Published in final edited form as:
J Dermatol Sci. 2014 June ; 74(3): 236–241. doi:10.1016/j.jdermsci.2014.02.003.

Human *in vitro* skin organ culture as a model system for evaluating DNA repair

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Abstract

Background—UV-exposures result in accumulation of genetic lesions that facilitate the development of skin cancer. Numerous pharmacologic agents are currently under development to both inhibit formation of DNA lesions and enhance repair. Drugs must be evaluated *in vitro,* currently performed in cell culture systems, before being tested on humans. Current systems do not account for the architecture and diverse cellularity of intact human skin.

Objective—To establish a novel, functionally viable, and reproducible *in vitro* skin organ culture system for studying the effects of various pharmacologic agents on DNA repair.

Methods—Human skin was obtained from neonatal foreskins. Intact skin punches derived from foreskins were cultured *in vitro* prior to exposure to UV-irradiation, and evaluated for DNA-damage using a DNA dot blot. Serial skin biopsies were obtained from patients with actinic keratoses treated with topical imiquimod. Expression of immune-stimulating and DNA repair genes was evaluated in *ex vivo* and *in vitro* samples.

Results—DNA dot blots revealed active repair of UV induced lesions in our *in vitro* skin organ culture. The photo-protective effect of sunscreen was detected, while imiquimod treatment did not enhance DNA repair *in vitro*. The DNA repair molecules XPA and XPF were up-regulated in the skin of imiquimod treated patients with actinic keratoses and imiquimod treated bone marrow-derived cell lines, but not keratinocytes.

Conclusion—Our *in vitro* human skin organ culture model detected repair of UV-induced DNA lesions, and may be easily adapted to investigate various photo-protective drugs intended to prevent or treat skin cancer.

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Keywords

Human skin organ culture; DNA repair; UV-irradiation

1. Introduction

The epidermis is the primary physical, immunological, and biochemical barrier of the body which protects internal processes from ongoing environmental insults. Solar ultraviolet (UV) irradiation is one of the most common and ongoing physical insults to the skin, as well as a major source of DNA damage, either through direct impact or via generation of oxidative stress [1]. Chronic UV exposure results in the accumulation of genetic lesions that facilitate the development of skin cancer. For example, UVB-induced immunosuppression depletes the skin's natural defenses against oncogenic processes by decreasing immunologic surveillance [2,3]. Direct damage to antigen presenting cells (APCs) such as Langer-hans' cells (LCs) inhibits their ability to present antigens, thus impairing Th1 type immunity [4,5]. Furthermore, UV irradiation induces immunosuppressive cytokines such as IL-10, which shifts the cutaneous microenvironment to an immunotolerant state, leading to loss of contact hypersensitivity [6,7]. Loss of sensitization to tumor antigens that form as a result of chronic UV exposure is thought to be a fundamental event in the development of skin cancer. Instead of activating the classic Th1 type response to tumor antigens, chronically sun exposed skin becomes immunotolerant and thus more permissive to neoplasm formation.

Following UVB irradiation, DNA damage can be detected in the form of photoproducts such as cyclobutane pyrimidine dimers (CBPDs) [8]. CBPDs may be repaired via the nucleotide excision repair (NER) mechanism, resulting in restoration of cell function and integrity [9]. The study of pharmacologic agents that may upregulate NER genes in the context of UV damage is valuable for understanding the pathogenesis of skin cancer as well as for developing various photoprotective therapies. Previous studies have assessed DNA damage and repair using human volunteers, animals, or culture of cell monolayers [10]. However, few studies have employed the use of whole human skin sections to examine the process of post-UV DNA repair, especially in the context of pharmacological modulators.

