

Importance of the EP₁ Receptor in Cutaneous UVB-Induced Inflammation and Tumor Development

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Chronic exposure to UV light, the primary cause of skin cancer, results in the induction of high levels of cyclooxygenase-2 (COX-2) expression in the skin. The involvement of COX-2 in the carcinogenesis process is mediated by its enzymatic product, prostaglandin E₂ (PGE₂). PGE₂ has been shown to have a variety of activities that can contribute to tumor development and growth. The effects of PGE₂ on different cell types are mediated by four E prostanoid (EP) receptors, EP₁–EP₄. While recent studies have demonstrated the importance of EP₁ in the development of colon and breast cancer, the extent of EP₁ involvement in the cutaneous photocarcinogenesis process is unknown. This study found that topical treatment with celecoxib or the specific EP₁ antagonist ONO-8713 decreased acute UVB-induced inflammation in the skin and significantly reduced the number of tumors per mouse following 25 weeks of UVB exposure and topical treatment. This study suggests that drugs designed to block EP₁ may have the potential to be used as anti-inflammatory and/or chemopreventive agents that reduce the risk of skin cancer development.

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INTRODUCTION

An abundance of evidence now exists demonstrating a role for cyclooxygenase-2 (COX-2) in the development of many types of cancer. One of the products of COX-2, prostaglandin E₂ (PGE₂), has been shown to be a critical player mediating the contribution of the COX-2 pathway to cancer development. PGE₂ plays a key role in normal skin homeostasis, but it can also act as a tumor promoter, controlling many of the behaviors typical of cancer cells (Lupulescu, 1978a, b). PGE₂ can stimulate increased proliferation, altered adherence, increased migration, and enhanced invasiveness of cancer cells (Vanderveen *et al.*, 1986; Tsujii and DuBois, 1995; Buchanan *et al.*, 2003; Kawamori *et al.*, 2003). A number of studies have demonstrated overexpression of COX-2 in chronically UVB-irradiated skin, as well as in UVB-induced premalignant lesions and squamous cell carcinomas (SCC) (Buckman *et al.*, 1998; Athar *et al.*, 2001; An *et al.*, 2002). A role for COX-2 in photocarcinogenesis is also supported by several studies, demonstrating that inhibition of COX-2 activity, by either general nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit both COX-1 and COX-2 or

specific COX-2 inhibitors, can partially block carcinogenesis induced by long-term UVB exposure (Fischer *et al.*, 1999; Pentland *et al.*, 1999; Orengo *et al.*, 2002; Wilgus *et al.*, 2003). While the roles of COX-2 and PGE₂ in UVB-induced skin cancer development have been well documented, the roles of the receptors that bind PGE₂ during this process have not been well characterized.

The effects of PGE₂ on different cell types depend on the E prostanoid (EP) receptor repertoire of the cell. Four distinct subtypes of EP receptors, designated EP₁–EP₄, have been cloned and sequenced (Negishi *et al.*, 1993; Narumiya *et al.*, 1999). These receptors, which were classified pharmacologically, are G-protein-coupled receptors that operate via different signaling pathways. EP₁ functions by increasing phospholipase C- β and is coupled to intracellular calcium. The remaining receptors signal by regulating adenylate cyclase activity. EP₃ decreases adenylate cyclase activity, while EP₂ and EP₄ increase its activity (Negishi *et al.*, 1995). Studies using EP₁-knockout mice as well as selective EP₁ antagonists have implicated this receptor in the development of colon and breast carcinogenesis (Watanabe *et al.*, 1999, 2000; Kawamori *et al.*, 2001, 2005). In addition, a recent report showed increased EP₁ levels in murine skin tumor cells and demonstrated that this receptor is critical for the mitogenic effects of PGE₂ on these cells *in vitro* (Thompson *et al.*, 2001), a finding that has also been reported in NIH-3T3 cells (Watanabe *et al.*, 1996). The development of selective EP₁ antagonists such as ONO-8711 and the more selective ONO-8713 has greatly enhanced our ability to study the relative contribution of this receptor to inflammatory and carcinogenic processes in the skin (Watanabe *et al.*, 2000; Kawamori *et al.*, 2001).

