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Endogenous Retinoic Acid Required to Maintain the Epidermis Following Ultraviolet Light Exposure in SKH-1 Hairless Mice‡

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Abstract

Ultraviolet light B (UVB) exposure induces cutaneous squamous cell carcinoma (cSCC), one of the most prevalent human cancers. Reoccurrence of cSCC in high-risk patients is prevented by oral retinoids. But oral retinoid treatment causes significant side effects; and patients develop retinoid resistance. Exactly how retinoids prevent UVB-induced cSCC is currently not well understood. Retinoid resistance blocks mechanistic studies in the leading mouse model of cSCC, the UVB exposed SKH-1 hairless mouse. To begin to understand the role of retinoids in UVB-induced cSCC we first examined the localization pattern of key retinoid metabolism proteins by immunohistochemistry 48 hours after UVB treatment of female SKH-1 mice. We next inhibited retinoic acid (RA) synthesis immediately after UVB exposure. Acute UVB increased RA synthesis, signaling, and degradation proteins in the stratum granulosum. Some of these proteins changed their localization; while other proteins just increased in intensity. In contrast, acute UVB reduced the retinoid storage protein lectin:retinol acyltransferase (LRAT) in the epidermis. Inhibiting RA synthesis disrupted the epidermis and impaired differentiation. These data suggests that repair of the epidermis after acute UVB exposure requires endogenous RA synthesis.

Graphical Abstract

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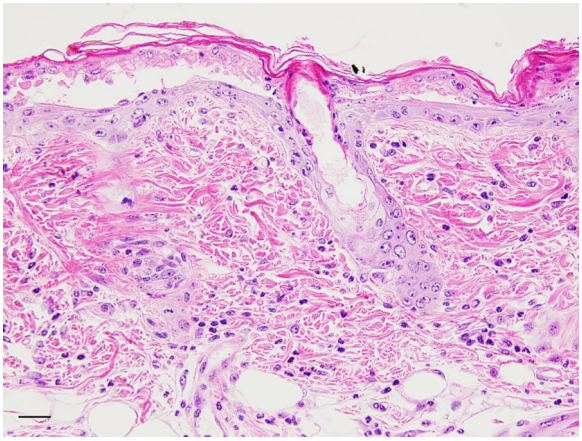

INTRODUCTION

UVB has a wavelength between 280-315 nm and causes sunburns (erythema) (1, 2). Acute UVB induces an inflammatory response characterized by increased neutrophils, monocytes, COX-2 activity, and PGE_2 (3, 4). Long-term exposure to UVB results in: photoaging; immunosuppression; and skin cancers, such as basal cell carcinoma (BCC), squamous cell carcinoma (cSCC), and melanoma (2). UVB causes: DNA damage; reactive oxygen species (ROS) formation; and induction of p53 (1, 2, 5). During acute UVB exposure, p53 arrests the cell cycle such that cyclobutane-pyrimidine and thymine dimers can be repaired (1, 2). When DNA cannot be repaired, p53 induces apoptosis (6). Aberrant DNA repair or mutations in p53 that fail to induce apoptosis lead to permanent DNA damage and cancer, especially cSCC (1). UVB exposure also increases proliferation and alters the expression and localization of differentiation markers (7, 8). Altered differentiation peaks 48 hours after UVB and is restored within 3-7 days. Very high doses of UVB also impair barrier function (9).

Retinoids are a class of natural compounds and synthetic drugs derived from vitamin A. Retinoids include: retinol; all-*trans* retinoic acid (atRA and the drug Retin A); 13-cis RA (isotretinoin); etretinate; and acretin. Exogenous retinoids produce variable effects on cSCC due to retinoid resistance. Both oral and topical RA reduce cSCC in the chemical carcinogenesis mouse model where 7,12-dimethylbenz[*a*]anthracene (DMBA) initiates and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) promotes tumorigenesis (10, 11). These chemicals cause defects in the RAS signaling pathway that account for only 21% of cSCC in humans (12). Retinoid treatments produced variable effects in the photocarcinogenesis model of cSCC using UVB or UVA&B treated hairless mice. Topical RA accelerated (13), inhibited (14), or had no effect (15) on photocarcinogenesis. These differential effects could be due to either the dose of RA or differences in the background strain. Both oral retinol and etretinate also failed to alter photocarcinogenesis when given at two high doses (16). In humans, low doses of oral retinoids prevent the reoccurrence of carcinomas in high-risk patients; but significant prevention by oral retinoids only occurs for a few years and relapse occurs upon stopping treatment (17-20). Topical RA failed to prevent carcinogenesis in

humans (21-23). Therefore RA has the potential for chemoprevention; but something is blocking RA's effectiveness.