Our previous studies have demonstrated that imiquimod, a TLR7/8 agonist, enhances the survival of epidermal bone marrow-derived cells (BMDCs) both *in vitro* and in an *in vivo* mouse model. Following UV irradiation, imiquimod-treated specimens show upregulation of NER genes, and increased CBPD resolution in a MyD88-dependent manner [11]. Toll-like receptors (TLRs) are a family of pattern recognition receptors that alter the immune response to pathogen associated molecular patterns, which are structurally conserved "danger" signals such as microbial glycolipids and viral ssRNA. TLRs are critical receptors in the innate immune response that trigger signaling cascades resulting in inflammation and destruction of damaged cells. Recently, there has been evidence that certain cytokines released by TLR pathways may play a role in DNA repair [12,13]. The topical drug imiquimod is a TLR7/8 agonist currently used in the treatment of actinic keratoses, superficial basal cell carcinomas, genital warts, and various other skin conditions [14,15]. Although imiquimod's exact mechanism of action has yet to be elucidated, it is known to

signal through the myeloid differentiation primary response 88 (MyD88) and nuclear factor kappa B (NF-κB) pathways via TLR7/8 activation. Interleukin-12 (IL-12) is one of the products of this pathway and is produced by LCs when treated with imiquimod [16]. IL-12 has been shown to reduce UVB-induced DNA damage in human cells [13], which is thought to be due to its upregulation of several NER genes, such as XPA and XPF [12]. Hence, we postulated that via activation of TLR7/8, imiquimod enhances DNA repair in APCs, thus allowing for proper maturation and function of the APC.

In this study we have developed a novel *ex vivo* model for the evaluation of DNA repair after UV irradiation and drug treatment using a human skin organ culture. The primary goal of this study was to create a viable *ex vivo* model that closely emulates the physical and biochemical properties of whole human skin, which can be easily adapted to evaluate various photoprotective drugs. Given recent publications highlighting its role in preventing UV-induced immunosuppression as well as its known use as an anticancer treatment, we utilized our human skin organ culture to study the effects of imiquimod in the context of post-UV DNA repair [11,16]. Additionally, we studied 3 patients treated with 5% imiquimod cream for actinic keratoses over a course of 2 weeks. Serial skin biopsies showed consistent upregulation of the NER genes XPA and XPF following treatment, demonstrating imiquimod's efficacy in stimulating DNA repair *in vivo*. We then set out to establish a comparable *ex vivo* model using human whole skin explants. Our *in vitro* human skin organ culture system closely resembles *in vivo* skin conditions, and reproduces observed *in vivo* biologic phenomena by preserving the integrity of normal skin structure.

2. Materials and methods

2.1. Reagents

Topical imiquimod (1-[2-methylpropyl]-1*H*-imidazo[4,5-c]quinolin-4-amine, R-837) 5% cream and vehicle were provided by Graceway Pharmaceuticals (Exton, PA). Imiquimod powder (Grace-way) was prepared as a stock solution in sterile water at 1 mg/ml and diluted to the appropriate working concentrations. DMEM (Dulbecco's Modified Eagle's Medium) was supplemented with 10% FCS (fetal calf serum), 1 mg/ml ciprofloxacin, and 200 mM L-glutamine. RPMI (Roswell Park Memorial Institute) 1640 medium was supplemented with 20% FCS and 1% penicillin/streptomycin. Keratinocytes were cultured in Epi-Life growth medium supplemented with epidermal growth factor and pituitary extracts (Cascade Biologics, Portland, OR). Dispase (BD Biosciences) was diluted in PBS to a working concentration of 15 U/ml.

2.2. Cell lines

HaCaT cells (spontaneously immortalized human keratinocyte cell line) were cultured in DMEM supplemented with 10% FCS, 1 mg/ml ciprofloxacin, and 200 mM L-glutamine. KG-1 cells (human monocytic acute myeloid leukemia cell line) were maintained in RPMI 1640 supplemented with 20% FCS and 1% penicillin/streptomycin. Primary KCs were prepared from newborn foreskins by dermis separation using diapase digestion then trypsinization of the epidermal layer. Use of newborn foreskins was approved by the

University of Maryland Institutional Review Board. All cells were cultured in a 37 8C in a humidified incubator with 5% CO_2.

2.3. Skin organ culture and epidermal isolation

All tissue samples for skin organ cultures were obtained from human neonatal foreskins following circumcision. Skin explants were divided into sections, rinsed with PBS, and cultured in an air-medium interface in at 37 8C, under 5% CO_2. The culture medium consisted of DMEM supplemented with 10% FCS 1 mg/ml ciprofloxacin, and 200 mM L-glutamine. Each experiment used skin samples from at least 4 different donors, with ratios from each donor normalized across experimental groups to reconcile for any individual differences or variations in skin type. For analysis of epidermal DNA damage following UV treatment, skin sections were incubated with dispase 15 U/ml for 1 h at 37° to promote separation of epidermis from dermis. Epidermis was then isolated using forceps and DNA was extracted from epidermis as described below.