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Abbreviations: COX-2, cyclooxygenase-2; MPO, myeloperoxidase; PGE₂, prostaglandin E₂; SCC, squamous cell carcinomas; EP, E prostanoid

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Although the effects of UVB on COX-2 expression and activity in the skin are well defined, the role that EP receptors play in photocarcinogenesis has not been explored. The current studies used a hairless mouse model to compare the effects of blocking EP₁, via topical application of ONO-8713, with or without topical application of the anti-inflammatory drug celecoxib, on UVB-induced cutaneous inflammation and tumor development.

RESULTS

Localization of EP₁ protein by immunohistochemistry

EP₁ protein is expressed at low levels in differentiated keratinocytes located in the stratum granulosum and stratum corneum of the epidermis (Figure 1a). Exposure to UVB increased the number of suprabasal layers containing EP₁-positive cells both at 48 hours following a single exposure to 2,240J/m² (Figure 1b) and at 25 weeks following three times

a week exposures (Figure 1c). Papillomas isolated following 25 weeks of UVB exposure demonstrated focal EP₁ expression in differentiated keratinocytes surrounding keratin pearls (Figure 1d). This pattern of staining was not altered in tumors that developed in mice treated topically with a combination of celecoxib and the specific EP₁ antagonist ONO-8713 (Figure 1e), or with either drug alone (data not shown). The specificity of the EP₁ antibody was demonstrated by preincubation of the antibody with an EP₁ peptide (Figure 1f) as well as through the use of an isotypic control antibody (data not shown). Preincubation of the EP₁ antibody with EP₂, EP₃, or EP₄ peptides had no effect on EP₁-specific staining (data not shown). Staining was confirmed using a second EP₁ antibody from Cayman Chemical that showed identical staining patterns (data not shown).

Inhibition of UVB-induced inflammation with celecoxib and/or an EP₁ antagonist

A specific antagonist of EP₁, ONO-8713, alone or in combination with the COX-2 inhibitor, celecoxib, was used to examine the contribution of signaling via the EP₁ prostaglandin receptor to acute UVB-induced cutaneous inflammation. Exposure to 2,240J/m² UVB resulted in a significant increase in the cutaneous inflammatory response, as measured by skin thickness ($P < 0.0004$) and neutrophil infiltration ($P < 0.002$) at 48 hours (Figure 2a and b). Topical treatment with 50 μg/mouse of ONO-8713, 500 μg celecoxib, or the combination of celecoxib and ONO-8713 immediately following UVB exposure significantly decreased vascular permeability, as measured by skin thickness at 48 hours following UVB exposure (Figure 2a). The decrease in edema correlated with significantly decreased MPO levels in the skin (Figure 2b) as compared to control irradiated skin (UVB/acetone). The MPO assay is used to determine the extent of neutrophil infiltration in tissues, and is an accurate measure of inflammation (Lundberg and Arfors, 1983). Immunohistochemical analysis using the LY-6G antibody, which specifically stains neutrophils, demonstrated a correlation between the decrease in MPO levels in treated skin and decreased dermal neutrophil infiltration (data not shown).

Effect of topical treatment with celecoxib and/or ONO-8713 on PGE₂ levels in the skin

Exposing Skh-1 hairless mice to UVB light resulted in a statistically significant increase in cutaneous PGE₂ levels (Figure 3; $P < 0.0004$). As reported previously, topical treatment with celecoxib significantly reduced UVB-induced increases in PGE₂ levels in skin at 48 hours following UVB exposure (Figure 3). Likewise, topical treatment with ONO-8713 significantly decreased PGE₂ levels in the skin. Treatment with the combination of celecoxib and ONO-8713 had no additional effect on cutaneous PGE₂ levels as compared with either treatment alone. The topical treatments had no effect on epidermal expression of COX-1 or COX-2 protein (data not shown). Decreases in PGE₂ levels correlated with decreases in UVB-induced edema and neutrophil infiltration (Figures 1 and 2).