Regulation of retinoid metabolism maintains precise levels of RA. Retinol enters the cell by either passive diffusion or the transport protein referred to as stimulated by RA6 (STRA6) (24), and binds cellular retinol binding protein (RBP1) within the cell (25). The majority of retinol that enters the keratinocyte is stored by the action of lectin:retinol acyltransferase (LRAT) (26, 27). The remaining retinol is reversibly oxidized to retinal by retinol dehydrogenases (28, 29). Retinal is subsequently oxidized to RA by retinal dehydrogenases 1-3 (ALDH1A1, 2, and 3)(30). RA then binds either Cellular RA binding protein 1 or 2 (CRABP1, CRABP2). CRABP1 directs RA to degradation enzymes cytochrome P450 26A1, B1, and C1 (CYP26A1, B1, and C1)(31). In contrast, CRABP2 protects RA from degradation by CYP26 enzymes and directs the transport of cytosolic RA to the nucleus where it binds to its receptors (RARA, RARB and RARG) (32). These receptors are RA inducible transcription factors of the nuclear hormone family (33, 34) that regulate the expression of >500 genes involved in: differentiation; cell cycle control; and apoptosis (35). RA also regulates its own metabolism and function by directly inducing LRAT, CRABP2, CYP26A1, and RARB (36-43).

UVA and UVB cause retinol and retinyl esters to degrade, leading to reduced concentration of these retinoids in the skin (44-46). In vitro, UVA/UVB causes retinoids to photoexcite and form reactive oxygen species (ROS) (47). But these effects are not seen in vivo. In cultured keratinocytes, UVB exposure also increases dehydroretinol synthesis (48). Thus, UVB exposure alters the ratio of retinol to dehydroretinol and RA to dehydroRA. UVB and UVA also change the skin expression of retinoid metabolism proteins. UVA treatment decreases RXRA, increases B-carotene dioxygenase activity, and increases CRBP1 (44, 46). Sun exposed skin and the precursor lesion actinic keratosis (AK) express higher levels of CYP26A1 (49). SCC cell lines contain reduced retinyl esters and LRAT activity (50-52). Patients with cSCC express less message levels of Rara, Rarg, and Rarb1' than patients with basal cell carcinoma (53).

To better understand retinoid resistance in UVB induced cSCC, we examined the effects of acute UVB exposure on key retinoid metabolism proteins. In the stratum granulosum, acute UVB reduced LRAT, and increased CRBP1, DHRS9, ALDH1A2, CRABP2 and CYP26A1. To better understand the role of this UVB-induced RA, we inhibited RA synthesis immediately after UVB exposure. Reducing RA damaged the suprabasal epidermis in a dose dependent manner. These results suggest that UVB-induced RA is important for the repair process after acute UVB exposure. In addition, some of these changes in retinoid metabolism and signalling proteins may contribute to retinoid resistance well before cancers arise in the UVB exposed skin.

MATERIALS AND METHODS

Animals

Pilot experiment—RA reporter mice that contain a retinoic acid response element (RARE) linked to beta galactosidase-(Tg(RARE-Hspa1b/lacZ)12Jrt/J) were wax stripped

and 3 days later topically treated with acetone or 2 mg/g BW of disulfiram daily for 3 days and sacrificed 24 hours after the last dose. Skin was fixed in Feketes acid-alcohol-formalin fixative (61% ethanol, 3.2% formaldehyde, 0.75N acetic acid) overnight, routinely processed, embedded in paraffin and sectioned at 5-6 μm, and placed on microscope slides (Superfrost/Plus Fisherbrand, Pittsburgh, PA). The Ohio State University Institutional Animal Care and Use Committee approved all procedures.

