

Immunosuppression, Inflammation, and Skin Cancer: Will Eczema Treatment Enhance
Ultraviolet Light-Induced Skin Cancer?

A Senior Honors Thesis

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by

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Abstract

Eczema, specifically atopic dermatitis (AD), is a common condition characterized by skin inflammation with erythema and pruritis. Protopic (tacrolimus) and Elidel (pimecrolimus) are immunosuppressant drugs that have been introduced for the treatment of AD. Using the Skh-1 mouse model of ultraviolet light B (UVB) induced inflammation, we examined the effects of local immunosuppression following topical treatment with Elidel and Protopic on levels of cutaneous ultraviolet light mediated inflammation including neutrophil infiltration, COX-2 expression and PGE₂ production. The goal of the studies was to determine whether topical treatment with these drugs in combination with UVB exposure would alter levels of inflammation, a process known to contribute to an increase in skin tumor development. The results of the current study demonstrate that the timing and number of topical treatments in relation to UVB exposure changes the inflammatory response in the skin. Repeated topical application of the immunosuppressants prior to UVB exposure resulted in changes in products that are indicators of future tumor development, including edema, neutrophil infiltration and activation as measured by myeloperoxidase (MPO) activity. The current study demonstrated that induction of COX-2 expression and PGE₂ production is not responsible for the observed changes in skin inflammation.

Introduction

Eczema is a general term for many types of skin inflammation. Among the types of eczema, atopic dermatitis (AD) is the most common. AD is difficult to define because it has a variable morphology and distribution. Typically, AD is a pruritic and highly inflammatory skin disorder commonly found in skin flexures [1]. Often it is characterized by poorly defined erythema with edema, vesicles, and weeping [2]. Acute lesions show all of the normal signs of eczema while chronic lesions display a heightened lichenification of the skin [3]. A criterion for the clinical diagnosis of AD has been put forth. The diagnosis requires the presence of itchy skin plus three or more of the following: history of involvement of the skin creases (e.g., backs of knees, fronts of ankles, areas around the eyes), history of asthma or hay fever, history of very dry skin in the past year, onset in a child under two years of age, or visible flexural dermatitis [4].

The course of AD is greatly variable with most cases running their course during infancy, although it can persist through adolescence or even appear in the elderly [3]. Most often AD is found in children and its onset substantially decreases with age. Globally, the onset in children of six or seven years of age in a one-year period ranged from 2 percent in China to about 20 percent in Australia, England, and Scandinavia [5]. Approximately 70 percent of AD cases start in children under five years of age, but 10 percent of cases that are seen in the hospital start in adults [6]. About 10 percent of all infants and young children experience symptoms, and 60 percent of these experience symptoms into adulthood. Allergic rhinitis develops in approximately 35 percent of children with AD, and asthma in 30 percent [7].

Various studies indicate that AD has a complex etiology, activating several inflammatory and immunological pathways [8]. The condition shows elevated IgE serum levels and peripheral eosinophilia. AD is the beginning of an “atopic march” leading to asthma and rhinitis in most patients [9]. AD patients usually display an elevated Th2 immune response with an increased frequency of allergen-specific T cells [9]. Through the study of cytokines, the course of AD is thought to be biphasic, with a Th2-predominant acute phase and a Th1 predominant chronic phase [10]. This concept has gained further support from skin lesions caused by atopy patch tests where aeroallergens are applied to cause eczematoid reactions in sensitive patients. In the early phases of these lesions there is an increase of the Th2 cytokine IL-4, whereas the later phase is mostly the Th1 cytokine IFN- γ [11]. The phenotype of AD, observed in the clinical setting, is a product of many complex gene interactions and has a high familial occurrence. However host environment (dust mites, *Staphylococcus aureus* infections, and foods), defects in the skin barrier function, and local immune responses play an intrinsic role. Analysis of cellular and cytokine gene expression in AD skin lesions in humans as well as mouse models has proved a very effective means to explain the pathogenesis of AD [9].

The affects of AD on children are of the utmost concern to their families. Due to the highly pruritic nature of AD, children are prone to scratching which thickens and breaks the skin, causing further pain and discomfort [12]. The disorder is a chronic, relapsing condition that can severely impair quality of life, especially in children. Sleep disturbances are a part of this impact. One of the most severe signs is the distress caused by disrupting sleeping patterns and routines while itching and scratching. This itching

may be caused by a neuropeptide-induced vasodilation causing erythema [13]. With hot and itchy skin, the child is likely to scratch the skin and bring on infection. The psychological impact of AD also cannot be discounted. Even though AD can be sporadic, it has been suggested that the psychological impact of severe AD on children and their families is greater than that of type I diabetes [14]. There is not an aspect of a child's life that is not somewhat affected by AD, whether it is in physical activity or social interactions. As a result, children become more irritable and need extra care [15]. Parental anxiety has a great impact on the family's quality of life as well. Mothers frequently cite problems such as disturbed sleep, pressing financial obligations, increased emotional stress, and paranoia [15]. These factors should be taken into consideration when developing a method of treatment.

Several treatment methods exist for the management of atopic dermatitis including topical corticosteroids, emollients, topical calcineurin inhibitors, oral antihistamines, refined-coal tar, topical doxepin, and oral corticosteroids [2]. The most popular or common treatment is the use of topical corticosteroids. Literature on topical corticosteroid use has shown that in vehicle-controlled studies of less than one month, about 80 percent report good, excellent, or clear responses, whereas 38 percent of control groups reported similar responses [16]. Use of these drugs gives rise to the potential for vasoconstriction. Generally, only mild preparations are used on the face and genital area while stronger preparations are used on other areas of the body. Milder preparations are always used for younger children. The biggest risks associated with topical corticosteroids are local side effects such as skin atrophy, striae, acne, and irreversible skin thinning [17, 18]. Skin thinning is often an overblown concern for parents that is not

in proportion to any true risk. Overdoses of steroids can cause thinning, but four 16-week randomized trials did not show any clinically significant skin thinning [19-22].

The new nonsteroid topical calcineurin inhibitors (TCIs) were developed specifically for the treatment of AD. Pimecrolimus (Elidel[®]) and tacrolimus (Protopic[®]) are two such TCIs, and are the focus of this senior honors thesis. Pimecrolimus is a macrolactam immunomodulator, along with tacrolimus and cyclosporine, belonging to the family of calcineurin inhibitors. Mechanistically, these work by binding to cytoplasmic proteins and the resulting complex binds to calcineurin, inhibiting its ability to dephosphorylate the nuclear factor of activated T-cells (NF-AT) [23]. Pimecrolimus and tacrolimus bind macrophilin-12, known as the FK506-binding protein. NF-AT, a nuclear transcription factor, facilitates the transcription of growth factors and inflammatory genes, but it must be dephosphorylated to translocate into the nucleus [24].

Pimecrolimus shows selective action on T cells and mast cells. The activation of these cells play a crucial role in the pathogenesis of inflammatory skin diseases like AD [23]. Pimecrolimus inhibits T-cell proliferation and production and release of several growth factors and pro-inflammatory cytokines including IL-2, IL-4, interferon- α and tumor necrosis factor- α . Also, pimecrolimus blocks mast cell release of pro-inflammatory mediators including histamine, cytokines, and eicosanoids [25, 26]. It does not have general antiproliferative activity on keratinocytes, endothelial cells, and fibroblasts, and it does not affect the differentiation, maturation, functions, and viability of human dendritic cells [27]. Studies using mice have shown that topical pimecrolimus does not affect epidermal Langerhans cells or antigen-presenting cells that play a role in immunosurveillance [28]. In animal models, pimecrolimus has a low potential to impair

systemic immune reactions and topical application in humans is not associated with the atrophogenic side effects observed with corticosteroids. Blood levels of pimecrolimus are consistently low after repeated topical use [29].

The tacrolimus ointment Protopic was approved in December 2000 and marketed in two strengths; the 0.03% ointment is approved for children 2 to 15 years of age whereas the 0.1% ointment is approved for adults. It is only approved as a second-line agent for patients whom alternative conventional therapies are inadvisable [30]. Tacrolimus inhibits the proliferation and activation of CD4⁺ T helper cells and, like pimecrolimus, binds to FK506-binding protein. Tacrolimus has been shown to inhibit mast cell adhesion, inhibit the release of mediators from mast cells and basophils, and down-regulate the expression of interleukin-8 receptor and FcγRI on Langerhans' cells [31-34]. A major advantage of topical tacrolimus is that it does not cause skin atrophy [30]. In fact, long-term use may reverse corticosteroid-induced skin atrophy.

Non-melanoma skin cancer is the most common human neoplasm. This year in the United States it is estimated that there will be one million new cases of diagnosable skin cancer. Fortunately, because of their high visibility, skin cancer is rapidly diagnosed and more easily treated than other forms of cancer [35]. Nonetheless, at an estimated cost of \$650 million and 2,200 American deaths each year, non-melanoma skin cancer is a devastating problem [36].