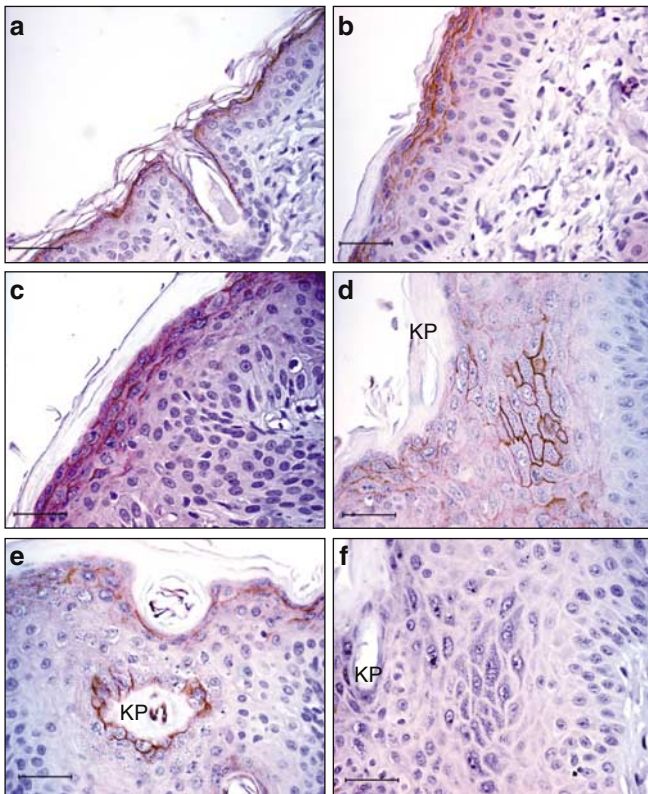


Figure 1. Immunohistochemical localization of EP₁ in dorsal skin and papillomas isolated from hairless mice. Histological skin sections from unirradiated mice (a), mice acutely exposed to UVB and euthanized at 48 hours following exposure and topical treatment with acetone (b), mice chronically exposed to UVB and treated topically with acetone for 25 weeks (c), 25-week papilloma isolated from mice chronically exposed to UVB and topical acetone (d), 25-week papilloma isolated from mice chronically exposed to UVB and topical 500 μg celecoxib + 50 μg ONO-8713 (e) demonstrate specific expression of the EP₁ receptor in more differentiated cells. The specificity of the EP₁ staining is demonstrated by the lack of staining of the 25-week papilloma shown in (d), after preincubation of the primary antibody with EP₁-blocking peptide (f). All photographs were taken at a magnification of × 60 (bar = 10 μm; KP = keratin pearl).

