angiogenesis. Interestingly, EP2 and EP4 are expressed in proliferating mammary glands during pregnancy as well as in mammary tumors (12). Indomethacin, an NSAID, inhibits not only expression of EP2 and EP4 receptors but also mammary tumor development (12). This suggests that these two receptors may be involved in the induction of mammary carcinogenesis and angiogenesis (12). In human embryonic kidney cells, PGE2 induced T-cell factor signaling via the EP2 receptor–mediated cAMP-protein kinase A (PKA) pathway (13). Recently, one group showed that increased EP2 receptor expression may induce PGE2-mediated VEGF production in endometrial adenocarcinoma cells through activation of the epidermal growth factor receptor and extracellular signal-regulated kinase 1/2 signaling pathways (14).

Previous studies showed that interleukin-1α (IL-1α), an important inflammatory cytokine in skin, can be induced by PGs; in addition, IL-1α can also induce PG synthesis (15). Several groups...
showed that the PGE2 level is increased dramatically in human colonic mucosal inflammation (16) and UV-irradiated or TPA-treated skin (17–20). In inflamed human colonic mucosa, PGE2 receptor was expressed significantly in lateral crypt epithelial cells (16). However, the possibility that TPA-mediated inflammation is via the EP2 receptor was previously unknown. The possibility that the EP2 receptor is involved in the development of skin tumors was also unknown. Based on previous observations, we hypothesized that activation of the EP2 receptor by PGE2 contributes to skin tumor development. To investigate this hypothesis and the role of EP2 in skin carcinogenesis, we used an EP2 knockout mouse model. Here we report our observations on the effects of EP2 deletion on skin tumor development using a chemical DMBA/TPA protocol, as well as the effects of EP2 ablation on keratinocyte proliferation, apoptosis, angiogenesis, and inflammation in mouse skin.

Materials and Methods

Materials. Monoclonal rat anti-bromodeoxyuridine (BrdUrd) antibody (Becton Dickinson, San Jose, CA); [3H]-thymidine ([3H]thymidine, 79.20 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA); anti-CD-31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA); bicinchoninic acid (BCA) kit (Bio-Rad, Richmond, CA), cAMP enzyme immunoassay system antibody (Santa Cruz Biotechnology, Santa Cruz, CA); bicinchoninic acid (Perkin-Elmer Life Sciences, Boston, MA); anti-CD-31 antibody; PGE2 (Cayman Chemical, Ann Arbor, MI); SQ 22,536 (Sigma Chemical, St. Louis, MO), terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) kit (Calbiochem, La Jolla, CA); EP1, EP2, and EP4 antibody (Cayman Chemical); goat anti-rabbit-immunoglobulin G–conjugated horseradish peroxidase and β-actin antibody (Santa Cruz Biotechnology); chemiluminescence detection system (enhanced chemiluminescence, Perkin-Elmer Life Sciences); and anti-macrophage antibody (F4/80 Macrophage, Serotec, Oxford, United Kingdom) were used.

Animals. EP2 and EP3 knockout mice on a 129 background were kindly provided by Dr. Beverly Keller (University of North Carolina-Chapel Hill). The genotypes of EP2 and EP3 knockout mice were determined as previously described (21, 22). Wild-type (WT) mice (129 background) were used as controls. WT mice were purchased from TacGermontown, NY). All mice were maintained at Science Park and housed in an air-conditioned facility that is Association for Assessment and Accreditation of Lab Animal Care accredited.

Western blot analysis. Total protein was isolated from the epithelium of female homozygous EP2 and EP3 knockout mice and WT (129 and FVB) mice with Triton X-100 buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.5% Triton X-100]. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted onto polyvinylidene difluoride membranes. Blots were blocked for 1 hour in 5% nonfat milk in PBS containing 0.25% Tween 20. EP1 (42 kDa), EP2 (40 kDa), EP3 (53 kDa), and EP4 (52 kDa) proteins were detected with anti-EP1, anti-EP2, anti-EP3, and anti-EP4 polyclonal antibodies as recommended by the manufacturer (Cayman Chemical, Ann Arbor, MI) and diluted at 1:500; in addition, goat anti-rabbit-immunoglobulin G–conjugated horseradish peroxidase (diluted 1:2,000) was used as a secondary antibody. Immuno blotting for β-actin for each blot was carried out to confirm equal loading.