2.4. Skin organ cultures with imiquimod-enriched medium

Circular 4 × 4 mM full-thickness punch biopsies were taken from foreskins and the sections were rinsed with PBS. Sections were incubated dermal side down and epidermal side exposed to air in 96-well plates containing DMEM or imiquimod-enriched DMEM (20 μg/ml) at 378 for 24 h to allow drug penetration. Sections were then rinsed with PBS and transferred to new wells filled with sterile gauze soaked in fresh medium, and irradiated with 70 mJ/cm^2 UVB. At 0, 6, and 12 h following UV treatment, epidermis was harvested for DNA extraction and dot blot.

2.5. Skin organ cultures with topical imiquimod

Foreskins were trisected and rinsed with PBS. The epidermal sides of each section were gently rubbed with 70% ethanol to dry. Sections were then rubbed with imiquimod 5% cream, vehicle, or left untreated. Sections were transferred dermal side down to 6-well plates filled with sterile gauze soaked in medium, and incubated at 37 °C for 24 h. Sunscreen was then applied to the untreated sections of skin for 1 h prior to UV irradiation. 70% ethanol was then used to remove any residual imiquimod cream, vehicle, or sunscreen from all skin sections. 4 × 4 mM punch biopsies were taken from each skin section, rinsed with PBS, and transferred to 96-well plates dermal side down on medium soaked gauze as described above. Samples were then irradiated with 70 mJ/cm^2 UVB and epidermis was harvested at 0 and 12 h for DNA extraction.

2.6. UV irradiation and light source

Skin organ cultures were irradiated with a panel of 48 Q-Sun light bank (Q-Panel Laboratory products, Cleveland, OH) (equipped with a UVC WG320 filter) at a distance of 12 inches from the light source. The spectral emission profile of this light source closely mimics that of natural sunlight (400–280 nm), emitting predominantly UVA (400–315 nm). A UVB (315–280 nm) radiometer (National Biologic Corporation, Twinsburg, OH) was used to determine UVB output, and calculate the time necessary to deliver the desired doses of UVB.

2.7. DNA extraction and dot blot

Whole genomic DNA was extracted from epidermal sections using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Extracted DNA was quantified using NanoDrop spectrophotometer and 500 ng of heat-denatured DNA was spotted onto a nitrocellulose membrane allowed to dry at room temperature for 2 h, then baked at 80 8C for 20 min. The membrane was then probed with an anti-CBPD antibody (Cosmo Bio cat no. NMDND001) and developed using secondary antibody and reagents from the WesternBreeze kit (Invitrogen cat no. WB7104) according to manufacturer's protocol. Image density quantification was performed using Image-Pro Plus software version 4.5.1.29 (Media Cybernetics).

2.8. RNA extraction, cDNA synthesis, and quantitative real-time PCR

Primary keratinocytes and HaCaT cells were cultured in monolayers whereas KG-1 cells were grown in suspension. Each cell type was treated for 24 h with 10 μg/ml imiquimod dissolved in medium. RNA was extracted from cells and cDNA synthesized for RT-PCR. Standard methods were used to extract total cellular or tissue RNA using the RNeasy Kit (Qiagen) following manufacturer's protocol. cDNA was then synthesized from 2 μg of total RNA using the First-Strand cDNA synthesis kit (GE Healthcare cat no. 27-9261-01) following manufacturer's protocol. Detection of XPA and XPF mRNA was performed using quantitative real-time PCR (Light-Cycler; Roche, Indianapolis, IN) according to previously published methods [17]. Primers to amplify human DNA repair genes were as follows: XPA (NM_011728.1) and XPF (NM_015769.1). All primers were purchased from SuperArray Biosciences (Qiagen, Valencia, CA). Relative levels of XPA and XPF mRNA were normalized to 18S mRNA (NM_X03205.1). The primers, PCR protocol, and product quantification for 18S mRNA were as reported previously [17]. The cDNA was assayed in triplicate, and mean values are depicted in the graphics for the quantitative real-time PCR.