The two main types of NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), with numbers increasing worldwide. BCC represents about 80% of all skin cancers whereas SCC accounts for 10-30% of all skin cancers. Unsurprisingly, both tend to appear on sun-exposed areas of the body. Chronic or intensive exposure to

ultraviolet-light is the most important risk factor for the development of NMSC. Of the 3 wavelengths of solar radiation, ultraviolet light A (UVA), ultraviolet light B (UVB), and ultraviolet light C (UVC), UVC (200-290nm) is filtered out of the earth's atmosphere by the ozone layer. UVB (290-320nm) is highly mutagenic and carcinogenic when compared to UVA (320-400nm) radiation [37]. While UVA will penetrate deeply into the skin, UVB is responsible for most of the epidermal skin damage. Studies suggest that solar UV radiation is responsible for development of skin tumors and contributes to localized immunosuppression [37]. There are several mechanisms of photocarcinogenesis, but they all commence with the absorption of UV by cellular DNA. Absorption of UV can cause lesions in the DNA or the formation of photoproducts like thymidine dimers. Exposure can cause deficiencies in DNA repair mechanisms resulting in un-repaired breaks and mutations in DNA. The mutations frequently occur in tumor suppressor genes or proto-oncogenes allowing these initiated cells to proliferate uncontrollably ultimately leading to the development of skin cancer. It has also been shown that UVB radiation alters the antigen-presenting activity of epidermal Langerhans cells and targets keratinocytes, which produce and release immunosuppressive mediators. The relative immunosuppression caused by these factors prevents tumor rejection [37-42].

In solid organ transplant recipients, there has been an established connection between skin cancer onset and long-term systemic immunosuppression induced by a combination of drugs, notably oral calcineurin inhibitors [43]. SCCs in transplant recipients are very aggressive, growing rapidly and showing a higher rate of reoccurrence and metastasis than those occurring in the general population [44]. BCCs are more

frequent in the general population, but the number is only increased tenfold with transplant recipients [44]. By 20-years after transplantation, about 40-50% of all Caucasian patients in western countries and 70-80% of Caucasian Australians will have developed a non-melanoma skin cancer (NMSC) [45-48]. Reports of melanomas are mixed: there are some reports of increased incidence [49, 50], and other reports failing to confirm these findings [51, 52]. The contribution of specific immunosuppressants to the development of skin cancer in transplant patients in humans and animals has led to findings with inconclusive results. It is suggested that the cumulative, rather than individual, immunosuppressant load is more important to cancer development, as well as age, sunlight exposure, and ethnicity [45, 49, 53]. In vitro studies using calcineurin inhibitors which induce a systemic immunosuppression have been found to have an effect in the local inhibition of DNA repair and apoptosis in skin cells [54], however the degree of immunosuppression required does not seem attainable with TCIs [55].

Recently, the US Food and Drug Administration (FDA) expressed concerns about the marketed Elidel[®] and Protopic[®] topical immunosuppressants. The FDA has approved revisions to the safety labeling for 1% pimecrolimus (Elidel) and tacrolimus (Protopic) 0.03% and 0.1% ointment advising of the potential risk of cancer associated with their use [56]. These revisions include a new boxed warning and medication guide for patients to minimize risk while using the products. The initial impetus to this labeling is based on a recommendation from the Pediatric Advisory Committee because the products posed, in their view, a potential risk for cancer [57]. The FDA's judgment was based mostly on theoretical concerns of immunosuppression and simian study. As a result, the American Academy of Dermatology, the Inflammatory Skin Disease Institute, and the topical

Calcineurin Inhibitor Task Force of the American College of Allergy, Asthma & Immunology (ACAAI) have shown disappointment in this action due to the immaturity of data collection and lack of a clear, causal relationship [57].

The carcinogenicity of tacrolimus and pimecrolimus has been studied using the multistage mouse model and a photocarcinogenesis model. In multistage mouse models of skin carcinogenesis, animals are first treated with the initiating agent 7,12-dimethylbenz[a]anthracene (DMBA) and then with a promoting agent such as 12-O-tetradecanoylphorbol-13-acetate (TPA). With this treatment carcinomas are initially seen at about 30 weeks [58]. Past literature has shown that daily topical application of a 0.006- μ mol formulation of tacrolimus ointment to CD-1 mouse skin over a 20-week period led to a significant increase in the development of skin tumors in mice between week 14 and 20 treated with DMBA + TPA + tacrolimus compared with DMBA + TPA-treated mice [59].

The effect of UVB radiation on hairless mouse skin treated with tacrolimus ointment 0.1% and pimecrolimus cream 1% has also been investigated [60, 61]. Mice were treated once daily over a 10-day period followed by UVB irradiation and it was found that neither tacrolimus nor pimecrolimus increased the formation of DNA damage following UVB irradiation. In contrast to these findings, it has also been found that calcineurin inhibitors decrease DNA repair and apoptosis in human keratinocytes following UVB irradiation [54]. The manufacturers of these drugs performed 52-week studies assessing these drugs in hairless, albino mice and found that topical administration of pimecrolimus had no influence on tumor development, indicating the active ingredient in pimecrolimus is not photo-carcinogenic [62]. The study found that

there was no decrease in the median time of onset of skin tumor formation for the tacrolimus formulations. In addition, long-term rodent trials indicate no dermal carcinogenic effects with use of tacrolimus or pimecrolimus. This information is included in the prescribing information sheet provided with the drugs however, these studies have never been published in peer reviewed journals so the treatment methodologies cannot be evaluated. Based upon some concerns raised in patient use of these drugs further studies are ongoing. However, the FDA notes that it may be many years before conclusive data on long-term safety is obtained [56]. It seems that at the moment, there is no conclusive evidence from rodent trials to indicate that long-term TCI use is photocarcinogenic [43].

The focus of this thesis is on evaluating the effect of TCIs, specifically tacrolimus and pimecrolimus, on UVB induced inflammation in the skin using an Skh-1 hairless mouse model. This is a widely accepted model for the study of ultraviolet light effects on the skin [63]. The studies designed in this senior honors thesis examined the effects of local immunosuppression by topical Elidel and Protopic on levels of cutaneous UVB-induced acute inflammation. Inflammation, “which includes the release of growth factors, pro-inflammatory cytokines and prostaglandins, the infiltration and activation of inflammatory cells, and the induction of oxidative DNA damage” is instrumental in the development of skin cancer [64]. Acute ultraviolet exposure induces edema, neutrophil infiltration and activation, release of arachidonic acid from the phospholipid membranes, and increased levels of cyclooxygenase-2 (COX-2) at both the message and protein levels [64]. Prostaglandin E₂ (PGE₂), the enzymatic product of COX-2, promotes the

carcinogenic process by contributing to uncontrolled proliferation of damaged cells that have the potential to form skin tumors [65].

It is my postulate that the immunosuppression that results from topical treatment with Elidel and Protopic will alter the levels of ultraviolet light induced inflammation in the skin. Because chronic UVB-induced inflammation can lead to an increase in skin tumor formation, it is imperative that the relationship between UVB exposure and topical application of the immuosuppressants Elidel and Protopic needs to be explored. As eczema may be severe and chronic, prolonged use of pimecrolimus and tacrolimus is likely to become common and widespread. With this in mind, it is important that the risk of skin cancer with prolonged use of these drugs be assessed [59].

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 (2,240J/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 (290-320nm).

Acute UVB studies were performed to examine the effects of pimecrolimus (Elidel[®], Novartis, East Hanover, NJ) and tacrolimus (Protopic[®], Fujisawa, Deerfield, IL) on cutaneous inflammation. The following experimental groups were examined: KY jelly vehicle control, Elidel drug control, 0.1% Protopic drug control, 0.03% Protopic drug control, UVB/KY, UVB/Elidel, UVB/0.1% Protopic, and UVB/0.03% Protopic. Three mice were assigned to all control groups while eight mice were assigned to all UVB groups. The mice were treated by three different methods in order to simulate the patterns of drug treatment and sun exposure of patients. In the post-treatment method, mice were irradiated dorsally followed by 100 µl of topical treatment with either the vehicle or the drugs immediately and at 24 hrs following exposure. In the pre-treatment method, mice were topically treated with the vehicle or drugs for five consecutive days followed by two days of no treatment. Animals were then irradiated with UVB and

sacrificed 48 hrs after exposure. In the concurrent treatment method, mice were treated with the vehicle or drugs for seven consecutive days. On the eighth day, the mice were irradiated followed immediately and at 24 hrs following UVB exposure with topical application of the vehicle or drugs and sacrificed at 48 hrs following exposure.

Following sacrifice, edema was assessed by measuring dorsal skin fold thickness using metric calipers, 10mm skin biopsies were harvested to assess myeloperoxidase (MPO) levels, 0.5cm² 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.

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 the extent of neutrophil infiltration. Skin punches were isolated from all mice with a 10-mm-diameter cork borer. Each punch was homogenized separately in 1.25 ml of 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. The skin was homogenized at 4°C and subjected to three cycles of sonification, freezing, and thawing. The samples were then centrifuged for 30 min at 14,000 rpm at 4°C. The supernatants were transferred to individual wells of a 96-well microtiter plate with 290µl of substrate (0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% H₂O₂ in 50 mM potassium phosphate buffer, pH 6.0). MPO activity was measured spectrophotometrically over a 5 min period at 450 nm with a programmable microplate reader (Molecular Devices, Menlo Park, CA). The data are expressed as mean units of

MPO activity, where the amount of MPO required to degrade 1 μ mol of peroxidase/min at 25°C is equal to 1 U of MPO activity. MPO activity in each sample was calculated based on a standard curve.

Quantitation of tissue PGE₂ levels: 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 min for 30 min, and then spun at 4°C for 10 min at 3,500 rpm. 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.

Detection of neutrophils (LY-6G) by immunohistochemistry: Immediately following sacrifice, skin sections were placed in 10% neutral buffered formalin for 2 hrs, washed with PBS, processed, and embedded in paraffin blocks. Tissue sections (5 μ m) were cut and mounted onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were deparaffinized with ClearRite 3 (Richard-Allen Scientific, Kalamazoo, MI) and rehydrated in a graded series of alcohols.