Tumor experiments. Thirty 6- to 8-week-old female homozygous EP2 and EP3 knockout mice and WT mice were initiated topically with 100 μg of DMBA in 200 μL of acetone 2 days after shaving the dorsal skin. Two weeks after initiation, the mice were topically treated with 2.5 μg TPA in 200 μL of acetone twice a week for a period of 30 weeks. Tumor incidence and tumor multiplicity were recorded weekly. Tumor multiplicity was calculated as the average number of skin tumors per mouse. Tumor incidence was calculated as the percentage of mice with skin tumors. Tumor size was measured with calipers at the end of the experiments. Tumor type was determined by histopathologic analysis of stained sections, as described below.

Bromodeoxyuridine incorporation. Four each of 8-week-old EP2 knockout mice and WT mice were shaved 2 days before treatment with TPA (2.5 μg)/200 μL acetone. BrdUrd (0.1 mg/g body weight) was injected i.p. 1 hour before sacrifice. The mice were euthanized 18 hours after a single or four (twice a week) TPA treatments and their dorsal skins were removed, fixed in formalin, and processed for paraffin embedding and immunohistochemical staining with a monoclonal rat anti-BrdUrd antibody by the Histology Core of the Science Park Research Division. BrdUrd-positive and BrdUrd-negative basal cells were counted in three to five randomly selected areas of each skin section and the mean percentage of BrdUrd-positive cells and SD for each treatment group were determined.

Cell culture. Primary skin keratinocytes from newborn EP2 knockout mice and WT mice were prepared as described by Yuspa et al. (23). Briefly, 1- to 2-day-old mouse pups were euthanized on ice and washed in 75% ethanol. The skin was stripped off and floated on 0.25% trypsin overnight at 4°C. The epidermis was separated from the dermis and chopped in Waymouth's medium containing 1.2 mmol/L calcium and 10% fetal bovine serum. The cells were filtered through a sterilized mesh and plated at 2 × 106 cells per dish for most purposes. Cells were incubated at 37°C with 5% CO2 for 2 hours in Waymouth's medium to allow them to attach to the plate. Cells were then washed with PBS and grown in keratinocyte growth medium (serum-free medium containing 0.03 mmol/L calcium, Cambrex,Walkersville, MD) at 37°C with 5% CO2 for experimental use and treated with 0.2% vehicle (methanol) or PGE2.

[3H]-thymidine incorporation assay. Primary cultures of skin keratinocytes from WT and EP2 knockout mice at about 85% to 90% confluence in six-well plates were treated in triplicate with 0 to 30 μmol/L PGE2 for 20 hours and pulsed with 1 μCi/ml [3H]-thymidine 2 hours before harvest. Cells were then washed twice with ice-cold PBS and trice with ice-cold 10% trichloroacetic acid. Cells were lysed with 0.3 N NaOH/1% SDS, and the [3H]-thymidine incorporated by cells was counted in a scintillation counter and normalized to protein concentration. Protein concentration was determined with the BCA kit.

Hyperplasia. Hyperplasia was assessed in three each of 8-week-old EP2 knockout mice and WT mice 48 hours after a single or 18 hours after four treatments with acetone or TPA (2.5 μg/200 μL acetone). The thickness of the epidermis (μm) was measured using an image system (Nikon ACT-1). The on-screen measurements of epidermis were obtained as raw data, and the actual thickness of epidermis was calculated by dividing the raw data by the magnification (× 650) in 15 fields per section.