Main experiment—Female SKH-1 mice (6-8 weeks old, 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 and fed Harlen Teklad diet #7912 (Harlen Teklad, Indianapolis, IN) ad libitum. Mice were exposed to (N=10) or not exposed to (N=3-4) one minimal erythemic dose of UVB (2240 J/m^2 as determined by a UVR meter; (UVP Inc., Upland, CA) emitted by Phillips FS40 UVB lamps (American Ultraviolet Company, Lebanon, IN) that were fitted with TA422 Kodacel filters (Eastman Kodak, Rochester, NY) to ensure the exclusion of UVC light and emission primarily of UVB light (290–320 nm). This was immediately followed by topical application of 1.5 or 2.0 mg disulfiram per g body weight or vehicle (acetone). Mice were sacrificed 48 hours later and dorsal skin was harvested and fixed in Feketes acid-alcohol-formalin fixative (61% ethanol, 3.2% formaldehyde, 0.75N acetic acid) overnight, routinely processed, embedded in paraffin and sectioned at 5-6 μm, and placed on microscope slides (Superfrost/Plus Fisherbrand, Pittsburgh, PA). The Ohio State University Institutional Animal Care and Use Committee approved all procedures.

Histological analysis: Hematoxylin and eosin (H&E) stained sections of skin (2-3/slide) were scored for lesions after consultation with a board-certified veterinary pathologist (Krista La Perle, DVM, PhD, DACVP) by methods modified from several published protocols (54-58). Clefting lesions between the stratum spinosum (SS) and stratum granulosum (SG) layers were scored for severity on a 3 point scale: 1 = thin, barely detectable cleft between the SG and SS (**Figure 3a, green arrow**); 2 = wider and taller cleft between the SG and SS containing cell debris (**Figure 3b, green arrow**); and 3 = very long and wide cleft +/− cell debris (**Figure 3c, green arrow**). A 6 point scale, incorporating the percent of the skin sections affected, was then created: 1 = ≤ 5% severity 1; 2 = > 5% severity 1; 3 = ≤ 5% severity 2; 4 = > 5% severity 2; 5 = ≤ 5% severity 3; 6 = > 5% severity 3. Lesions characterized by clefting with retention of viable keratinocytes within the SG and SS were semi-quantitatively scored as none (0), mild (1= ≤ 5%), moderate (2= 6-20%), or severe (3= > 20%) (**Figure 3g, blue arrow**). The presence of parakeratotic hyperkeratosis within the stratum corneum was noted but not analyzed as this lesion was simply increased by the UVB treatment as expected. Hypereosinophilic keratinocytes with pyknotic nuclei were counted in subcorneal layers (**Figure 3g, black arrow**). Sub-basilar clefting (**Figure 3g, arrowhead**) was scored on a three-point scale similar to the clefting between the SS and SG. The percentage of the skin sections in which intracellular edema was present was also recorded. Skin fold thickness, measured using digital calipers on whole skin, was confirmed by measuring epidermal thickness between the outer surface of the stratum granulosum and the inner edge of the stratum basale in digital photomicrographs. Photomicrographs were taken of 6 random 200× fields with an Olympus BX51 microscope and attached DP71

camera (Olympus, Tokyo, Japan). The minimum and maximum thickness in each image was measured with cellSens standard version 1.11 (Olympus, Tokyo, Japan) and averaged. The number of keratinocyte cell layers were also counted in both the minimum and maximum regions from each image and averaged.