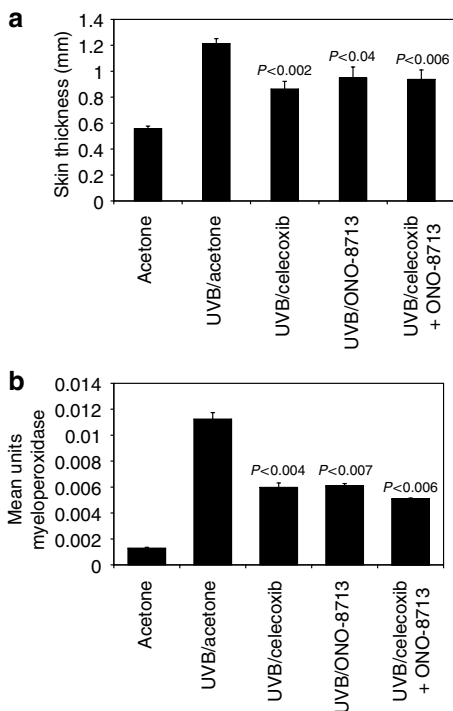


Figure 2. Characterization of the extent of UVB-induced inflammation. (a) Graphical representation of the extent of vascular permeability as measured by skin fold thickness demonstrates the ability of 500 μ g celecoxib, 50 μ g ONO-8713, or a combination of these two drugs to significantly inhibit UVB-induced edema. (b) Treatment with celecoxib and/or ONO-8713 significantly inhibited UVB-induced dermal neutrophil infiltration, a hallmark of UVB-mediated inflammation.

Decreasing PGE₂ levels or blocking the EP₁ receptor decreases the number of p53-positive cells following UVB exposure

Induction of p53 allows for cell cycle arrest, at which time damaged DNA can be repaired. UV light exposure results in both direct DNA damage as well as indirect DNA damage as a result of the production of reactive oxygen species in the skin. Consequently, p53 can be used as an indirect marker of both direct and indirect DNA damage in the skin. P53 protein expression was measured in the skin at 24 hours, 48 hours, and 1 week following UVB exposure, and was found to be maximal at 24 hours. Figure 4 represents the percent of p53-positive cells in the epidermis of mice that were unirradiated (acetone) or irradiated, followed by topical application of acetone, celecoxib, ONO-8713, or a combination of celecoxib and ONO-8713. A single exposure to UVB resulted in a significant increase ($P<0.00001$) in p53 levels in the epidermis 24 hours later, as compared to acetone-treated control skin (Figure 4). Topical treatment with either celecoxib or ONO-8713 immediately following UVB exposure significantly decreased the number of p53-positive cells, suggesting a decrease in the levels of indirect epidermal DNA damage. Topical treatment with the combination of celecoxib and ONO-8713 was not any more effective than either treatment alone.

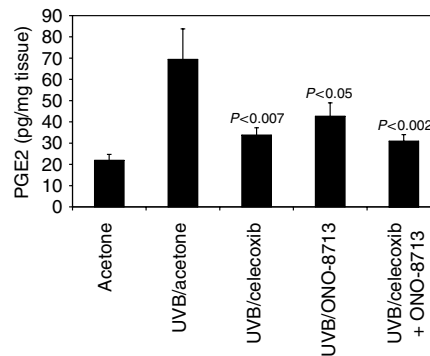


Figure 3. Effect of topical application of celecoxib, ONO-8713, or the combination of celecoxib and ONO-8713 on UVB-induced PGE₂ production. Exposure to UVB induced a significant increase in PGE₂ levels that were significantly inhibited by topical application of celecoxib. The EP₁ receptor antagonist ONO-8713 was slightly less effective than celecoxib at blocking UVB-induced PGE₂ production. The combination of celecoxib and ONO-8713 showed a decrease in UVB-induced PGE₂ production that was similar to the inhibitory effects of either drug alone.

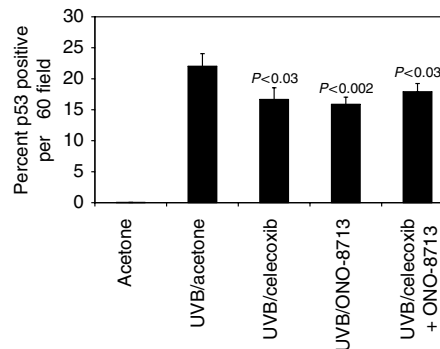


Figure 4. Extent of p53 induction in response to acute UVB exposure. Graphical representation of the percent of p53-positive cells in the epidermis of mice that were unirradiated (acetone) or UVB-irradiated followed by topical application of acetone, 500 μ g celecoxib, 50 μ g ONO-8713, or a combination of celecoxib and ONO-8713. UVB increased the percentage of cells expressing p53 protein in the epidermis. Topical application of either celecoxib or ONO-8713 significantly decreased UVB-induced p53 expression 24 hours following exposure. Topical treatment with the combination of celecoxib and ONO-8713 was not any more effective than either treatment alone.