Immunohistochemistry. At the end of the experiments, tumors were removed from the animals, fixed immediately in formalin, and embedded in paraffin. Sections were stained with H&E for light microscopy. The Science Park Histology Service did the work involved in embedding, sectioning, and staining for light microscopy. All tissues were examined histologically to determine whether the epithelium was normal, dysplastic, invasive, or had the characteristic papilloma or carcinoma patterns. Angiogenesis was assessed by staining of endothelial cells in papilloma samples of similar sizes from WT and EP2 knockout mice with an anti-CD-31 antibody. The number of CD31-positive vessels was counted in eight fields at a magnification of 40×. Apoptosis was determined by caspase-3 staining in papilloma samples of similar sizes from WT and EP2 knockout mice. The number of apoptotic-positive cells was counted in eight fields at a magnification of 40×. Inflammation was assessed by antibody staining of macrophages in three each of 8-week-old EP2 knockout mice and WT mice treated with acetone or TPA for 48 hours. The number of macrophage-positive cells was counted in 15 fields at a magnification of 40×.

Cyclic AMP analysis. cAMP measurement was carried out using a kit from Perkin-Elmer Life Sciences. Dorsal epidermis from EP2 knockout and WT mice was removed quickly 1 hour after treatment with 100 μmol/L PGE2 with as little manipulation as possible and immediately snap-frozen in liquid nitrogen, placed in ice-cold 0.05 mol/L Tris-HCl buffer (pH 7.4) containing 5 μg/mL indomethacin and stored at −70°C. In some groups of mice, the adenylate cyclase inhibitor SQ 22,536 (100 μmol/L) was topically applied 30 minutes before the application of PGE2. Calculation of cAMP concentrations (pmol/mg protein) was based on a standard curve for each experiment.

Northern blot analysis. Total RNA was extracted from whole skin of EP2 knockout and WT mice treated with 100 μmol/L PGE2 or TPA...
(2.5 μg/200 μL acetone) with Tris reagent (Medical Research Council, Cincinnati, OH) following the manufacturer's protocol. Ten micrograms of total RNA of each sample were denatured and separated on 1% agarose/6% formaldehyde gel and then transferred to nylon membranes. A [32P]dCTP-labeled cDNA probe for IL-1a was hybridized to the blots at 65°C for 2 hours. The blots were then washed twice each for 15 minutes in 0.1% SDS/2× NaCl/sodium citrate solution (where 1× is 0.15 mol/L NaCl/15 mmol/L sodium citrate) at room temperature and once for 30 minutes with 0.1% SDS/0.1× NaCl/sodium citrate solution at 60°C and exposed to X-ray film at −80°C. The blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA as a loading control.

**Statistical analysis.** Data are shown as the means ± SD. Statistical differences between means were determined using one-way ANOVA.

**Results**

**EP2 deficiency suppressed skin tumor development.** Western blot analysis was done to confirm ablations of EP2 and EP3 expression in EP2 and EP3 knockout mice (Fig. 1A). Our observation showed that EP2 and EP3 proteins were expressed in the skin of WT 129, but EP2 and EP3 expression were lacking in EP2 knockout mice (Fig. 1A). To determine whether TPA treatment alters expression levels, we used anti-EP1, anti-EP2, anti-EP3, and anti-EP4 polyclonal antibodies to examine their expression at 18 hours after treatment (Fig. 1B). We found that all four receptors were expressed in murine epidermis and that TPA up-regulates the expression only of the EP2 receptor (Fig. 1B). This was verified by real-time PCR (data not shown). To test the role of the EP receptors in mouse skin carcinogenesis, we initially used EP3 and EP2 knockout mice and WT mice in a DMBA/TPA two-stage carcinogenesis protocol. As several groups reported that the EP3 receptor is not important in several cancer tissues, including mammary gland and colon (10, 12), we hypothesized that EP3 was also not likely to be important in skin carcinogenesis. As we expected, there was no difference between EP3-null mice and WT mice in tumor multiplicity (Fig. 1C, left) or tumor incidence (Fig. 1C, right) after 30 weeks of TPA promotion. The tumor size distribution of EP3 knockout mice was almost the same as that of WT mice (data not shown) at week 30. Unlike EP3 knockout mice, the EP2 knockout mice had significantly fewer tumors (Fig. 2A) and

