# Biopositive effects of low-dose UVB on the epidermal permeability barrier reinforcement and anti-microbial peptides expression

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# Biopositive effects of low-dose UVB on the epidermal permeability barrier reinforcement and anti-microbial peptides expression

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# Abstract

Biopositive effects of low-dose UVB on the epidermal permeability barrier reinforcement and anti-microbial peptides expression

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While high-dose UVB is detrimental to the epidermal permeability barrier, suberythemal doses of UVB are used to treat atopic dermatitis, which is characterized by defective permeability barrier and antimicrobial function. Because epidermal permeability barrier and antimicrobial peptides (AMP) expression are co-regulated and interdependent functions, we hypothesized that suberythemal doses of UVB exposure could regulate AMP expression in parallel with permeability barrier function.

Hairless mice were exposed to 40 mJ/cm$^2$ UVB (about 1/2 MED) daily for 1 or 3 days. Twenty-four hours after the last exposure, epidermal barrier function was assessed and skin specimens were taken for Western blotting, immunohistochemistry, and quantitative RT-PCR for mBD2, mBD3 and CRAMP. mRNA levels of the vitamin D receptor (VDR), 1α-hydroxylase and key epidermal lipid synthetic enzymes were also quantified.

After 3-days of UVB exposure, acceleration of barrier recovery and augmentation in expression of epidermal differentiation markers (e.g., involucrin and filaggrin) occurred in

parallel with increased mBD2, mBD3 and CRAMP expression at both the mRNA and protein level. VDR, 1α-hydroxylase, and the major epidermal lipid synthetic enzymes were also up-regulated. When an inhibitor of 1α, 25 dihydroxyvitamin $D_3$ formation, ketoconazole, was applied immediately after UVB exposure, the cutaneous vitamin D system was inhibited, which in turn, blocked epidermal lipid synthesis, AMP expression, and permeability barrier homeostasis, suggesting that the beneficial effect of low-dose UVB depend, at least in part, on activation of the cutaneous vitamin D system. Our results provide new insights into the mechanisms whereby low-dose UVB comprises effective therapy for atopic dermatitis.

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Key words: antimicrobial peptides, beta-defensin, cathelicidins, epidermal permeability barrier, vitamin D, ultraviolet B

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# I. Introduction

Atopic dermatitis (AD) is characterized by abnormal immune responses, impaired barrier function and an increased susceptibility to bacterial and viral infections. Expression levels of both hBD2 and LL-37 are significantly decreased in lesional skin[1]; i.e., up-regulation does not occur as in other inflammatory or infectious dermatoses[2]. Accordingly, the increased susceptibility to colonization and invasion by microbial pathogens that occurs in atopic dermatitis patients could be related to a combined deficit in permeability barrier function, coupled with low AMP expression. Accordingly, stratum corneum (SC) expresses a number of antimicrobial peptides (AMP), such as β-defensins, cathelicidins, RNase 7, and psoriasin, that both recognize and kill invading microorganisms, while functioning as distal sensors of the innate immune system[3]. Two important AMP families are the β-defensins (hBD1, 2, 3, and 4) and the cathelicidin carboxyterminal fragment (LL-37), whose murine homologues include mBD3 (hBD2) and CRAMP (LL-37), respectively. Two of these AMP, hBD2 and LL-37, are

expressed at low levels in normal skin, but higher levels are induced in wounded and/or diseased skin[2], with the important exception of atopic dermatitis[1]. Both hBD2 and LL-37 are co-packaged along with lipids within lamellar bodies (LB), and apparently delivered to the SC interstices by LB secretion[4, 5]. Acute permeability barrier perturbations also up-regulate mBD3 and CRAMP expression[6]. Such barrier perturbations initiate a cytokine cascade that up-regulates not only permeability barrier repair, but also hBD2 generation[7]. In contrast, LL-37 expression is not regulated by cytokines, but rather by ligands of the vitamin $D_3$ receptor[3, 8], which also are known stimulators of epidermal differentiation[9]. Notably, certain AMP (i.e., LL-37) are essential for the homeostasis of the permeability barrier[6]. Together, these results suggest a close interrelationship between the permeability and antimicrobial barriers that could be regulated in part through inflammatory cytokine signaling[3, 10].