Following rehydration, tissues were rinsed in automation buffer (Biomedica Corp, Foster City, CA) and nonspecific binding was blocked with Casein Solution (Vector Laboratories, Burlingame, CA) for 30 min. After every step that follows, slides were rinsed in automation buffer. The tissue was incubated with a 1:400 dilution of

monoclonal rat anti-mouse Ly-6G (PharMingen, San Diego, CA), or appropriate isotypic control antibody for 1 hr. The tissue was then incubated with a 1:200 dilution of biotinylated anti-rat secondary antibody (Vector Laboratories) for 30 min. The chromagen DAB (Vector Laboratories) was placed on the tissue for 10 min. DAB development was stopped by rinsing with distilled water, and then the tissue was counterstained with Harris hematoxylin (Shandon, Pittsburgh, PA) for 1 min. Slides were then dehydrated, mounted, viewed, and photographed.

RNA isolation: RNA from dorsal skin that had been frozen in liquid nitrogen was isolated using TRIzol (Life Technologies). Briefly, tissue was homogenized in 1 ml TRIzol reagent, after which 0.7 ml chloroform was added and the samples were centrifuged to facilitate the separation of the organic and aqueous phases. The aqueous phase was then transferred into a clean tube, and RNA was precipitated using isopropanol. The RNA was pelleted by centrifugation, washed with ethanol, centrifuged, then air dried. After the pellets were completely dry, they were resuspended in Molecular Biology Grade water (5 prime to 3 prime, Boulder, CO) and heated to facilitate complete resuspension. Spectrophotometric analysis was used to quantitate the RNA and ethidium bromide staining of 2 μ g of RNA electrophoresed on a 1.2% agarose formaldehyde gel, and was used to determine the integrity of the 18S and 28S ribosomal RNA.

Reverse transcription and polymerase chain reaction (RT-PCR): RNA (2 μ g/ 10 μ l) was heated for 5 min at 60°C to release any secondary structure, after which cDNA was reverse transcribed using a mixture containing a final concentration of 1X PCR Buffer II

(Life Technologies), 5 mM MgCl₂, 1 mM dNTP's, 2.5 mM Oligo dT's, 1 unit Rnasin (Life Technologies) and 100 units Maloney Murine Leukemia Virus-Reverse Transcriptase (Life Technologies). An aliquot of 30 µl of the PCR buffer mixture was added to 10 µl of RNA and a total of 40 µl sample volume was incubated for 60 minutes at 37°C to initiate elongation, followed by a 5 minute incubation at 95°C to heat inactivate the enzymes. The HPRT and COX-2 genes were amplified from each sample using 1 x PCR Buffer II, MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI), 200 pmol 5' primer, 200 pmol 3' primer, and 0.25 units Platinum Taq DNA polymerase (Life Technologies). Amplification was carried out using the following protocol: 1) initial denaturation: 95°C for 30 sec; 2) cycles of a 95°C 30 sec denaturation, cycle number varying depending on which gene is used, and a 72°C 30 sec elongation; 3) final elongation: 72°C for 7 min. The amplified gene products were visualized with ethidium bromide on 2.0% agarose gels. Images of the gels were captured using a Kodak DC120 digital camera (Eastman Kodak, Rochester, NY) and analyzed using Kodak 1D image analysis software (Eastman Kodak). The net intensities of the PCR products of COX-2 were compared to those of the housekeeping gene HPRT to give a ration of gene expression compared to the housekeeping gene.

Results

Effect of TCIs on Skin Thickening

Edema is a strong indicator of UVB-induced inflammation and can be quantitated by measuring skin thickness. The effects of pimecrolimus and tacrolimus are shown in the post-treatment group in Fig. 1A. In this group, Skh-1 mice were irradiated dorsally followed by 100 μ l of topical treatment with either the vehicle or the drugs immediately and at 24 hrs following UVB exposure. UVB/KY significantly increased skin edema compared to KY alone. Additionally, topical treatment with both Protopic and Elidel following UVB exposure significantly inhibited the UVB-induced edematous response. Finally, UVB/0.03% Protopic was significantly thinner than UVB/Elidel. The pre-treatment group is shown in Fig 1B. In this group, mice were topically treated with the vehicle or drugs for five consecutive days followed by two days of no treatment. Animals were then irradiated with UVB and sacrificed 48 hrs after UVB exposure. As can be seen, there were no significant differences in skin thickness across all treatment groups and a slight average increase in thickness in the UVB groups. The concurrent-treatment group is shown in Fig. 1C. In this group, mice were treated with the vehicle or drugs for seven consecutive days. On the eighth day, the mice were irradiated followed immediately and at 24 hrs following UVB exposure with topical application of the vehicle or drugs and sacrificed at 48 hrs following UVB exposure. All UVB drug groups were statistically significantly different compared to UVB/KY, with Elidel being thinner and Protopic being thicker. Additionally, both UVB/Protopic groups were significantly thicker than UVB/Elidel.

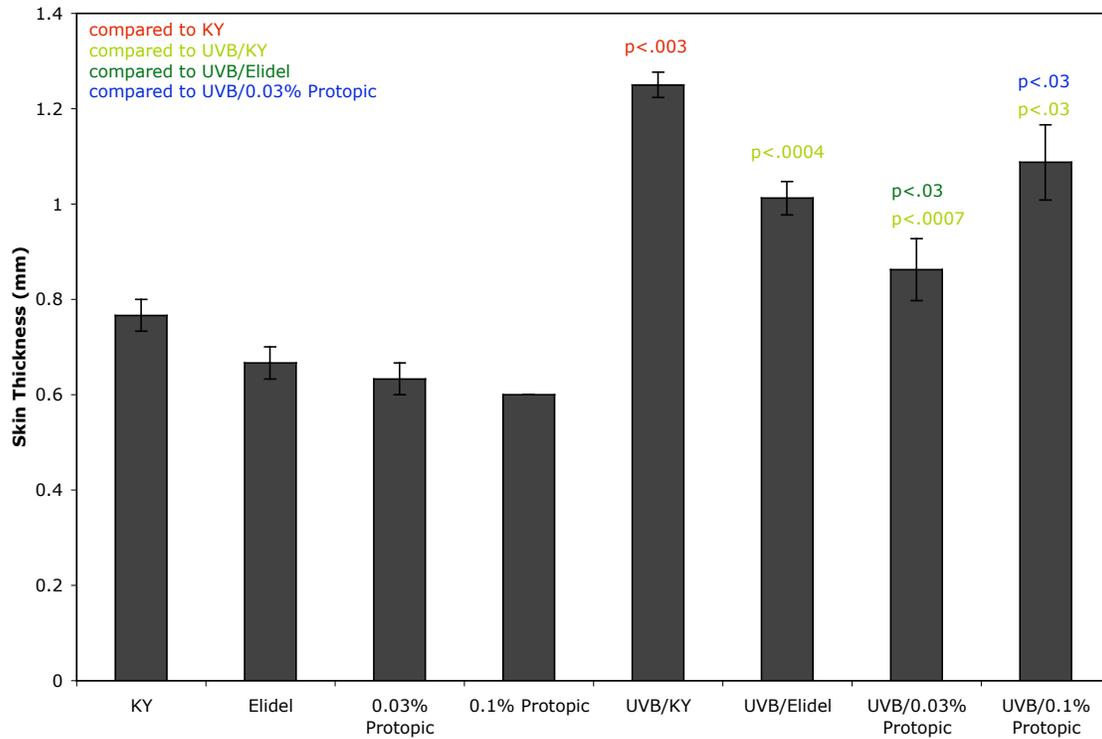


Figure 1A. Average skin thickness (mm) \pm standard error of mice in the post-treatment group. Three mice were present in all non-UVB groups, while 8 mice are represented in all UVB groups. Statistically significant differences ($p < 0.05$) are highlighted in color.

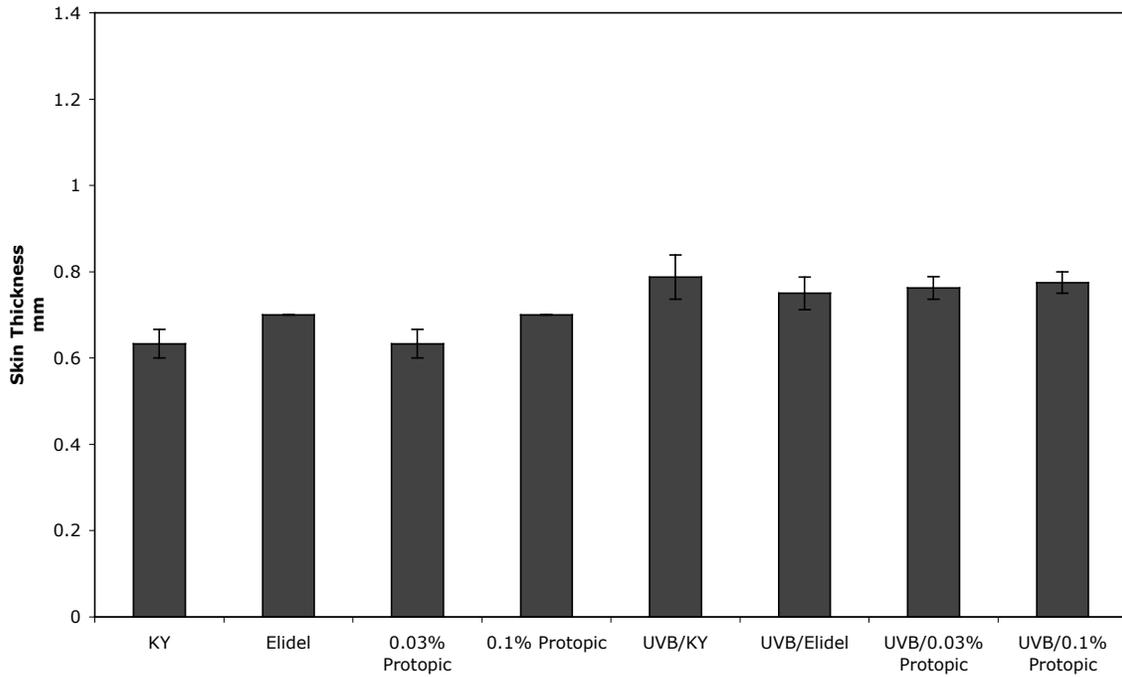


Figure 1B. Average skin thickness (mm) \pm standard error of mice in the pre-treatment group. Three mice were present in all non-UVB groups, while 8 mice are represented in all UVB groups. Note there were no significant differences between any treatment groups.