![Figure 1.](image-url) **Figure 1.** The EP3 receptor was not involved in DMBA/TPA-induced skin carcinogenesis. A two-stage DMBA/TPA skin carcinogenesis experiment was done with WT and EP3 knockout mice (EP3−/−) for 30 weeks. Thirty female mice were used for each group. Tumor numbers included both benign papillomas and malignant squamous cell carcinomas in the WT and EP3 knockout mice. A, ablations of the EP2 and EP3 expression in the epidermis of EP2 and EP3 knockout mice were verified by Western blot analysis. B, TPA up-regulated only the expression of the EP2 receptor. Western blot of epidermal proteins from WT FVB (WT) treated with acetone or TPA for 18 hours visualized with antibodies against EP1, EP2, EP3, EP4, and β-actin. C, left, tumor multiplicity (the average number of tumors per mouse); right, tumor incidence (the percentage of mice with tumors).

![Figure 2.](image-url) **Figure 2.** The EP2 knockout mice had significantly fewer tumors and reduced tumor incidence compared to WT mice. A two-stage DMBA/TPA skin carcinogenesis experiment was done with WT and EP2 knockout mice (EP2−/−) for 30 weeks. Tumor numbers for the WT mice included benign papillomas and malignant squamous cell carcinomas; the EP2 knockout mice had no carcinomas. A, tumor multiplicity (the average number of tumors per mouse). B, tumor incidence (the percentage of mice with tumors).
reduced tumor incidence (Fig. 2B) compared with WT mice. At 30 weeks, the mean number of tumors per mouse was 5.9 for WT and 2.9 for EP2 knockout mice. Tumor size distribution for the EP2 knockout mice was similar to that of WT mice in the small (≤ 2 mm) and medium (2 to < 5 mm) size range; however, the EP2 knockout mice produced fewer large size (approximately = 5 mm) tumors than WT mice (data not shown). Additionally, no carcinomas were produced in the EP2 knockout mice, whereas six carcinomas were observed in the WT mice. These data suggest that the EP2 receptor may play an important role in chemically induced skin carcinogenesis.

**EP2 deficiency resulted in decreased cell proliferation and hyperplasia.** PGE2 has been reported to regulate cell proliferation, apoptosis, and the expression of the Bcl-2, matrix metalloproteinase, and VEGF genes in various cell types including skin (8). Therefore, we examined whether a proliferative effect of PGE2 could be mediated by the EP2 receptor. To study the role of the EP2 receptor in cellular proliferation of mouse skin keratinocytes *in vivo* and *in vitro*, we did BrdUrd and [3H]-thymidine incorporation assays using EP2 knockout mice and WT mice. Results showed that BrdUrd incorporation 18 hours after a single TPA treatment was significantly reduced in the skins of EP2 knockout mice compared with that in WT mice (Fig. 3A: 48.8% for EP2−/− versus 64.6% for WT; *P* < 0.001). We next examined whether the EP2 receptor could affect epidermal hyperplasia 48 hours after TPA treatment. Our data showed that epidermal thickness of EP2 knockout mice was significantly reduced compared with that of WT mice 48 hours after TPA treatment (21.2 μm for EP2−/− versus 30.3 μm for WT; *P* < 0.01; Fig. 3B and C).

We also investigated whether multiple treatments with TPA could produce similar changes in cell proliferation and epidermal thickness between WT and EP2 knockout mice. As shown in Fig. 4A and B, EP2 knockout mice showed significantly reduced cell proliferation (47.7% for EP2−/− versus 69.8% for WT; *P* < 0.001) and epidermal thickness (39.1 μm for EP2−/− versus 58.1 μm for WT; *P* < 0.01) compared with those of WT mice 18 hours after the last of four treatments with TPA. Taken together, the EP2 receptor may play an important role in mediating TPA-induced cell proliferation and hyperplasia in mouse skin.