Effects on tumor number following chronic UVB exposure

Skh-1 hairless mice were exposed to 2,240J/m² UVB three times a week for 25 weeks. Immediately following each UVB exposure, mice were treated topically with either acetone as a control or 500 μ g celecoxib, 50 μ g ONO-8713, or a combination of the two. Tumors larger than 1 mm first appeared in all groups by week 13 of exposure. The number of tumors per mouse was counted on a weekly basis until the animals were euthanized at 25 weeks. By week 15, 60% of mice in all treatment groups had at least one tumor larger than 1 mm. All groups reached 100% tumor incidence by week 18. As we demonstrated previously, topical treatment with 500 μ g celecoxib (UVB/CX) significantly decreased the number of tumors at 20 weeks of treatment. By 25 weeks, treatment with celecoxib alone decreased the number of

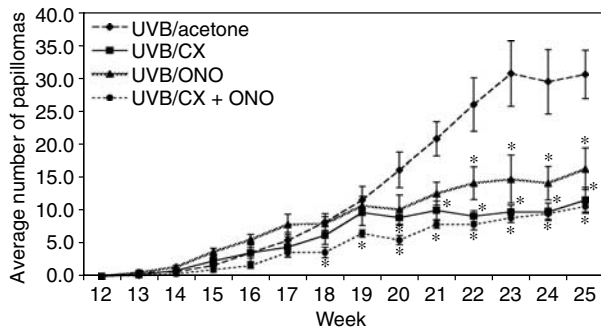


Figure 5. Effect of topical application of celecoxib, ONO-8713, or the combination on UVB-induced tumor development. Exposure to UVB three times a week resulted in the development of papillomas beginning at 13 weeks after exposure. Topical treatment with celecoxib, ONO-8713 or a combination of the two compounds for 25 weeks significantly ($P < 0.05$) decreased tumor number compared to vehicle-treated mice. Tumor multiplicity was calculated as the average number of tumors per mouse (mean \pm SE).

tumors by approximately 60% compared to vehicle treatment (UVB/acetone; Figure 5). Topical treatment with the specific EP₁ antagonist ONO-8713 (UVB/ONO) demonstrated a significant decrease in tumor number compared to vehicle control, beginning at week 22. Similar to what was seen with celecoxib treatment, topical treatment with ONO-8713 reduced tumor number by approximately 50% in comparison to vehicle control mice (UVB/acetone). Mice treated with the combination of the two compounds (UVB/CX + ONO) showed a statistically significant decrease in tumor number by week 18 when compared to UVB/acetone mice, 2 weeks earlier than either drug alone. While mice treated topically with the combination of the two compounds displayed fewer tumors at every time point examined, by 25 weeks this decrease was not significantly different from that seen with either drug alone.

DISCUSSION

The involvement of the COX-2 enzyme and its product, PGE₂, in skin carcinogenesis is well established, with much of the evidence based on studies utilizing specific COX-2 inhibitors. While COX-2 inhibitors can reduce inflammation and the formation of skin tumors in response to UVB, they do not completely block PGE₂ production or tumor development (Fischer *et al.*, 1999; Pentland *et al.*, 1999; Wilgus *et al.*, 2000, 2003; Orengo *et al.*, 2002). The inability to completely block tumor formation may be due, at least in part, to the fact that any residual PGE₂ can bind to EP receptors to stimulate proliferation of the transformed keratinocytes that form SCC. Thus, EP receptors are likely to be key elements determining SCC susceptibility, and a better understanding of the expression patterns of these receptors may improve our chemopreventive and chemotherapeutic strategies.