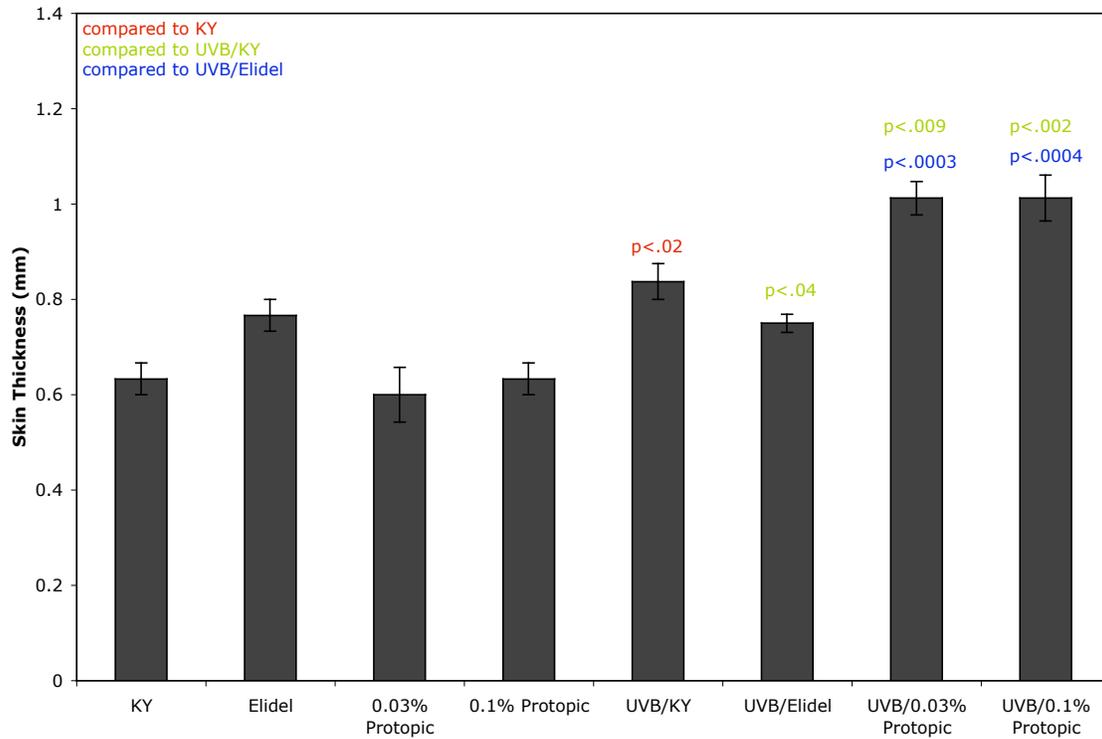


Figure 1C. Average skin thickness (mm) \pm standard error of mice in the concurrent-treatment group. Three mice were present in all non-UVB groups, while 8 mice are represented in all UVB groups. Statistically significant differences ($p < 0.05$) are highlighted in color. Protopic is shown to be significantly thicker ($p < 0.003$, $p < 0.004$) than Elidel.

Neutrophil Infiltration

Monoclonal rat anti-mouse LY-6G was utilized to detect the levels of neutrophil infiltration in all treatment groups. Histological sections of the post-treatment group are shown in Fig. 2A. There was a marked increase in the number of infiltrating cells to the dermis in the UVB/KY group when compared to the KY group, indicating increased levels of inflammation in the irradiated skin. The pre-treatment group is shown in Fig. 2B. Pre-treatment groups KY, UVB/KY, 0.03% Protopic, and UVB/0.03% Protopic are represented. There was a slight increase in neutrophil infiltration when comparing UVB/KY to KY. The 0.03% Protopic group and UVB/0.03% Protopic group are very much alike in their staining and leukocyte infiltration. All groups show similar epidermal skin thickness, illustrating the low effects of UVB on the pre-treatment method. Histological sections of the concurrent-treatment group are shown in Fig. 2C. Box (B) shows a large increase in the number of neutrophils in the UVB/KY group over the KY group. This same pattern is seen with 0.03% Protopic. Also, the epidermis is noticeably thicker in the UVB groups (B) and (D). The concurrent-treatment group shows more dramatic changes in epidermal thickness and neutrophil infiltration than the post-treatment or pre-treatment groups.

Levels of Tissue Myeloperoxidase (MPO)

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 the extent of neutrophil

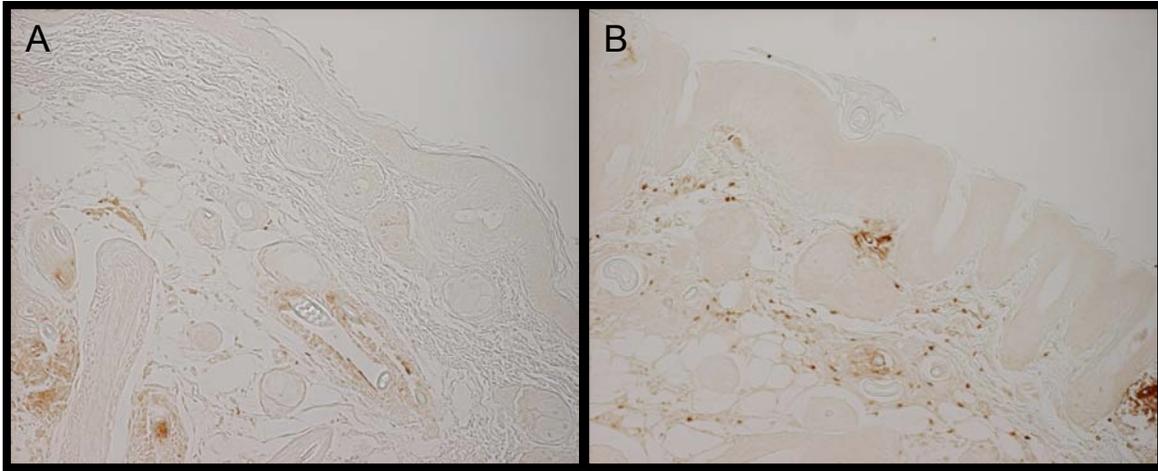


Figure 2A. Histological sections of LY-6G stained tissue in the post-treatment KY group (A) and post-treatment UVB/KY group (B). Infiltrating cells are marked brown. Epidermal thickness is greatly increased in (B) as well as dermal neutrophil infiltration. Photographs were taken at a magnification of x 20.

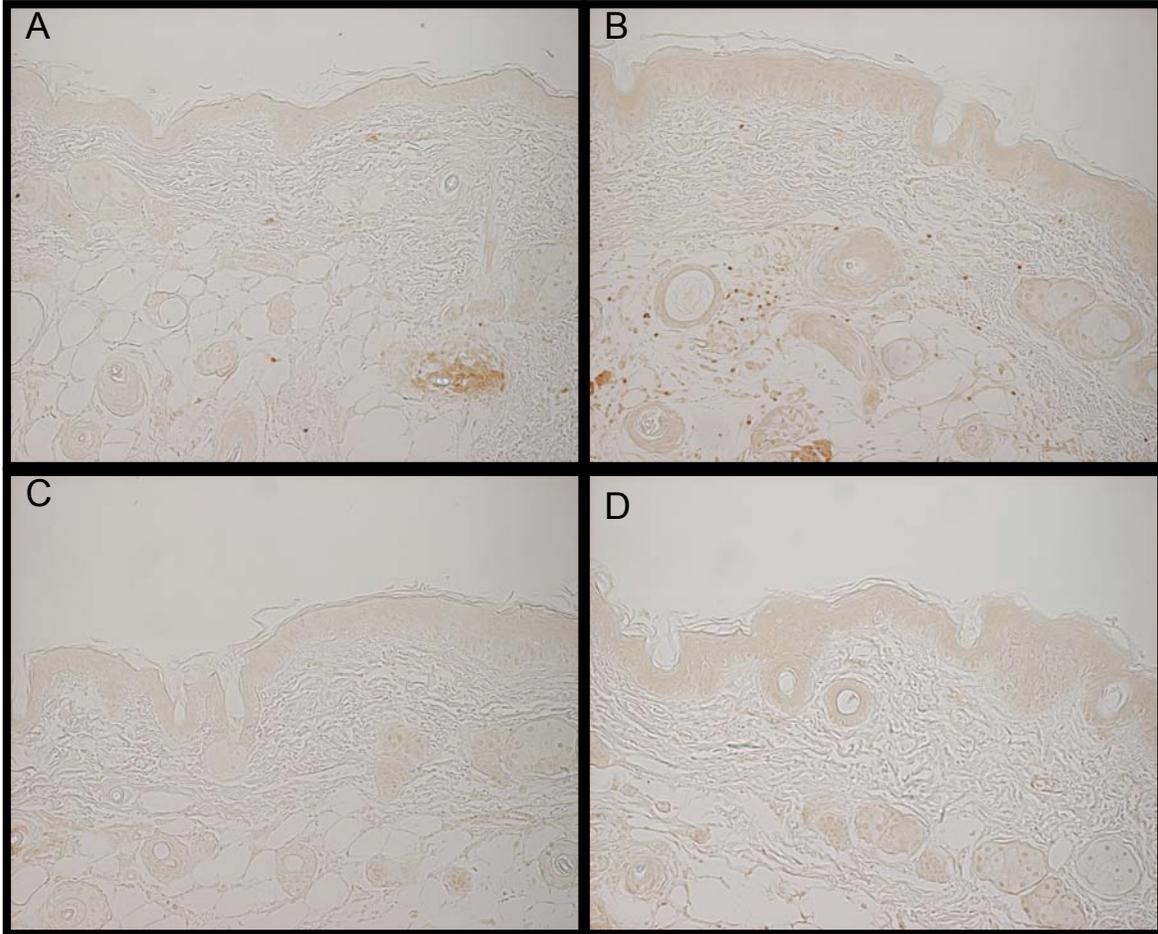


Figure 2B. Histological sections of LY-6G stained tissue in the pre-treatment KY group (A), UVB/KY (B), 0.03% Protopic (C), and UVB/0.03% Protopic (D). Increased neutrophils seen in (B) are not present in any other group. Skin thickness is consistent. All photographs were taken at a magnification of x 20.