Cultures of primary skin keratinocytes from EP2 knockout mice also showed a significantly decreased ability to incorporate [3H]-thymidine 20 hours after PGE2 treatment compared with WT keratinocytes (Fig. 4C). These data suggest that PGE2 directly mediates a proliferative response in keratinocytes; that is, PGE2-induced mediators from the dermis are not necessary.

**EP2 deficiency affects 12-O-tetradecanoylphorbol-13-acetate–induced inflammation.** To determine whether TPA-induced inflammation was also mediated by the EP2 receptor, we immunostained the skins of EP2 knockout mice and WT mice 48 hours after acetone or TPA treatment with an antibody against macrophages. There was no difference between EP2 knockout mice and WT mice with acetone treatment (Fig. 5A). However, the number of macrophages was significantly, although not completely, reduced after TPA treatment in the skins of EP2 knockout mice compared with that in WT mice. (53.1 ± 1.6 for WT versus 32.8 ± 3.0 for EP2−/−; *P* < 0.001; Fig. 5A). This suggests that the EP2 ablation reduces TPA-induced inflammation in mouse skin.

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**Figure 3.** The EP2 receptor deficiency was associated with decreased cell proliferation *in vivo* and resulted in decreased epidermal hyperplasia. A, BrdUrd incorporation into basal epidermal keratinocytes was determined in four WT and four EP2 knockout mice treated with acetone or 2.5 μg TPA for 18 hours. Skin sections were immunostained with antibody against BrdUrd and [3H]-thymidine incorporation assays using EP2 knockout mice and WT mice. Results showed that BrdUrd incorporation 18 hours after a single TPA treatment was significantly reduced in the skins of EP2 knockout mice compared with that in WT mice (Fig. 3A: 48.8% for EP2−/− versus 64.6% for WT; *P* < 0.001). We next examined whether the EP2 receptor could affect epidermal hyperplasia 48 hours after TPA treatment. Our data showed that epidermal thickness of EP2 knockout mice was significantly reduced compared with that of WT mice 48 hours after TPA treatment (21.2 μm for EP2−/− versus 30.3 μm for WT; *P* < 0.01; Fig. 3B and C).

**Figure 4.** A, incorporation of [3H]-thymidine into basal epidermal keratinocytes (Fig. 4A) and cell proliferation (47.7% for EP2−/− versus 69.8% for WT; *P* < 0.001) compared with those of WT mice 18 hours after the last of four treatments with TPA. Taken together, the EP2 receptor may play an important role in mediating TPA-induced cell proliferation and hyperplasia in mouse skin. B, differences in epidermal thickness between WT and EP2 knockout mice. The thickness of the epidermis (μm) was calculated as described in the methods section. Columns, means; bars, ±SD. *P* < 0.01 versus WT. C, H&E-stained sections from WT and EP2 knockout. a, acetone-treated skin of WT; b, 48-hour TPA-treated skin of WT; c, acetone-treated skin of EP2 knockout; d, 48-hour TPA-treated skin of EP2 knockout.
Because IL-1α is an important inflammatory cytokine induced by PGs in skin (15), we examined IL-1α expression in TPA-treated EP2 knockout and WT mice. As shown in Fig. 5B, the induction of IL-1α mRNA was significantly reduced in EP2 knockout mice compared with WT mice. Cotreatment with PGE2 and TPA enhanced IL-1α expression above that seen with TPA alone in WT mice; this enhancement by PGE2 was not observed in EP2 knockout mice. Unexpectedly, PGE2 alone was not sufficient to induce IL-1α mRNA expression, even at 100 μmol/L, in vivo. Collectively, these data suggest that part of the mechanism by which TPA elicits inflammation is via EP2 activation by PGE2. The reduced inflammation in EP2 knockout mice may also explain the observed differences in tumor development between WT and EP2 knockout mice.