Immunohistochemistry: Immunohistochemistry (IHC) was performed as per Everts et al (2007) (59). Antibodies against STRA6, CRBP, DHRS9, ALDH1A1, ALDH1A2, CRABP2, and LRAT were made in David Ong's laboratory, while antibodies against CYP26A1 (Alpha Diagnostics Intl,San Antonio, TX), β-galactosidase (Abcam, Cambridge, MA), RARA (Santa Cruz Biotechnology, Dallas, TX), Loricrin (LOR), and Keratin 6 (KRT6, Biolegend, Dedham, MA) were purchased. Biotinylated anti-rabbit secondary antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA) while a horseradish peroxidase conjugated anti-biotin tertiary antibody was purchased from Bethyl Laboratories (Montgomery, TX). All antibodies were mixed in blocking solution consisting of 3% Bovine Serum Albumin (Fraction V, Fisher BioReagents #BP1600, Pittsburg, PA) in 100mM TBS and 1.28% normal goat serum (Vector, Burlingame, CA). The red 3-amino-9-ethylcarbazole plus enhancers (AEC+) substrate chromogen and aquamount were purchased from Dako (Carpinteria, CA). Gils Hematoxylin III was purchased from Poly Scientific (Bay Shore, NY). A nonspecific IgG, produced during affinity purification, was used as a negative control. Immunoreactivity in the epidermis was semi-quantitatively scored in a blinded fashion on a 4-point scale for both intensity (negative (0), weak (1), moderate (2), strong (3), or very strong (4)) and percent of cells (0 = 0, 1-25 = 1, 26-50 = 2, 51-75 = 3, 76-100 = 4). An IHC level (IHCL) was calculated as the sum of intensity plus percent of cells as previously validated (60, 61). An IHCL of 8 is the maximum. Lesions and abnormally thin areas of the epidermis were scored on a similar scale of 0-8 for IHCL.

Statistics

Effects of UVB on retinoid metabolism—Mann Whitney tests were performed with GraphPad Prism version 5.0d (GraphPad Software, Inc., La Jolla, CA) or SPSS, version v21 (IBM; Armonk, NY). A p value less than 0.05 was considered significant.

Disulfiram study—Skin fold thickness, epidermal thickness, number of keratinocyte layers, Loricrin and Krt 6 expression were analysed by a 2 × 3 analysis of variance, followed by Tukey post hoc tests when appropriate using SPSS, version v22 (IBM; Armonk, NY). When a significant interaction occurred this was followed by a One-Way ANOVA and Tukey post hoc test. Lesions were analyzed in only the UVB treated mice by Kruskal-Wallis analysis, followed by Tukey post hoc tests when appropriate using SPSS, version v22 (IBM; Armonk, NY). Independent T-tests were performed to compare the IHCL in the lesions to normal skin in disulfiram treated samples for KRT6 and LOR using SPSS, version v22 (IBM; Armonk, NY).

RESULTS

Acute UVB exposure increased proteins involved in RA synthesis, signaling, and degradation; while reducing storage within the upper epidermis

SHK-1 hairless mice begin to lose hair after the first growth cycle (3). The hair follicles then become deformed and utriculus and dermal cysts form, leading to a very heterogeneous tissue. We found that retinoid metabolism proteins localized to specific cell layers of the hair follicle and epidermis that changed throughout the hair cycle (59). Because of these issues we chose IHC to examine the expression and localization of retinoid metabolism proteins 48 hours after UVB exposure. Immunoreactivity was then semi-quantitatively scored. In the upper epidermis (stratum corneum and stratum granulosum) acute UVB significantly increased: STRA6; CRBP (RBP1); DHRS9; ALDH1A2; CRABP2; RARA; and CYP26A1 (**Figure 1a-q**). STRA6, CRBP, DHRS9, ALDH1A2, CRABP2, and RARA localized closer to the stratum corneum. In contrast, CYP26A1 localized lower in the granulosum layer and extended into the stratum spinosum. In addition, UVB treatment reduced the expression of LRAT throughout the epidermis (**Figure 1p-r**).

Reducing RA synthesis altered differentiation and damaged the upper epidermis 48 hours after acute UVB treatment