Using immunohistochemical analysis, we have demonstrated in the present studies that EP₁ is expressed by differentiated keratinocytes in murine epidermis and that this receptor continues to be expressed following acute and chronic UVB exposure. Recently, Konger *et al.* (2005)

demonstrated a similar pattern of EP₁ immunolocalization in adult human epidermis. Furthermore, using cultured human primary keratinocytes, they verified that the epidermal EP₁ receptor was functional.

Previously, we showed that the anti-inflammatory effects of topically applied celecoxib following acute UVB exposure (Wilgus *et al.*, 2000) correlated with a reduction in UVB-induced SCC development (Wilgus *et al.*, 2003). However, this single treatment modality did not completely inhibit tumor growth. The current study was designed to compare the effects of a specific COX-2 inhibitor, a specific EP₁ antagonist, and a combination of the two compounds on UVB-induced inflammation and tumor development. Previous studies have demonstrated the effectiveness of selective EP₁ antagonists in reducing inflammation in other animal models (Nakayama *et al.*, 2002; Omote *et al.*, 2002). In our study, we found that blocking the EP₁ receptor through topical application of a specific EP₁ antagonist, ONO-8713, successfully decreased the infiltration of neutrophils into the skin in response to acute UVB exposure. In the present study, we also demonstrated that blocking signaling through the EP₁ receptor using the specific antagonist ONO-8713 significantly reduced UVB-induced tumor development. This establishes a role for the EP₁ receptor in the photocarcinogenesis process. Several recent studies have indicated the importance of these receptors in the development of other types of cancers, including both colon and breast cancer, and have also demonstrated the effectiveness of specific EP receptor antagonists as chemopreventive agents (Watanabe *et al.*, 1999, 2000; Kawamori *et al.*, 2001, 2005). In addition, based upon the recently reported negative clinical side effects of selective COX-2 inhibitors (Couzin, 2004; Vanchieri, 2004, 2005), our study suggests the need for a closer examination of EP₁ antagonists to determine if blocking signaling via the prostaglandin receptor would provide anti-inflammatory benefits similar to those seen with COX-2 inhibition, but with fewer negative side effects.

As expected, both decreasing PGE₂ levels via celecoxib treatment and blocking PGE₂ signaling through the EP₁ receptor via ONO-8713 treatment significantly decreased several parameters of acute UVB-induced inflammation and reduced the number of tumors that developed during the 25-week carcinogenesis study. However, while we had anticipated that combining inhibitors such as a COX-2 inhibitor that blocks PGE₂ production and an EP₁ antagonist to block signaling would result in a more effective tumor chemopreventive strategy, the combination of celecoxib and ONO-8713 was not more effective than using either compound alone. Taken together, these data suggest that, in addition to EP₁, other EP receptors, such as EP₂, may be involved in the cutaneous carcinogenesis process. The presence of the EP₂ receptor in epidermal cells has been demonstrated, and there is a strong suggestion that this receptor may also play a key role in modulating tumor growth in the skin (Konger *et al.*, 2002). However, the role of this receptor in preventing UVB-induced tumor development has, to date, not been described. Furthermore, the potential importance of signaling via the higher affinity receptors, EP₃ and EP₄, in skin carcinogenesis

is also not known. Modulating more than one EP receptor may be key to more effective chemoprevention strategies for SCC. Alternatively, a combination treatment strategy, blocking EP₁ signaling and a secondary target not related to the prostaglandin pathway, may prove to be a more effective chemopreventive and/or chemotherapeutic strategy. Data in a variety of cancer types suggest greater efficacy in treating tumors with combination therapies. In the skin, we found that topical treatment with 5-fluorouracil and celecoxib together was up to 70% more effective in reducing the number of UVB-induced skin tumors than 5-fluorouracil treatment alone (Wilgus *et al.*, 2004). Similarly, Fischer *et al.* (2003) demonstrated that targeting PGE₂ production and ODC activity had strong therapeutic effects against UVB-induced murine skin tumors. In addition, recent studies carried out by Han and Wu (2005) suggest that combining agents targeting EP₁ and EGFR may be an effective cancer therapeutic strategy in preventing cholangiocarcinoma cell growth and invasion.