EP2 deficiency reduced cyclic AMP levels following prostaglandin E2 treatment in vivo. EP2 receptor–dependent signaling leads to an increase in cAMP levels (8). cAMP is an important second messenger in the EP2-mediated signaling pathway, because cAMP activates PKA that stimulates phosphorylation of cAMP response element binding protein (CREB; ref. 24). Thus, we hypothesized that cAMP levels would be reduced in the epidermis from EP2 knockout mice compared with WT mice following 100 μmol/L PGE2 treatment. PGE2 treatment resulted in a more than a 2-fold induction of cAMP in WT mice (Fig. 6A). However, PGE2 failed to induce cAMP production in the epidermis of EP2 knockout mice. As expected, PGE2-induced cAMP production was blocked in the epidermis from WT mice treated with 100 μmol/L SQ 22,536, an adenylate cyclase inhibitor. This suggests that activation of the EP2 receptor can induce cAMP production and that cAMP-dependent PKA signaling may affect expression of genes related to cell proliferation, apoptosis, and angiogenesis in murine skin cancer.

EP2 deficiency affects apoptosis and angiogenesis. PGE2 is a possible inducer of angiogenesis and induces the expression of proangiogenic genes such as VEGF (11, 12, 14). Several groups...
have shown that PGE2 increased VEGF expression via a cAMP-dependent mechanism (25). CREB regulates cell cycle–related genes such as cyclin D1 (26) and cyclin A (27), proangiogenic genes such as VEGF, and antiapoptotic factors such as bcl-2 via a CRE element on their gene promoters (24), and it has been shown that CREB plays an important role in inducing proliferation and angiogenesis (24–31). Thus, we hypothesized that PGE2 elevates cAMP levels, which in turn induces VEGF, in murine keratinocytes and possibly other cell types in the skin. PGE2 also plays an important role as an antiapoptotic mediator in several tissues (32–35). Here, we have shown that the antiapoptotic effects of PGE2 are mediated at least in part by the EP2 receptor. To determine whether the extent of tumor angiogenesis or apoptosis correlates with the reduced tumor numbers in the EP2 knockout mice, we immunostained papilloma samples of similar sizes from EP2 knockout and WT mice with an anti-CD31 antibody, a marker of endothelial cells, and by TUNEL staining for apoptosis. Significant differences in blood vessel number were observed in tumors from WT and EP2 knockout mice (75.6 ± 5.5 for WT versus 37.6 ± 6.3 for EP2−/−, P < 0.001; Fig. 6B). For apoptotic cells, there was also a significant difference, with an average of 44.4 ± 9.3 and 73 ± 7.4 (P < 0.001) observed in tumors from WT and EP2 knockout mice, respectively (Fig. 6C). These data suggest that alterations in angiogenesis and apoptosis could also explain the observed differences in tumor growth between WT and EP2 knockout mice.

Discussion

In several studies, a role for PGs in keratinocyte proliferation has been shown (5). PGE2 was regarded as a comitogen for phorbol ester–induced hyperproliferation (7) or mechanical wounding of epidermis (6). In cultured human keratinocytes, the NSAID indomethacin inhibited cell proliferation, which was countered by adding back PGE2 (9). Our current results are in agreement with this previous study in that EP2 knockout mice showed a reduction in cell proliferation compared with WT mice after a single TPA treatment in vivo. In vitro, primary keratinocytes cultured from EP2 knockout mice also showed a reduced proliferation response to PGE2. Additionally, epidermal thickness of EP2 knockout mice was significantly reduced compared with that of WT mice after TPA treatment. Taken together, these results strongly support a model in which tumor promoter-induced cell proliferation and hyperplasia are at least partially mediated by the production of PGE2 and its subsequent activation of the EP2 receptor in keratinocytes.