2.9. Immunofluorescence

CBPDs were identified on frozen cryostat sections of specimens from skin organ cultures using specific antibodies. Skin sections from organ cultures were embedded in OCT and frozen overnight at −20 °C. Cryostat sections (12 μm) were fixed with acetone/methanol (1:1) for 10 min at 20 8C and dried at room temperature. Sections were incubated with a primary monoclonal mouse anti-CBPD antibody (Cosmo Bio cat no. NMDND001) overnight. Sections were then washed and incubated with secondary Ab conjugated with goat-anti-mouse rhodamine conjugated Ab (Santa Cruz) for 1 h. Slides were visualized using a Nikon Eclipse E600 microscope and the images were documented using SpotTM imaging system (Diagnostic Instruments, Sterling Heights, MI).

2.10. Human volunteers

Three patient volunteers with actinic keratoses were treated with 5% imiquimod cream applied to the affected areas twice per week at bedtime. Serial punch biopsies were taken at baseline, 1 week, and 2 weeks of treatment. These serial biopsies were taken from the same affected area on each patient. This study was approved by the local IRB at University of Maryland, Baltimore.

2.11. Statistical analyses

GraphPad Instat and Prism software programs (GraphPad, San Diego, CA) were used to compare quantitative data from experimental groups for statistical significance. For comparisons between multiple groups, ANOVA was used; $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Imiquimod upregulates NER genes in patients following topical treatment

To evaluate the effect of imiquimod on DNA repair *in vivo*, we studied 3 patients with actinic keratoses who were treated with topical 5% imiquimod cream over a course of 2 weeks. Serial skin biopsies were taken at baseline, 1 week, and 2 weeks of treatment and skin sections were evaluated with RT-PCR for expression of the NER genes XPA and XPF. We selected these two genes because they are expressed early in the DNA repair process [18]. Their importance is clinically evident, as defects in these two NER genes result in the severe xeroderma pigmentosa phenotype [19]. There was a 2 to 4 fold increase in expression of XPA (Fig. 1A) and XPF (Fig. 1B) in patients 2 and 3 following imiquimod treatment compared to baseline values. However, since it is infeasible to continue experimentation on patient volunteers, our next step was to establish an *ex vivo* skin organ culture model to further investigate the DNA repair properties of imiquimod.

3.2. Skin organ cultures showed active DNA repair following UVB

Skin organ cultures were pretreated with varying doses of imiquimod (20–100 μg/ml) for 6 h. RT-PCR was then performed on RNA extracted from the tissue samples to assess for IL-6 and IL-8 expression, which have been previously shown to increase in keratinocytes treated with imiquimod [20–22]. There was a 1.8 fold upregulation of IL-6 and a 2-fold upregulation of IL-8 at the dose of 20 μg/ml imiquimod, which became the effective dose for subsequent experiments (Fig. 2A). Higher concentrations of imiquimod did not result in further upregulation of these two genes. Two sets of experiments were then performed using skin organ cultures pretreated with imiquimod to study the drug's effects on post-UV DNA repair, quantified by resolution of CBPDs on dot blot. In the first set of experiments, skin sections were pretreated with either medium alone or imiquimod 20 μg/ml for 24 h prior to UVB irradiation (70 mJ/cm^2). After irradiation, DNA extracted from epidermis at 0, 6, and 12 h was blotted for CBPDs. There was a gradual resolution of CBPDs over the post-UV time course, signifying a viable and functional skin organ culture (Fig. 2B). In the second set of experiments, skin sections were pretreated with either topical vehicle or commercially formulated imiquimod 5% cream rubbed on the epidermal surface for 24 h prior to UVB irradiation (70 mJ/cm^2). After irradiation, DNA extracted from epidermis at 0 and 12 h was blotted for CBPDs. There was again resolution of CBPDs over the post-UV time course (Fig. 2C). While there was marked repair over time in the *in vitro* cultured skin sections, imiquimod did not significantly enhance DNA repair as was observed in imiquimod treated actinic keratosis patients.