Although narrowband UVB or UVA phototherapy is a mainstay of treatment, both natural and artificial UVB irradiation are commonly employed in the treatment of AD[11, 12]. Although the benefits of UVB are attributed generally to its immunomodulating activity, we hypothesized here that suberythemal UVB therapy could improve permeability barrier function and antimicrobial defense in parallel. In contrast, single, high doses of UVB (e.g., $\geq$ 4 MED) instead are known to exert detrimental effects on permeability barrier function[13-15]. Moreover, low-dose UV phototherapy is useful for the treatment of various skin disorders, including psoriasis and atopic dermatitis[11, 12, 16]. Our hypothesis is also supported by the observations that 1) 1.5 MED UVB irradiated skin becomes relatively resistant to irritants[17], and 2) irradiation with suberythemal dose of UVB stimulates epidermal ceramide production[18, 19]. Recently, repeated low-dose UVB has also been demonstrated to provide a limited protective effect against subsequent UV-induced inflammatory responses[20]. Since AMP expression is regulated by UVB irradiatio[21-23], we also hypothesized that expression of hBD2 and LL-37, the two barrier-linked AMP that are decreased in AD, could be up-regulated by suberythemal UVB, further accounting for its therapeutic benefits.

UVB also promotes production of biologically active 1α,25-dihydroxy-vitamin $D_3$ [$1,25(OH)_2D_3$], which has various roles including induction of differentiation and regulation of apoptosis, in keratinocytes as well as immunomodulatory effects in the skin (Lehmann, 2005). The benefits of vitamin $D_3$ could be related to both its known effects on epidermal differentiation and lipid synthesis[24, 25], coupled with enhanced innate immunity (i.e., increased toll-like receptor and AMP expression)[8, 22, 26]. We hypothesized further that vitamin $D_3$ could provide a link between the permeability barrier and antimicrobial barriers, hypothesizing that low-dose UVB irradiation could improve permeability barrier function in parallel with increased AMP expression, mediated through increased cutaneous vitamin $D_3$ production. To date, there are no published studies that have assessed the effects of suberythemal UVB on either permeability barrier homeostasis or epidermal AMP expression. Herein, we report that UVB irradiation up-regulates permeability barrier function and AMP expression in parallel in hairless mouse epidermis, and that cutaneous vitamin $D_3$ plays a major role in mediating this process.

# II. **Materials and Methods**

## 1. Animals and UVB irradiation

Female hairless mice (8-12 weeks old) were purchased from the animal laboratory of Yonsei University. Mice were kept under controlled humidity (40%) and temperature (22±2°C). To observe the changes in AMP expression after UVB irradiation, the dorsal skin of each mouse was irradiated with 40 mJ/cm$^2$ UVB, equivalent to 1/2 MED, once a day for 1 or 3 days (n=6 each for functional study; n=3 each for immunohistochemistry, Western blot and PCR studies). UVB irradiation was delivered with Philips TL20W/12 fluorescent lamps (Eindhoven, Netherlands) emitting 280-320 nm. One MED, previously determined using the same species, equals approximately 80 mJ/cm$^2$. A control group was exposed to sham light (ordinary fluorescent lamp, DULUX®-L, Oslam Korea, Ansan, Korea) for the same exposure time. Epidermal barrier function was assessed and skin specimens for analysis of epidermal AMP and differentiation marker expression were obtained from the central dorsum 24 h after the last exposure. Additionally, to assess the effect on cutaneous vitamin $D_3$ synthesis and mRNA expression of epidermal lipid synthesis related enzymes, dorsal skins of mouse were obtained at 6 h or 16 h after 3-day UVB exposure. Finally to elucidate the effect of vitamin D activated by UVB on epidermal permeability and antimicrobial barrier, ketoconazole (known as an inhibitor of 1,25$(OH)_2D_3$ synthetic enzymes) was used. A cream containing 2% ketoconazole (Nizoral®, Janssen) or vehicle cream (hydrobase) as control were topically applied about 4 mg/cm$^2$ to skin immediately after each UVB irradiation for 3 days as previous report[27]. Functional study for permeability barrier, immunohistochemical stain for AMP, and real-time RT-PCR for vitamin D system and epidermal lipid enzyme were done as described.

## 2. Assessment of epidermal permeability barrier function

To determine whether UVB irradiation influenced the kinetics of barrier recovery, we first assessed baseline permeability barrier status and recovery after acute disruption by tape-stripping (TS). The dorsal surface of skin was disrupted by TS with cellophane until TEWL levels reached 50 g/m$^2$h (normal basal TEWL < 10 g/m$^2$h), and measurements were repeated 3 and 6 h following TS using a Tewameter TM210 (Courage and Khazaka, Cologne, Germany). The barrier recovery rate was expressed as percent recovery calculated by the following formula: (TEWL immediately after barrier disruption-TEWL at indicated time point)/(TEWL immediately after barrier disruption-baseline TEWL) X 100%.