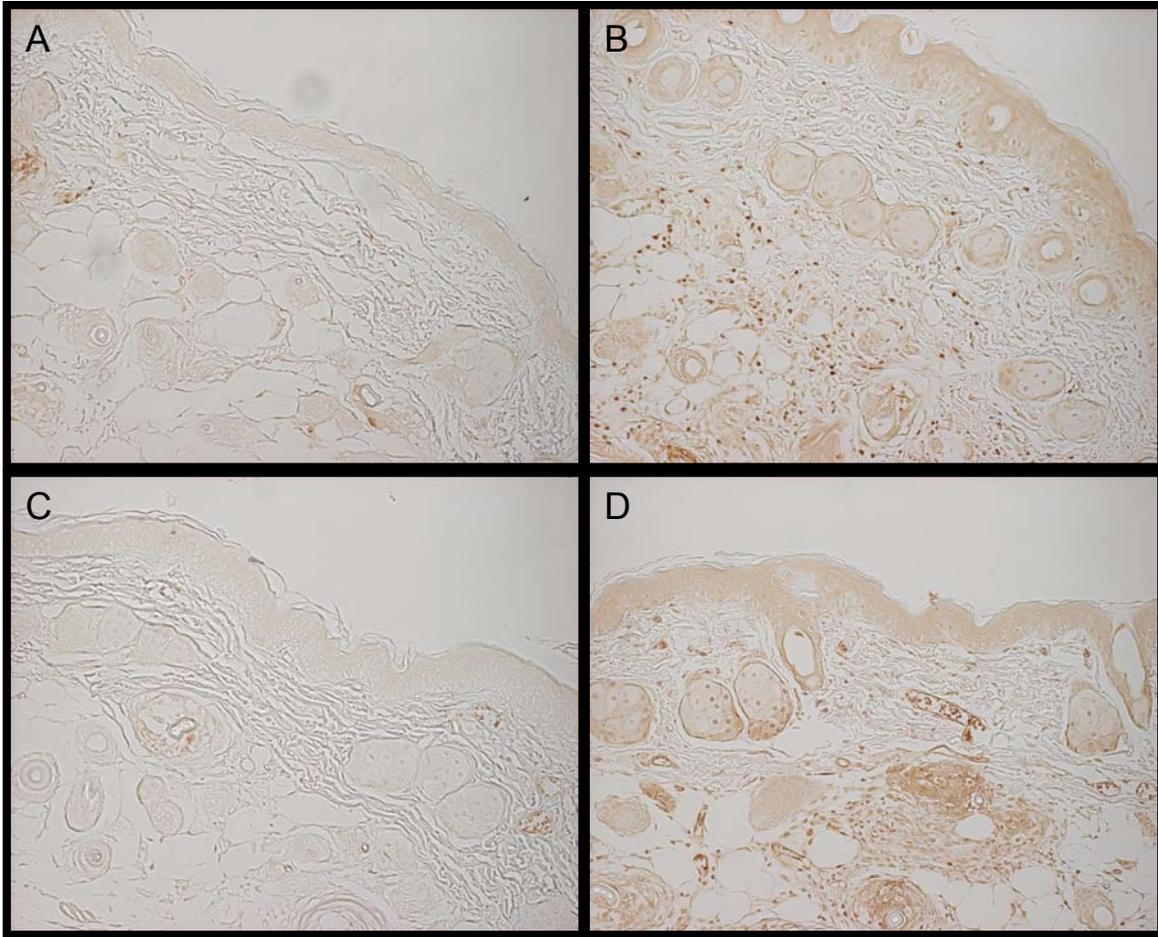


Figure 2C. Histological sections of LY-6G stained tissue in the concurrent-treatment KY group (A), UVB/KY (B), 0.03% Protopic (C), and UVB/0.03% Protopic (D). Neutrophils are prevalent in UV groups (B) and (D). All photographs were taken at a magnification of x 20.

activation. Mean units of MPO for the post-treatment group are shown in Fig. 3A. UVB/KY was significantly higher as compared to KY ($p < 0.0000002$) and both UVB/Protopic groups were significantly lower than UVB/Elidel ($p < 0.000007$ and $p < 0.0002$) and UVB/KY. All non-UVB groups show a consistent level of MPO below that of the UVB groups. Mean units of MPO for the pre-treatment group are shown in Fig. 3B. Here, a reversal is seen in the levels of MPO for the two drug groups. UVB/Elidel is significantly lower than UVB/KY, UVB/0.03% Protopic ($p < 0.000009$) and UVB/0.1% Protopic ($p < 0.0002$). Also, there is a significant reversal in MPO levels between UVB/0.03% Protopic and UVB/0.1% Protopic when comparing the post-treatment and pre-treatment groups. Mean units of MPO for the concurrent-treatment group are shown in Fig. 3C. Here, again, UVB/Elidel is lower than the UVB/Protopic groups. And, similarly to the post-treatment group, UVB/0.1% Protopic is significantly higher ($p < 0.0002$) than UVB/0.03% Protopic.

Effect of Elidel and Protopic on PGE₂ Levels in the Skin

Prostaglandin E₂ (PGE₂), the enzymatic product of COX-2, promotes the carcinogenic process by contributing to uncontrolled proliferation of damaged cells that have the potential to form skin tumors [65]. PGE₂ levels (pg/mg) for the post-treatment group are shown in Fig. 4A. UVB/KY is significantly higher than KY ($p < 0.032$) and UVB/0.1% Protopic is significantly lower than UVB/KY ($p < 0.032$). PGE₂ levels for the pre-treatment group are shown in Fig. 4B. UVB/0.03% Protopic is significantly higher ($p < 0.037$) than UVB/KY and UVB/0.1% Protopic is significantly lower ($p < 0.021$) than UVB/0.03% Protopic. Finally, PGE₂ levels for the concurrent-treatment groups are

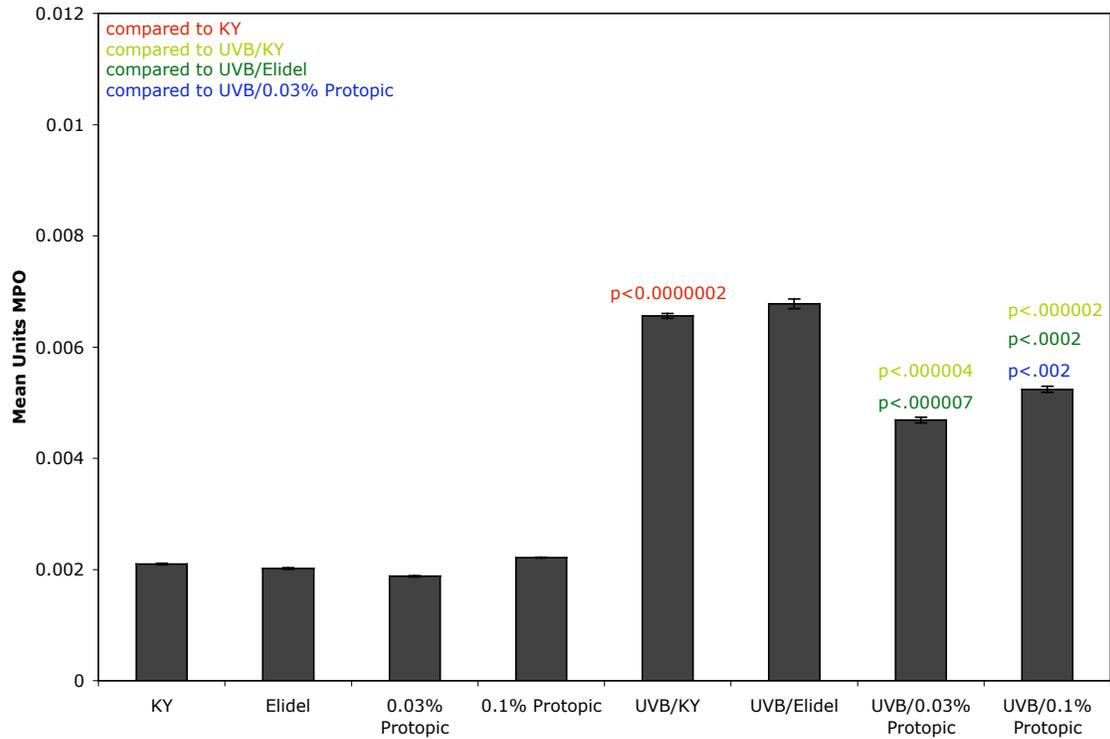


Figure 3A. Average mean units MPO \pm standard error in the post-treatment group. Three mice were present in all non-UVB groups, while 8 mice are represented in all UVB groups. Statistically significant differences ($p < 0.05$) are highlighted in color.