3.3. Immunofluorescent staining shows visible resolution of CBPDs over 6 h time course in skin organ culture

Histopathological studies further confirmed the viability and functionality of our skin organ culture model to measure DNA repair. Skin samples from experiments were sectioned and stained with a rhodamine-labeled CBPD antibody. Immediately following UV irradiation, extensive epidermal DNA damage was observed (Fig. 3A). However, 6 h following irradiation, there was a marked resolution of CBPD-positive cells in the epidermis (Fig. 3B). These findings verify the presence of active DNA repair in our skin organ cultures.

3.4. Imiquimod did not enhance DNA repair in skin organ culture

Although the skin organ cultures were viable and showed active DNA repair over the post-UV time course, there was no significant difference in rate of repair between imiquimod-treated and medium/vehicle-treated samples (Fig. 4A–C). Densitometry analysis performed on dot blots did not reveal an effect of topical or medium-enriched imiquimod on DNA repair (Fig. 4A and B). Similarly, the number of CBPD-positive cells from staining experiments decreased at equitable rates in the treated and untreated samples post-UV. However, CBPD were significantly decreased when sunscreen was applied to the skin organ culture before irradiation (Fig. 4B), which further supports the efficacy of our *in vitro* human skin organ culture system.

4. Discussion

Our DNA repair model using skin organ culture utilized the preserved structural integrity of whole skin to study the biochemical and immunological processes following UV irradiation. Here, we have demonstrated a viable, functional, and reproducible method for examining the effects of various pharmacologic agents on post-UV DNA repair. The skin sections were able to maintain many basic functions of normal skin as well as intact DNA repair machinery for up to 4 days in culture and 12 h after UV treatment. Thus, this model serves as a useful tool for evaluating the effect of drugs on DNA repair in the epidermis.

These studies did not demonstrate enhancement of DNA repair in the skin organ cultures following treatment with imiquimod. This apparent discordance with our other studies using human volunteers, animals, and bone marrow-derived cells may be due to a variety of factors. The epidermis is comprised primarily of keratinocytes (>95%), which do not express TLR7/8 [23]. Since our dot blots used DNA from whole epidermis, UV-damaged keratinocytes accounted for the majority of CBPDs detected. On the other hand, bone marrow-derived cells such as LCs express TLR 7/8 and respond robustly to imiquimod by increasing levels of IL-12 mRNA transcripts [24]. We initially postulated that using our culture system we may be able to detect an imiquimod effect, as LCs activated by imiquimod may potentially influence neighboring keratinocytes via cytokine diffusion and thus provide an indirect protection against UV damage. In addition, several other cell types in the skin such as mast cells, myeloid dendritic cells, and plasmacytoid dendritic cells express TLR7/8 [25]. Upon TLR activation, these cells produce a variety of inflammatory cytokines including IL-12 [26]. Thus, they may also be able to offer protection to neighboring keratinocytes. Finally, some studies have shown that despite lacking TLR7/8,

keratinocytes can still respond to imiquimod by upregulation of IL-6 and IL-8, suggesting the presence of an alternative TLR7/8-independent pathway [20–22]. Schön et al. found that imiquimod directly antagonizes the adenosine A_{2A} receptor, which they proposed as a method for activating NF-kB in keratinocytes, which is also activated by the TLR pathways [27].

Considering these findings, it is surprising that the skin organ culture did not respond to imiquimod by enhancing DNA repair. A possible explanation of our findings is that LCs migrate out of the epidermis and towards draining lymph nodes shortly after UV irradiation [28]. In addition, imiquimod alone has been shown to alter LC morphology and promote migration to local lymph nodes [29]. When skin is subjected to both UV irradiation and imiquimod, a majority of LCs are depleted from the epidermis. *in vivo*, this corresponds to the initiation of the cutaneous immune response as inflammatory cells are recruited from regional lymph nodes to act in the skin. This basic response is essential in defending the body against viruses, foreign antigens, tumors, and perhaps stimulates DNA repair. However, our skin organ culture did not accommodate for the migration of APCs to the local lymph nodes and is thus limited in this regard. As migratory cells are depleted from the epidermis following imiquimod and UV, the remaining keratinocytes cannot benefit from ambient IL-12 production in the skin organ culture because there is no recruitment of systemic immune cells. Lastly, although keratinocytes may respond directly to imiquimod in a TLR7/8-independent fashion [27], they do not upregulate DNA repair genes. Our additional studies did not show an increase in XPA or XPF gene expression when keratinocyte monolayers (HaCaT and primary keratinocytes) were treated with imiquimod (data not shown). However, monolayers of KG-1 cells, a monocytic cell line, treated with imiquimod did exhibit upregulation of NER gene expression (data not shown). This further illustrates the differential effects of imiquimod on bone marrow derived cells versus keratinocytes, where DNA repair is directly enhanced by the drug in the former but not the latter.