## 3. Immunohistochemical staining

For the light microscopic examination, full-thickness skin specimens were fixed in 10% formalin solution and embedded in paraffin. Sections of 5 μm thickness were cut and stained with antibodies for the mBD2 (Santa Cruz, CA, USA), mBD3 (Santa Cruz, CA, USA) and CRAMP (Santa Cruz, CA, USA) as AMP, and filaggrin (Covance, CA, USA) and involucrin (Covance, CA, USA) as differentiation markers.

After de-paraffinization, sections were rehydrated sequentially with 100%, 90% and 70% ethanol and incubated for 5 min in Tris-buffered saline (TBS) to inactivate endogenous peroxidases. Samples were then blocked for 10 min with blocking serum solution (DAKO, Carpinteria, CA, USA) and incubated overnight at 4°C with primary antibody. After several washes in TBS, they were incubated for 30 min with a secondary biotinylated antibody. Antigen was detected with the avidin-biotin complex system (Vector, Burlingame, CA, USA), according to the manufacturer's instructions by using 3,3'-diaminobenzidine

tetrahydrochloride as substrate. Then they were examined under a light microscope.

## 4. Total RNA isolation and cDNA Synthesis

Skin was excised from the irradiated area. Subcutaneous fat was removed with a surgical blade and epidermis was obtained by EDTA (Sigma, St. Louis, USA) separation. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA), resuspended in RNase-free water and quantified by means of UV spectrophotometer (Perkin Elmer Ltd, Shelton, USA). Single-stranded cDNA was prepared from 1 μg of total RNA in a 20 μl reaction volume using Oligo-dT primer (TaKaRa, Osaka, Japan), which contains 5mM $MgCl_2$, 1 mM dNTP mixture, 1 U/μl RNase inhibitor and 0.25 U/μl AMV reverse transcriptase.

## 5. Classic Reverse Transcription-PCR (RT-PCR)

PCR was performed using primers for AMP (Table 1). Primers were designed based on retrieved GeneBank sequences using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/, Massachusetts Institute of Technology, Cambridge, MA). Each reaction was prepared with a Taq DNA polymerase buffer, 0.2 mM of each deoxy-NTP, 2.5 mM $MgCl_2$, 0.2 mM of each forward and reverse primer, 5 U/μl Taq DNA polymerase and 2 μl cDNA, in a total volume of 20 μl. Reactions were cycled at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at the annealing temperature (Table 1), and for 2 min at 72°C. All RT-PCR products were subsequently separated by electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide and visualized with UV light.

## Table 1. Classic RT-PCR primers of antimicrobial peptides

| Target gene | Primer Sequence | Annealing Temperature | Product size |
|---|---|---|---|
| GAPDH | 5’-AATggTgAAggTCggTgTgA-3’<br>5’-CTggAAgATggTgATgggC-3’ | 56℃ | 229 bp |
| mBD2 | 5’-gCCATgAggACTCTCTgCTC-3’<br>5’-TgCAACAggggTTCTTCTCT-3’ | 58℃ | 205 bp |
| mBD3 | 5’-TTgAgTCATgAggATCCATT-3’<br>5’-CTTTgTTTAACgggATCTTg-3’ | 56℃ | 215 bp |
| CRAMP | 5’-TCCCAAgTCTgTgAggTTCC-3’<br>5’-ACCAATCTTCTCCCCACCTT-3’ | 56℃ | 241 bp |

GAPDH: glyceraldehydes-3-phosphate dehydrogenase, mBD: mouse beta-defensin, CRAMP: cathelicidin related antimicrobial peptide

## 6. Quantitative Real-time PCR

Real-time RT-PCR was performed using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) for mBD2 and CRAMP measurement and Rotor-Gene™ 3000 (Corbett Life Science, NSW, Australia) for VDR, 1α-hydroxylase, FAS, SPT and HMG-CoA according to the manufacturer's instructions.