There are a number of studies associating PGE2 with angiogenesis in many tissues (28, 29), as well as studies showing a reduction in angiogenesis by COX inhibitors (31). Previous reports also showed that the EP2 receptor is involved in angiogenesis in several cancer tissues including colon and mammary gland (10, 12). It has been reported that among the EP receptors, PGE2-mediated signaling is most likely transduced through the EP2 receptor. Additionally, one group showed that PGE2 increased VEGF expression via a cAMP-mediated mechanism (27). In support of this, we have shown that there is a reduced cAMP response to PGE2 application in the epidermis of EP2 knockout mice and that tumors from the EP2 knockout mice have decreased vascularization. Thus, our data are in agreement with a model in which PGE2 elevates cAMP levels through EP2 activation, which in turn induces angiogenesis.

PGE2 has also been shown to play an antiapoptotic role in several tissues. In chicken embryo fibroblasts, PGs were also shown to inhibit apoptosis (33). Other studies have suggested that PGE2 can induce expression of apoptosis inhibitors such as bcl-2 and cellular inhibitor of apoptosis protein 2 (32–35). Here, we have shown that the antiapoptotic effects of PGE2 are mediated at least in part by the EP2 receptor because tumors from EP2 knockout mice showed a significantly increased number of apoptotic cells compared with WT tumors. These results support our conclusion that deletion of EP2 causes a significant decrease in skin tumor development due to a reduction in cell proliferation, a reduction in angiogenesis, and increased apoptosis.

Topical TPA has been well shown to induce inflammation in skin (36) and inhibition of inflammation inhibits skin tumor development (36). All categories of anti-inflammatory agents prevent/inhibit skin tumor development including agents targeted against the edema caused by histamine (36), COX inhibitors (37, 38), and reactive oxygen species (39). Finally, the ability of chronic inflammation to promote skin carcinogenesis was definitively shown in a recent genetic model (40). COX inhibitory NSAIDs in particular contributed to both reduce TPA-induced progression and TPA-mediated inflammation (1). Our results show that
TPA-induced inflammation in skin is mediated in part via the EP2 receptor. It suggests the possibility that PGE2 may play an important role as a comitogen for phorbol ester–induced hyper-proliferation of epidermis as well as a coinducer for TPA-induced inflammation in skin.

A noteworthy finding in our studies is that whereas the EP2 receptor seems to play a major role in skin tumorigenesis, the EP3 receptor does not. There are several possible explanations for why EP3 has no effect on skin tumor development. First, in contrast to EP2/EP4 receptors, EP3 receptor–mediated signaling decreases cAMP levels via inhibition of adenylyl cyclase (22) and thus likely EP3 has no effect in skin tumor development. First, in contrast to that of COX-2-deficient APC716 mice, which have flat and regressive polyps (10).

Recent studies showed that the EP1- and EP4-selective antagonists, ONO-8711 and ONO-AE2-227, respectively, reduced the number of intestinal polyps in the Min mouse model (41, 42). Combination treatment of these two antagonists also contributed to the decrease in the number of intestinal polyps in the APC gene knockout mice (43). The EP1 receptor deficiency was associated with inhibition of azoxymethane-induced colon cancer development using EP1 knockout mice (44). Recently, one group showed that cell growth rate of malignant keratinocytes is mediated by the EP1 receptor (45). The potential role of EP1 and EP4 receptors in skin carcinogenesis also needs to be assessed. We can not rule out the possibility that the EP4 receptor may contribute to tumor development, because activation of EP4 should also elevate CAMP (8). Thus, further studies are needed to investigate whether EP1 and EP4 receptors play an important role in the induction of skin tumor development. The EP1 and EP4 receptors may mediate at least part of the PGE2 pro-tumorigenic action in mouse skin, which may explain why deletion of the EP2 receptor did not have more significant effects on DMBA/TPA-induced tumor incidence or tumor multiplicity in this study.

In summary, we have shown that deletion of expression of the EP2, but not the EP3 receptor, for PGE2 results in suppression of skin tumor development and is associated with decreased proliferation, angiogenesis, inflammation, and cell survival. Further studies are needed to elucidate the underlying molecular mechanisms.

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