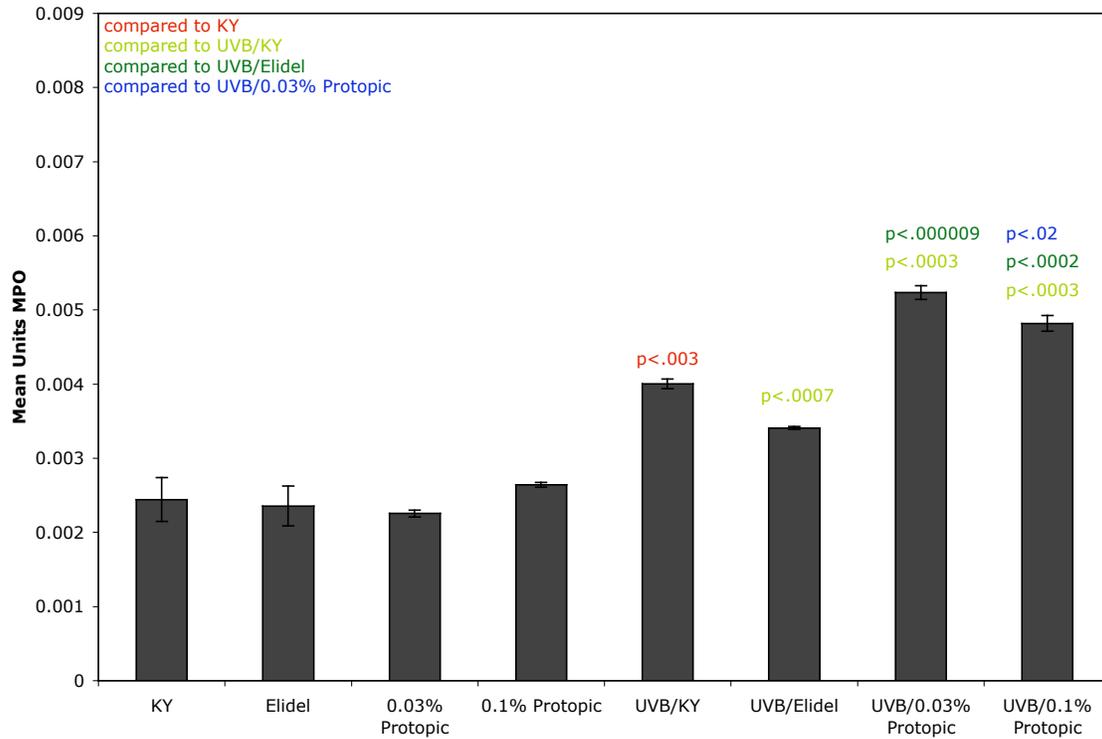


Figure 3B. Average mean units MPO \pm standard error in the pre-treatment group. Three mice were present in all non-UVB groups, while 8 mice are represented in all UVB groups. Statistically significant differences ($p < 0.05$) are highlighted in color.

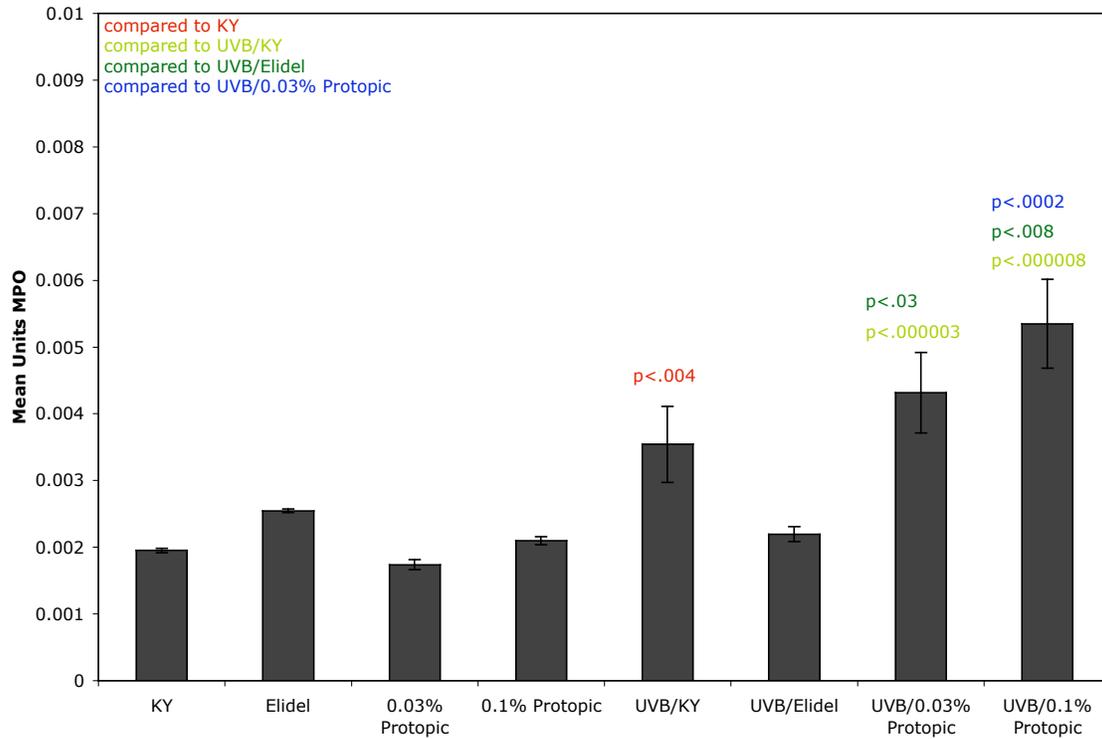


Figure 3C. Average mean units MPO \pm standard error in the concurrent-treatment group. Three mice were present in all non-UVB groups, while 8 mice are represented in all UVB groups. Statistically significant differences ($p < 0.05$) are highlighted in color.

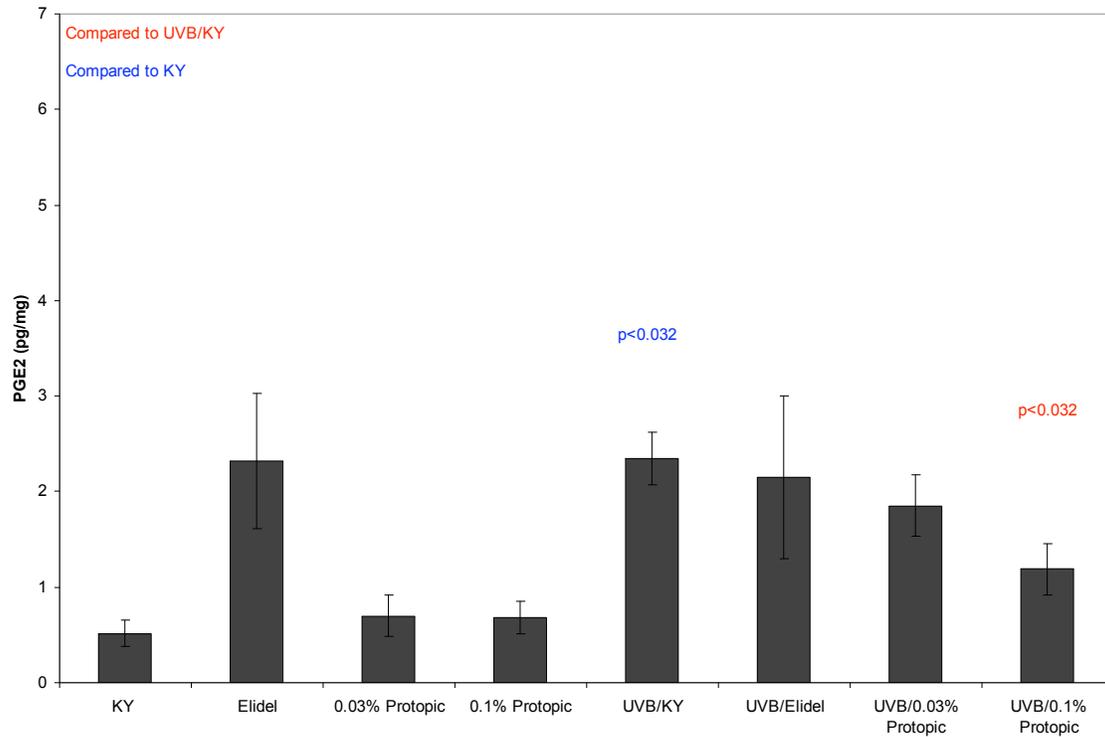


Figure 4A. Average pg/mg of PGE₂ ± standard error in the post-treatment group. Two mice are represented in each non-UVB group. Statistically significant differences (p<0.05) are highlighted in color.