A pilot study was performed first in RA reporter mice to confirm that a retinal dehydrogenase inhibitor (disulfiram) is effective at reducing RA synthesis *in vivo* when applied topically. **Figure 2** shows that disulfiram reduced RA synthesis in these mice to undetectable levels, as indicated by reduced β-galactosidase expression. To determine the role of UVB-induced RA, SKH-1 mice were topically treated with two doses of disulfiram immediately after the UVB exposure. Disulfiram treatment caused focal clefting of the epidermis between the spinous and granular layers in a dose dependent manner (**Figure 3a-d, green arrow**). Other areas of the epidermis were reduced to a single layer of keratinocytes. Disulfiram significantly reduced skin fold thickness and the minimal epidermal thickness (**Figure 3e-f**). The minimal number of keratinocyte layers ranged from 1-5, but the average of all 6 sites only trended to decrease with disulfiram ($p = 0.074$, data not shown). Disulfiram also did not alter the maximum epidermal thickness or number of cell layers, which ranged from 2-8 (data not shown). UVB treatment significantly increased all measures of epidermal thickness and cell layers ($p < 0.01$, **Figure 3e-f** and data not shown). UVB treatment also significantly increased clefting between the basal layer and the dermis (**Figure 3g, arrowhead**) and intracellular edema ($p < 0.001$); but disulfiram did not alter either of these lesions (data not shown). Disulfiram also dose dependently increased the number of hypereosinophilic keratinocytes with pyknotic nuclei (**Figure 3g, black arrow, i**). Both doses of disulfiram caused clefting with retention of viable keratinocytes subjacent to the stratum corneum (**Figure 3g-h, blue arrow**). LOR expression significantly increased with dislufiram dose in the stratum corneum (**Figure 4a-i**); but LOR was significantly reduced within regions containing viable keratinocytes compared to normal cells in both the stratum corneum and granulosum ($p < 0.05$; **Figure 4d, k**). UVB exposure significantly increased LOR IHCL in the spinous and basal layers (**Figure 4a-c, e-g**). KRT6 expression was used to assess epidermal hyperplasia (62). In epidermis thick enough to define all four layers, disulfiram and UVB treatments increased KRT6 IHCL in the granular and spinous

layers (**Figure 4j, k, m-t**). UVB also increased KRT6 IHCL in the basal layer (data not shown). In thin epidermis, disulfiram trended to decrease KRT6 in skin unexposed to UVB (Control verse high dose, $p = 0.062$); but KRT6 remained high in UVB exposed skin regardless of disulfiram (**Figure 4l**).

DISCUSSION

This study highlights the role of endogenous RA synthesis in UVB exposed skin. Acute UVB exposure changed the localization patterns and/or intensity of all proteins tested. Forty-eight hours after UVB exposure RA synthesis and signaling proteins localized to the upper stratum granulosum with increased intensity. Acute UVB increased CYP26A1 further down in the epidermis. Acute UVB also reduced retinoid storage by reducing LRAT in the epidermis. Inhibiting RA synthesis caused epidermal hyperplasia and cell death in the suprabasal cells; but left some live cells just under the stratum corneum. These results suggest that endogenously synthesized RA is important in epidermal differentiation and repair following UVB exposure.

To our knowledge, this is the first study to examine the effects of acute UVB on the localization of the complete system for retinoid metabolism. Sorg et. al. (2002)(44) found that CRBP expression increased in the epidermis upon UVA treatment. The same group found a non-significant decrease in CRBP expression with UVB treatment (45). This study, however, used homogenized epidermis and protein extractions, which do not account for the differences in expression between the skin layers. Here, we used IHC and were able to evaluate protein expression in the various epidermal layers. Our finding of increased CYP26A1 in the epidermis is consistent with reports in humans of greater expression of CYP26A1 in areas of skin exposed to sun and in actinic keratosis (49). The drop in LRAT expression is also consistent with previous findings of reduced retinyl esters with UVA and UVB exposure (44-46) and reduced LRAT expression and activity in SCC (50-52). It has been argued that a decrease in retinyl esters may contribute to retinoid deficiency in cancer cells, and LRAT expression may reduce cancer cell survival (51, 63). But by also showing that RA synthesis enzymes and binding proteins are increased we paint a different picture. CRABP2 and CYP26A1 are both induced by retinoic acid in the skin, and their increased expression in this study is an indication of increased RA synthesis and signaling with acute UVB exposure (42, 64, 65). Taken together, the changes in these key vitamin A metabolism proteins indicate that acute UVB exposure increased RA synthesis and signaling in the upper epidermis.