While our findings demonstrate an important role for the PGE₂-EP₁ signaling pathway in the photocarcinogenesis process, these data also illustrate the complexity of PGE₂ signaling in the skin. From studies described in the literature, it is clear that the effects of PGE₂ signaling through its receptors are cell type dependent. For example, in cholangiocarcinoma cells, signaling of PGE₂ through EP₁, but not EP₂, EP₃, or EP₄, induces crosstalk between EGFR and EP₁, resulting in upregulation of Akt (Han and Wu, 2005). However, in dendritic cells, Akt upregulation occurs via signaling of PGE₂ through both EP₂ and EP₄ receptors (Vassiliou *et al.*, 2004). It is clear that further *in vitro* and *in vivo* studies are needed to determine the effects of PGE₂ signaling through each of the four EP receptors on the various cell types that play a role in cutaneous photocarcinogenesis.

MATERIALS AND METHODS

Animal treatments

Female Skh-1 hairless mice (Charles River Laboratories, Wilmington, MA) were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. Prior to beginning all studies, procedures were approved by the appropriate Institutional Animal Care Utilization Committee. Irradiated mice were exposed dorsally to one minimal erythemic dose of UVB (2,240 J/m² as determined by a UVX radiometer (UVP Inc., Upland, CA)) emitted by Phillips FS40UVB lamps (American Ultraviolet Company, Lebanon, IN) that were fitted with Kodacel filters (Eastman Kodak, Rochester, NY) to ensure the emission of primarily UVB light (290–320 nm).

Acute studies were performed to examine the expression of EP₁ in response to short-term UVB exposure and to investigate the effects of inhibiting EP₁ signaling on UVB-induced inflammation. For these studies, eight mice per treatment group per time point were examined. Mice were irradiated and then immediately treated topically with vehicle control (acetone 200 μ l), 500 μ g celecoxib, 50 μ g ONO-8713 (a generous gift from ONO Pharmaceuticals, Japan), or a combination of celecoxib and ONO-8713. Unirradiated control mice were treated topically with 200 μ l of the vehicle control, acetone. The first and second groups of mice were exposed

to a single dose of UVB and topical treatment and killed at either 24 or 48 hours, respectively, following exposure. The final group of mice was irradiated and topically treated on nonconsecutive days (Monday, Wednesday, Friday) for a total of four treatments and killed at 24 hours following the final treatment. Following the killing, edema was assessed by measuring dorsal skin fold thickness using a metric calipers, 10 mm skin biopsies were harvested to assess myeloperoxidase (MPO) levels, 0.5 cm² skin sections were harvested and fixed in 10% neutral buffered formalin for immunohistochemical analysis, and the remaining skin tissue was snap-frozen in liquid nitrogen for PGE₂ analysis.

Chronic UVB studies were carried out to evaluate the expression of EP₁ during UVB-induced tumorigenesis and to determine the effects of inhibiting EP₁ signaling on this process. Mice (10 per group) were irradiated three times a week for 25 weeks, followed immediately by topical application of the vehicle control (acetone 200 μ l), 500 μ g celecoxib, 50 μ g ONO-8713, or a combination of celecoxib and ONO-8713. In order to track changes in tumor number and size, tumors were measured weekly, using a digital calipers, beginning at 12 weeks. Tumor incidence was calculated as the percentage of mice with tumors larger than 1 mm. Tumor multiplicity was calculated as the average number of tumors per mouse. Unirradiated control mice were treated topically with 200 μ l of the vehicle control, acetone. Mice were killed 24 hours following the final UVB exposure and topical treatment. Following the killing, edema was assessed by measuring dorsal skin fold thickness using a metric calipers, 0.5 cm² skin sections and multiple tumors were harvested and fixed in 10% neutral buffered formalin for immunohistochemical analysis, and the remaining skin tissue was snap-frozen in liquid nitrogen for PGE₂ analysis.