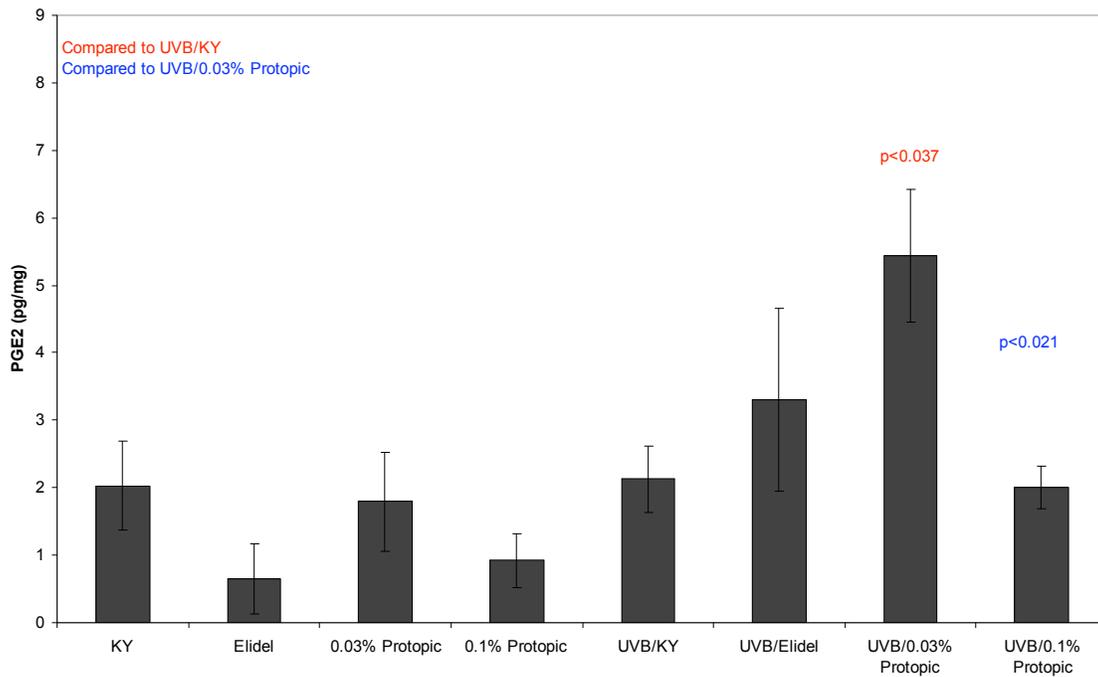


Figure 4B. Average pg/mg of PGE₂ ± standard error in the pre-treatment group. Two mice are represented in each non-UVB group. Statistically significant differences (p<0.05) are highlighted in color.

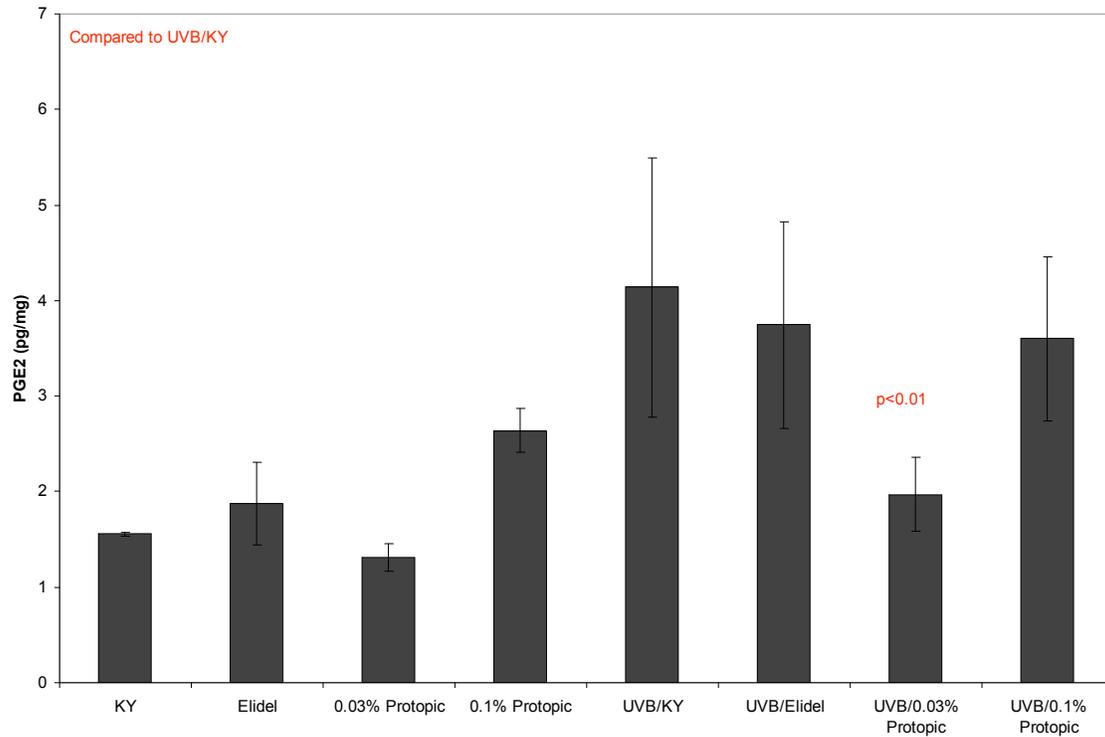


Figure 4C. Average pg/mg of PGE₂ ± standard error in the concurrent-treatment group. Three mice are represented in each non-UVB group except 0.1% Protopic, which has two. Seven mice represent all UVB groups. Statistically significant differences (p<0.05) are highlighted in color.

shown in Fig. 4C. UVB/0.03% Protopic is significantly lower than UVB/KY ($p < 0.01$) and is the only statistically significant difference amongst the data.

Quantitative RT-PCR Assessment of COX-2 Gene Expression

Cyclooxygenase-2 (COX-2) is an inducible enzyme involved in the immediate-early gene response to inflammatory stimuli, including UVB light [66-69]. Its induction results in increased prostaglandin production and inflammatory cell infiltration. HPRT is a common, stable housekeeping gene. To see if Elidel or Protopic induced higher levels of COX-2 in the mice, RT-PCR analysis was performed. Average ratios of COX-2 to HPRT are shown for the post-treatment group in Fig. 5A, the pre-treatment group in Fig. 5B, and the concurrent-treatment group in Fig. 5C. As can be seen in the figures, there is no significant difference in the levels of COX-2 gene expression between the treatment groups.

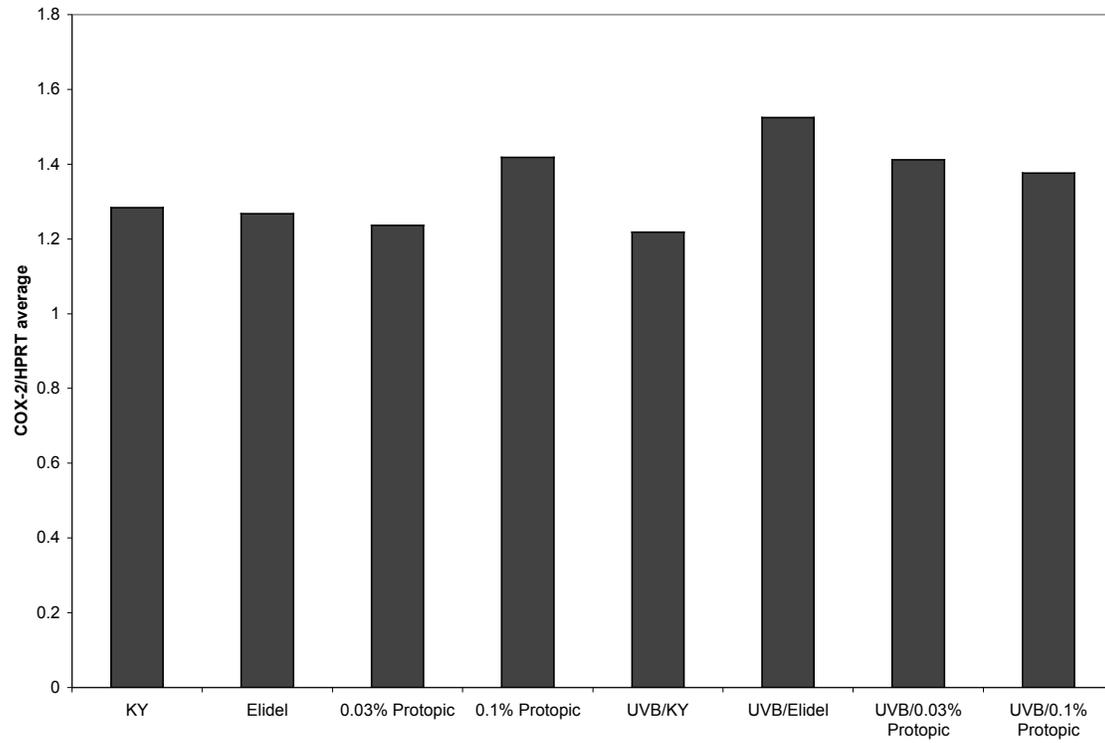


Figure 5A. Semi-quantitative RT-PCR assessment of COX-2 gene expression in the post-treatment group. Average ratios of COX-2 to HPRT are displayed.