The epidermal lesions seen with disulfiram treatment are consistent with a role for this endogenously synthesized RA in epidermal differentiation during the repair of UVB exposed skin. The role of endogenous RA on epidermal differentiation has been elusive due to mixed results in genetically modified mice (66-72) and opposite results of exogenous RA in studies performed *in vivo* verses *in vitro* (73). The reduction of the suprabasal layers seen at the higher dose of disulfiram were also seen in two studies with dominant-negative RARA mice (68, 72). In addition, the current short-term study was able to show patches of skin with active apoptosis/necrosis of these layers in addition to patches with just the thin epidermis. This suggests that RA is involved in the maintenance of these layers after UVB

treatment. The lack of histological abnormalities with disulfiram treatment in skin not exposed to UVB is consistent with results in RARA/G double null mice (67). LOR is a marker of terminal differentiation and is normally expressed in the granular layer (74). The reduction of LOR in the viable cells just under the stratum corneum suggests that these cells are not terminally differentiated. Similar cells were also seen in skin specific conditional CYP26B1 null mice (71), suggesting that both RA synthesis and degradation are important for differentiation and formation of the cornified envelop. The overall increase of LOR throughout the corneum with disulfiram is also consistent with skin specific conditional CYP26B1 null mice (71).

Increased KRT6 was also seen in two studies with dominant-negative RARA mice (70, 72), in mice lacking CYP26B1 (71), and with exogenous RA treatment in vivo (73). KRT6 is a marker of epidermal hyperproliferation (62). However, Imakado et al (1995)(70) argue that increased KRT6 is also associated with impaired barrier function, as they saw an increase in KRT6 in the absence of increased proliferation in their dominant-negative RARA transgenic mice. In both their study and in mice lacking CYP26B1 (71) Krt6 is also increased after embryonic day 17.5, when the cornified envelope replaces the periderm in maintaining barrier function. Repair after UVB treatment may be another time when the cornified envelope needs to be reestablished. The drop in KRT6 seen in the thin skin in the absence of UVB exposure is consistent with a reduction of RA by disulfiram; as KRT6 is argued to be a RA target gene (73). The lack of such a drop in the thin skin of UVB exposed skin suggests that epidermal hyperplasia and potential effects due to defective barrier function are dominant over RA in the regulation of KRT6. Retinoid metabolism message levels are also increased during spontaneously regressing cutaneous keratoacanthoma (KA) in mice; and pharmacological doses of RA induced differentiation and led to regression of KAs (75). Thus, RA may prevent tumor formation by assisting in the repair of UVB exposed skin.

In conclusion, this study suggests that UVB exposure causes significant changes in retinoid metabolism throughout the epidermis, which impacts epidermal differentiation. Future studies are needed to investigate the role retinoids may be playing in the repair of UVB damage and restoration of barrier function. Promoting the differentiation of the epidermis and repairing UVB damaged skin are essential to prevent skin cancer.

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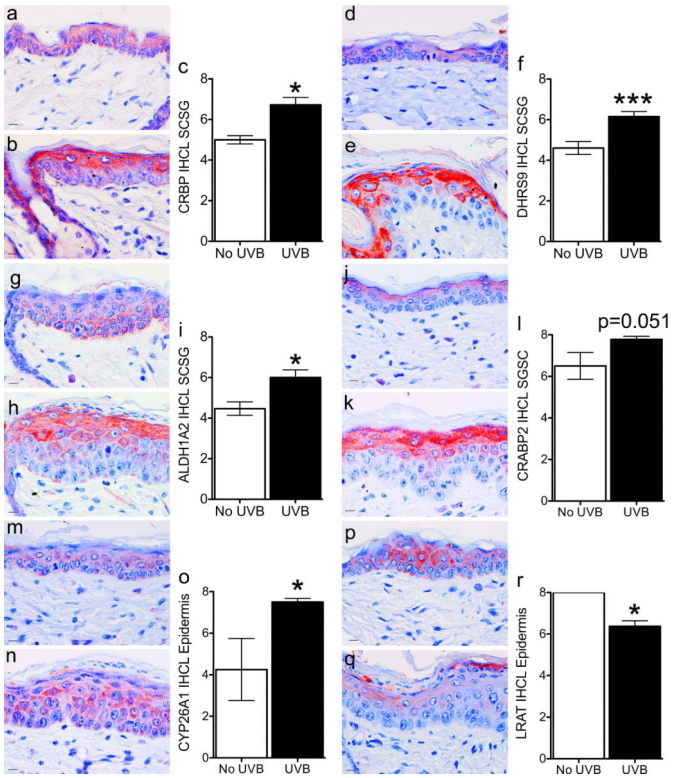