When sunscreen was applied in our *in vitro* skin organ culture model, we found that CBPDs were significantly decreased immediately following and 24 h after UV, as compared to untreated skin. From this finding we can conclude that our model system is useful for determining the effectiveness of DNA damage blockers, such as sunscreen. Our system will serve as a powerful tool to measure the efficacy of such compounds on intact human skin, thus limiting exposure of volunteers to new and potentially harmful chemicals during drug development.

5. Conclusions

This study serves as a preliminary investigation of the time course of post-UV epidermal DNA repair in response to imiquimod, using CBPD resolution as an endpoint. Here we have established a novel, functionally viable, and reproducible *in vitro* skin organ culture system for studying the effects of various pharmacologic agents on DNA repair. Imiquimod did not enhance repair in the skin organ culture, likely due to the requirement of an *in vivo* system for mounting the full cutaneous immune response. However, this model may be easily

adapted to investigate various other photo-protective drugs intended to prevent or treat skin cancer, or to detect phototoxic properties of experimental drugs.

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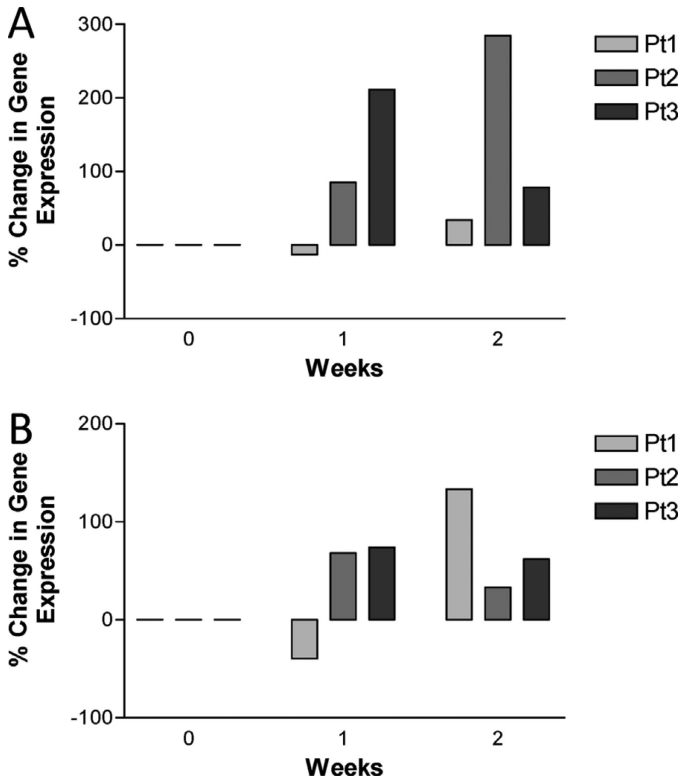

Fig. 1.

Imiquimod enhanced gene expression of the DNA repair enzymes XPA and XPF in human volunteers. Serial skin biopsies were taken at baseline, 1 week, and 2 weeks of treatment with 5% imiquimod cream for actinic keratoses. *Ex-vivo* skin sections were analyzed with RT-PCR. (A) XPA expression increased 2–4 fold in patients 2 and 3 over course of treatment. (B) XPF expression increased 2–3 fold in patients 2 and 3 over course of treatment.