Quantitation of tissue MPO levels

MPO, an enzyme that converts hydrogen peroxide to hypochlorous acid, is released by activated neutrophils during inflammatory events. The levels of MPO in cutaneous tissue were determined biochemically and used as a measure of neutrophil infiltration, as described previously (Wilgus *et al.*, 2003).

Quantitation of tissue PGE₂ levels

Acute UVB-irradiated skin that had been snap-frozen in liquid nitrogen was ground in liquid nitrogen using a mortar and pestle. The powdered skin was then placed in 1 ml of methanol and the tissue weight was recorded. Tissue was vortexed in the methanol every 10 minutes for 30 minutes, and then spun at 4°C for 10 minutes at 3,500 r.p.m. The supernatant was retained and 25 μ l was dried in a CentriVap Centrifugal Concentrator (Labconco, Kansas City, MO) and resuspended in EIA buffer (Cayman Chemical, Ann Arbor, MI) at 1:10 dilution. PGE₂ levels were assessed using the PGE₂ ELISA kit from Cayman Chemical according to the manufacturer's instructions.

Immunohistochemical detection of the EP₁ receptor

Immediately following the killing, skin sections (0.5 cm²) or tumors were placed in 10% neutral buffered formalin for 2 hours, washed with PBS, and then processed and embedded in paraffin blocks. Tissue sections (5 μ m) were mounted onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were deparaffinized using Clear-Rite 3 (Richard-Allan Scientific,

Kalamazoo, MI) and rehydrated in a graded series of alcohols followed by a 5-minute soak in distilled water. Tissue was then subjected to antigen retrieval as follows: antigen unmasking fluid (Vector Laboratories, Burlingame, CA) was heated for 15 minutes in a steamer, at which time the tissues were placed in the prewarmed unmasking fluid for 7 minutes and then cooled at room temperature for 10 minutes. The sections were washed in automation buffer (Biomedica Corp., Foster City, CA) followed by a 30-minute incubation in 1 × casein blocking solution (Vector). The tissue was incubated with primary anti-EP₁ antibody (28 μg/ml; Alpha Diagnostics International, San Antonio, TX) diluted in 1 × casein solution overnight at 4°C. Negative controls included replacing either the primary or secondary antibody with 1 × casein, incubating the tissue with equal amounts of EP₁ antibody and either EP₁ (Alpha Diagnostics), EP₂, EP₃, or EP₄ (Cayman Chemical) blocking peptide, incubating the tissue with equal amounts of rabbit IgG (28 μg/ml; Vector) and EP₁ blocking peptide, and replacing the primary EP₁ antibody with rabbit IgG (28 μg/ml; Vector). Following the overnight incubation, tissue was washed with automation buffer, then incubated with rabbit link solution (Biogenex, San Ramon, CA) and rabbit label solution (Biogenex) for 30 minutes each, with an automation buffer wash in between. The tissue was incubated with diaminobenzidine solution (Vector) for 10 minutes, with a final wash in distilled water, counterstained with hematoxylin 2 (Richard-Allan), dehydrated, and mounted. Photographs were taken using a Nikon Eclipse E400 microscope with a DXM1200 digital camera.

Immunohistochemical detection of p53

Staining was carried out as described previously (Wilgus *et al.*, 2003). Ten 60 × fields per section from each animal in each treatment group were examined. P53-positive cells and total cells were counted and data were expressed as the percent of p53-positive cells per field.

Statistical analysis

Microsoft Excel (Microsoft) was used to statistically analyze differences in the data by the Student's *t*-test, with statistical significance referring to a *P*-value <0.05.

CONFLICT OF INTEREST

The author states no conflict of interest.

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