Figure 1. Acute UVB exposure localizes retinoid metabolism proteins to the upper epidermis

SKH-1 mice were unexposed (a, d, g, j, m, p) or exposed (b, e, h, k, n, q) to 1 MED of UVB light and samples collected 48 hours later. Immunohistochemistry (IHC) was performed with antibodies against CRBP (a-c), DHRS9 (d-f), ALDH1A2 (g-i), CRABP2 (j-l), CYP26A1 (m-o), or LRAT (p-r). An IHC level (IHCL) was determined as the sum of intensity (0-4) plus percent of cells (0-4) for a maximum IHCL of 8. Bar = 10.1 uM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

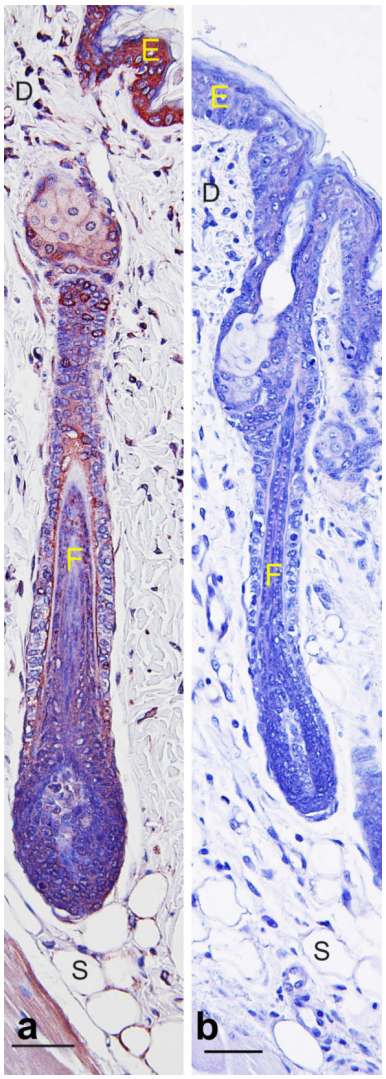

Figure 2. Disulfiram inhibits RA synthesis in RA reporter mice

RA reporter mice that contain a retinoic acid response element (RARE) linked to beta galactosidase-(Tg(RARE-Hspa1b/lacZ)12Jrt/J) were wax stripped and 3 days later topically treated with acetone (a, control), or 2 mg/g BW of disulfiram (b) daily for 3 days and sacrificed 24 hours after the last dose. Skin was fixed, routinely processed, paraffin embedded, and sectioned. IHC was performed with an antibody against beta-galactosidase using a red chromagen. Bar = 50 μM, n = 5. E=epidermis, D=dermis, F= hair follicle, S=subcutaneous fat.