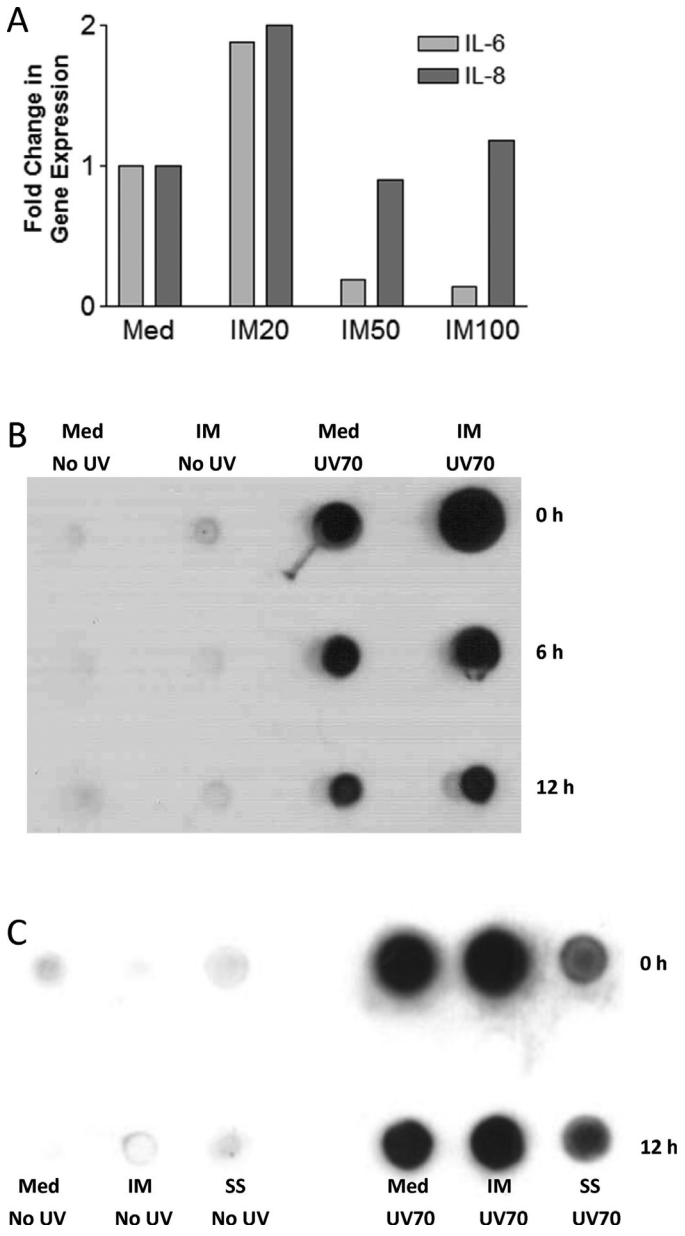

Fig. 2.

Active DNA repair was observed during the post-UV time course in skin organ cultures treated with topical or medium enriched imiquimod. (A) The effective concentration of imiquimod (IM) 20 μg/ml was determined based on upregulation of IL-6 and IL-8 with this dose. (B) In skin organ cultures treated with medium alone (MED) or imiquimod 20 μg/ml prior to UV irradiation, resolution of CBPDs was observed at 6 and 12 h. (C) In skin organ cultures treated with topical vehicle, imiquimod 5% cream, or sunscreen (SS) prior to UV irradiation, resolution of CBPDs was observed at 12 h. In both sets of experiments, unirradiated samples served as additional negative controls.

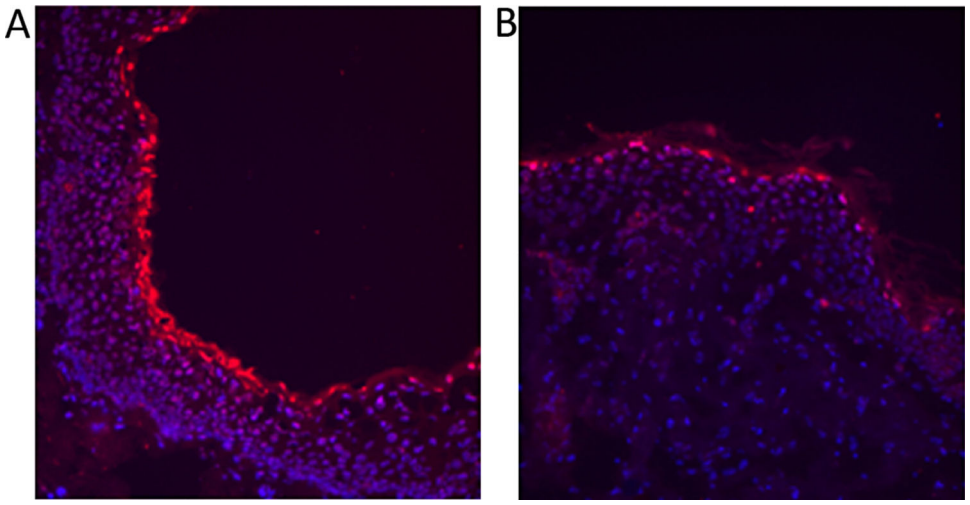

Fig. 3.