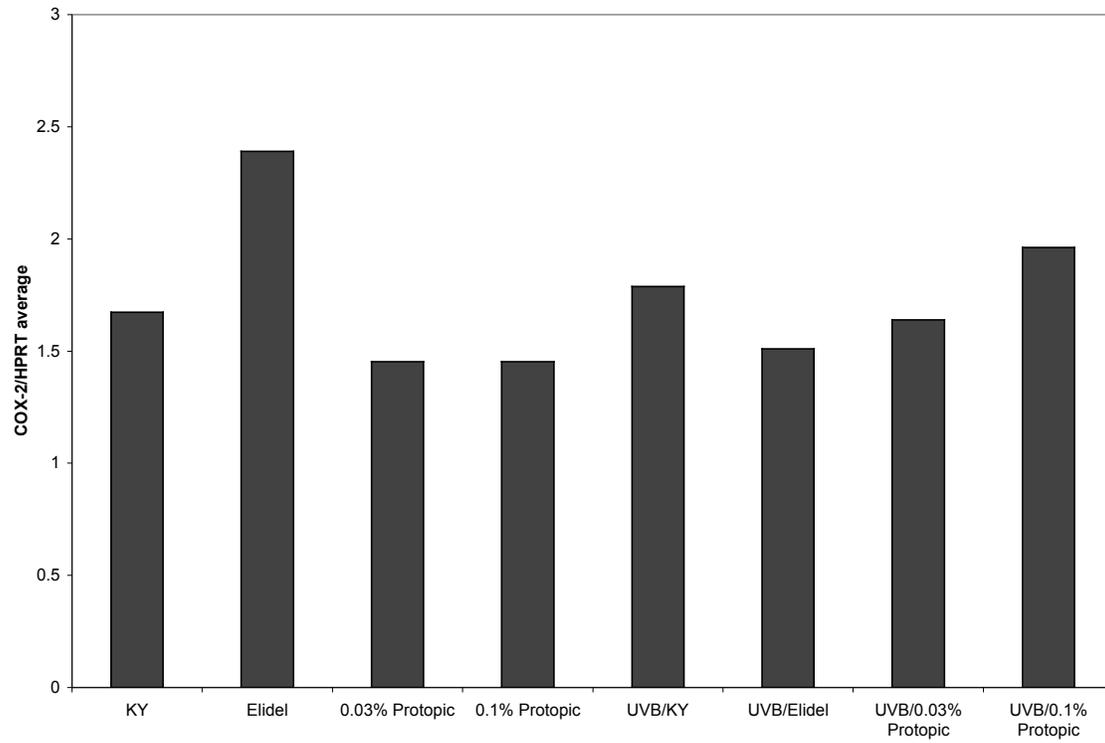


Figure 5B. Semi-quantitative RT-PCR assessment of COX-2 gene expression in the pre-treatment group. Average ratios of COX-2 to HPRT are displayed.

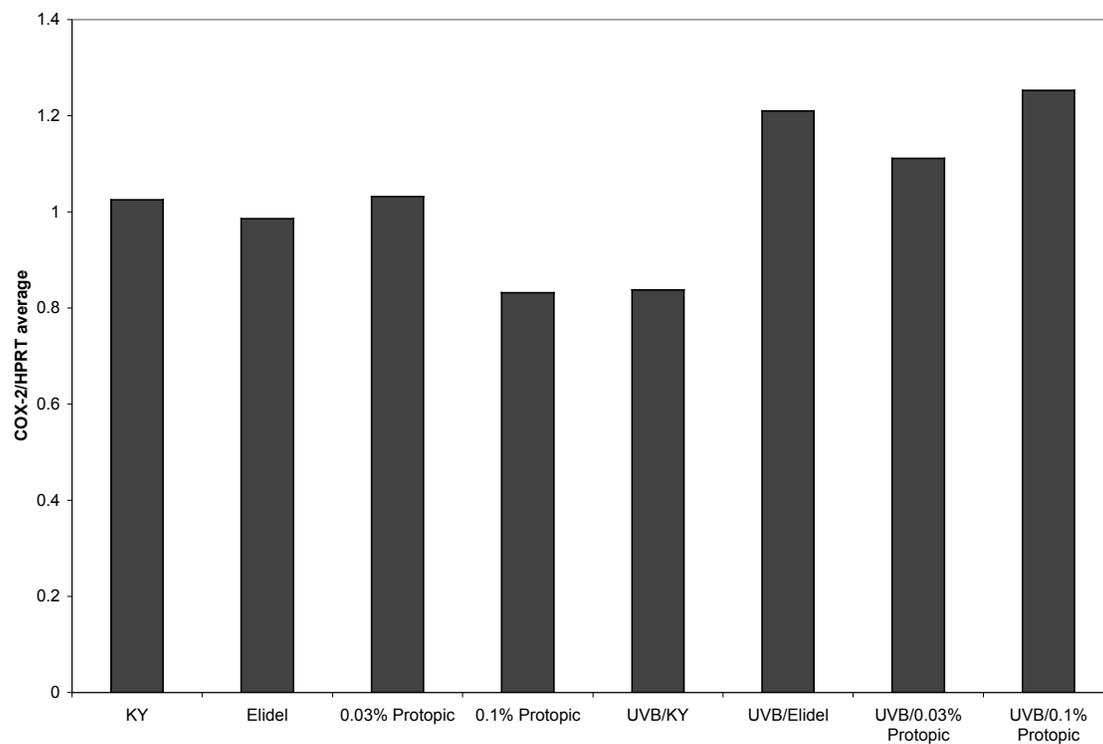


Figure 5C. Semi-quantitative RT-PCR assessment of COX-2 gene expression in the concurrent-treatment group. Average ratios of COX-2 to HPRT are displayed.

Discussion

Newer therapies for the treatment of AD include topical treatment with the calcineurin inhibitors tacrolimus and pimecrolimus. Since oral use of these same drugs by solid organ transplant recipients has been linked to an increase in the development of SCC of the skin, there is some concern that prolonged topical use of these drugs in combination with UVB exposure may result in increased development of skin cancers in AD patients. A small number of animal studies as well as a small number of reports of cancers in children and adults treated with Elidel or Protopic resulted in the FDA adding a “black box” warning of safety concerns associated with the use of these drugs [59]. However, the need for this type of warning remains controversial [43].

The current studies were designed in an attempt to understand the effect of treatment with TCIs on the acute UVB-induced inflammatory response in the skin. There is a clear link between inflammation and the development of a variety of cancers [70]. Previous studies in our laboratory have found that decreasing UVB induced inflammation by topical treatment with the anti-inflammatory drug celecoxib resulted in a 50% decrease in UVB induce skin tumor [71]. This data suggests that sustained inflammation in the skin may result in increased tumor development. The present study was designed to examine any potential changes in the acute inflammatory response in the skin following TCI treatment in an attempt to understand the potential mechanism by which these drugs could play a role in promoting the development of SCC.

In trying to relate the results from the murine model to humans it is important to determine the clinical relevancy of the post-treatment, pre-treatment, and concurrent-treatment groups. In the post-treatment method, mice were irradiated dorsally followed

by 100 μ l of topical treatment with either the vehicle or the drugs immediately and at 24 hrs following exposure. This method is not as clinically relevant as the pre-treatment and concurrent-treatment groups due to the timing of application. Most patients will first have an outbreak and then apply the drugs for a few days afterward. Then, patients will go out in the sun or use it while being out in the sun.

From our studies it can be seen that the time of treatment and UVB exposure changes the inflammatory response to UVB exposure in the skin. The data from Fig. 3A-C suggests that Protopic heightens the inflammatory response to UVB with increased applications. The more potent 0.1% preparation also increased MPO levels (Fig. 3C). Interestingly, while two doses of Elidel increased the UVB-induced inflammatory response (Fig. 3A, 4A), increasing the number of applications of Elidel actually decreased the inflammatory response to UVB in the skin (Fig. 3B,C and 4B,C). Immunohistochemical analysis of infiltrating neutrophils told the same story. Neutrophil infiltration was not dramatic in the pre-treatment LY-6G sections. The concurrent-treatment showed more dramatic infiltration, attributed to more drug applications. There were greater increases in skin thickness in the concurrent-treatment as well, implying greater edema. Over a long period of time, one would expect a greater accumulation of inflammatory products in Protopic-treated skin. Elidel still poses these risks, but to a lesser extent. This accumulation signifies possible tumor development as continuous damage is being done to cells and DNA.

Based upon our previous studies we chose to evaluate the contribution of COX-2 and PGE₂ to the inflammatory response observed in the treated skin. It appears that the observed inflammatory changes in the skin of Skh-1 mice are most likely not being

mediated by COX-2 expression and subsequent PGE₂ production. Fig. 4A-C shows a great fluctuation in PGE₂ levels between the two drugs with little statistically significant data points. Looking at Fig. 5A-C also shows no real significant difference between non-UVB and UVB treatments. It is known that calcineurin inhibitors can affect Th1 and Th2 cytokines that have been implicated in the development of AD [72]. It is most likely that Elidel and Protopic are affecting cytokine pathways that are mediating the observed altered inflammatory response not linked with COX-2 gene expression. Alternatively the observed increase in inflammation may be a result of altered trafficking of T-cells into the skin. In unpublished studies carried out in our lab we have shown that selective depletion of CD4 positive T cells results in a significant increase in UVB induced acute inflammation in the skin, and increased tumor development. While we did not examine the numbers of T-cells infiltrating into the skin in the present study, it is well established that these drugs work by inhibiting calcineurin-mediated gene transcription associated with T-cell activation and cytokine production [73]. It is therefore possible that the altered levels of inflammation were a result of changes in CD4 positive T-cell migration into the skin following drug treatment and UVB exposure.

In conclusion, the current study suggests that the timing and number of UVB exposures and drug applications can influence inflammatory levels in the skin. Repeated application of the immunosuppressant drugs prior to and during UVB exposure resulted in the infiltration of neutrophils and increased MPO levels that have been linked with increased tumor development when present in the skin chronically. COX-2 expression and PGE₂ production does not seem to be directing inflammatory levels in the skin in the current study, even though they are intimately involved in many inflammatory processes.

Future studies of benefit should explore potential mechanisms for the role of Elidel and Protopic in promoting inflammation with UVB exposure. Of course, the results of long-term clinical human studies will ultimately determine the fate of these drugs. However, it may be several years before clear data is available to assess the long-term safety and efficacy of Elidel and Protopic. In the mean time careful monitoring of UVB exposure during treatment is necessary.

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