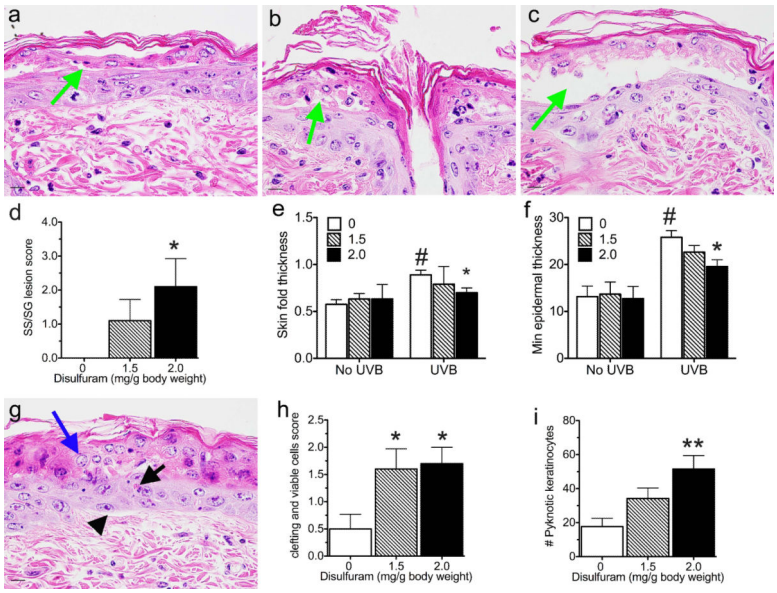

Figure 3. Reducing RA synthesis damages the epidermis

SKH-1 mice were unexposed or exposed to 1 MED of UVB light, treated with disulfiram, and samples collected 48 hours later. Clefting (green arrows) between the spinous and granular layers was scored as 1 (a), 2 (b), or 3 (c) and a 6 point scale was developed based on the amount of involvement (d). Skin fold thickness was measured on whole mice (e), while epidermal thickness was measured from photomicrographs (f). Retention of viable keratinocytes associated with clefts (g blue arrow) were scored on a 3 point scale based on skin involvement (h). Pyknotic keratinocytes (g black arrow) were counted (i). Arrowhead, sub-basilar clefts. Bar = 10.1 uM. * $p < 0.05$, ** $p < 0.005$ disulfiram verses control within UVB treatment group. # $p < 0.001$ all UVB treatment groups verses control groups.

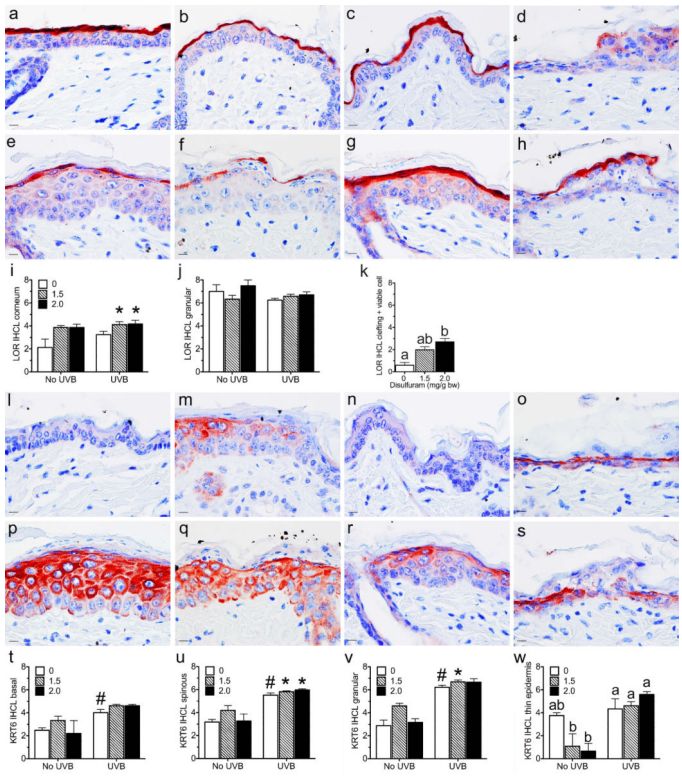

Figure 4. Reducing RA synthesis and UVB light altered LOR and KRT6 expression

SKH-1 mice were unexposed (a-c, l-n) or exposed (d-h, o-s) to 1 MED of UVB light, treated with 0 (a, e, l, p) 1.5 (b, f, m, q), or 2.0 (c, d, g, h, n, o, r, s) mg disulfiram per g body weight, and samples collected 48 hours later. Immunohistochemistry (IHC) was performed with antibodies against LOR (a-k), or KRT6 (l-w). An IHC level (IHCL) was determined as the sum of intensity (0-4) plus percent of cells (0-4) for a maximum IHCL of 8. Bar = 10.1 uM. * $p < 0.05$ disulfiram in both exposed and unexposed skin verses control groups. # $p < 0.001$ all UVB treatment groups verses control groups. Different letters are significantly different $p < 0.05$.