Immunofluorescent staining for CBPDs in tissue sections confirmed active DNA repair. (A) At 0 h following UV irradiation, several CBPD-positive cells were detected within the epidermis (red). (B) However, at 6 h post-UV, there was a marked decrease in CBPD-positive cells, indicating active DNA repair. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

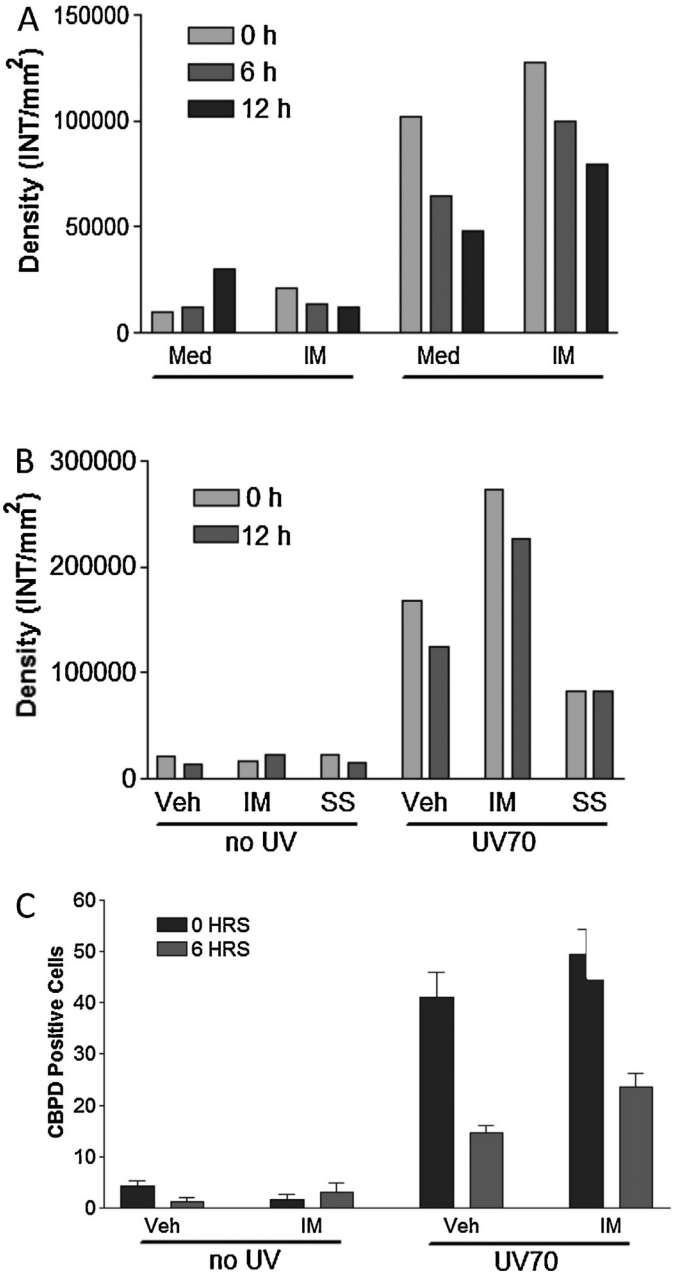

Fig. 4.

Imiquimod did not enhance DNA repair in skin organ cultures. Densitometry analysis of dot blots shown in Fig. 2 did not reveal any additional benefit in DNA repair for samples treated with (A) medium-enriched (Med), or (B) topical imiquimod (IM). Sunscreen (SS) pre-treatment is shown to decrease CBPD. (C) Similarly, counts of CBPD-positive cells from tissue staining experiments shown in Fig. 3 did not show enhanced DNA repair in imiquimod-treated samples, relative to vehicle control.