Briefly, real-time PCR was carried out by rapid cycling in a reaction volume of 20 μl. The reaction mixture consisted of a master mix containing Taq DNA polymerase, dNTP mixture, and reaction buffer (LightCycler-FastStart DNA Master Hybridization Kit) (Roche Diagnostics, Mannheim, Germany). 5 mM $MgCl_2$, 0.5 mM of each primer (Table 2), 0.2 mM of each probe, and 2 μl of template cDNA were used in all assays. The amplification and detection were carried out as follows: after an initial preincubation and denaturation step of 10 min at 95°C, amplification was performed in a three-step cycle procedure (denaturation 95°C, 10 sec; annealing 60°C, 10 sec; and elongation 72°C, 5 sec) for 55 cycles and the final cooling. Quantification of target gene expression was obtained by direct comparison with external standards amplified in parallel reactions in the same run. The LightCycler instrument system needs two fluorescence resonance energy transfer (FRET) probes, but the Rotor-Gene™ 3000 system use a dual-labeled probe. Sequence-specific probes were designed depending on each PCR machine system. The sequences of primers and hybridization probes are listed in Table 2.

## Table 2. Oligonucleotide primer and hybridization probe sequences for real-time PCR

| Target gene | Oligonucleotides | Sequence |
|---|---|---|
| GAPDH | Forward primer | 5’-ATCCCAgAgCTgAACG-3’ |
| | Reverse primer | 5’-gAAgTCgCAggAgACA-3’ |
| | Fluorescence-labeled probe | 5’-TggCCTTCCgTgTTCCTACC-3’ |
| | LC-red 640-labeled probe | 5’-CCCACAAAgTCAgCCg-3’ |
| mBD2 | Forward primer | 5’-TCTCTgCTCTCTgCTgCTgAT-3’ |
| | Reverse primer | 5’-CTggCAgAAggAggACAAAT-3’ |
| | Fluorescence-labeled probe | 5’-TTggATACgAAgCAgAACTTgACCAC-3’ |
| | LC-red 640-labeled probe | 5’-gCCACACCAATggAgggTACTgT-3’ |
| CRAMP | Forward primer | 5’-CAAggAACAgggggTgg-3’ |
| | Reverse primer | 5’-TCCggCTgAggTACAAgTTT-3’ |
| | Fluorescence-labeled probe | 5’-CgAgCCTggTgCACAgCCC-3’ |
| | LC-red 640-labeled probe | 5’-TTCggTTCAAgAAAATTTCCCggCTgg-3’ |
| VDR | Forward primer | 5’-CCTggggTACAggATgCTAA-3’ |
| | Reverse primer | 5’-ggATCATCTTggCgTAgAgC-3’ |
| | Dual-labeled probe | 5’-TTgAAgCCATTCAggACCgCCTATC-3’ |
| 1α-hydroxylase | Forward primer | 5’-gACCCCACACAgTTTCCAgA-3’ |
| | Reverse primer | 5’-CAAAgCCGAAgggAAgAgAT-3’ |
| | Dual-labeled probe | 5’-TCTTTTAATCCAgCTCgCTggCTgg-3’ |
| FAS | Forward primer | 5’-CTgAAgAgCCTggAAgATCg-3’ |
| | Reverse primer | 5’-TgTCACgTTgCCATggTACT-3’ |
| | Dual-labeled probe | 5’-TgAgCTTTgCTgCCgTgTCC-3’ |
| SPT | Forward primer | 5’-gAgAgATgCTgAAgCggAAC-3’ |
| | Reverse primer | 5’-TggTATgAgCTgCTgACAgg-3’ |
| | Dual-labeled probe | 5’-TgggATTTCCTgCTACCCCg-3’ |
| HMG-CoA | Forward primer | 5’-CCgAATTgTATgTggCACTg-3’ |
| | Reverse primer | 5’-ggTgCACgTTCCTTgAAgAT-3’ |
| | Dual-labeled probe | 5’-CTTgATggCAgCCTTggCAg-3’ |

GAPDH: glyceraldehydes-3-phosphate dehydrogenase, mBD2: mouse beta-defensin 2, CRAMP: cathelicidin related antimicrobial peptide, VDR: vitamin D receptor, 1α-hydroxylase: 25-hydroxyvitamin D-1α-hydroxylase, FAS: fatty acid synthase, SPT: serine palmitoyl transferase, HMG-CoA: 3-hydroxy-3-methylglutaryl-Coenzyme A(HMG-CoA) reductase

## 7. Western Blot Analysis

For western blotting of mBD2, mBD3 and CRAMP, the peptides were extracted from epidermis separated from full-thickness mouse skin. The proteins were separated by 4-12% SDS-PAGE with MOPS buffer (Invitrogen, Carlsbad, CA, USA) and transferred to PVDF membranes. After blocking with 5% non-fat dry milk, membranes were incubated overnight at 4°C with anti-mBD2, anti-mBD3, or anti-CRAMP antibodies (SantaCruz, CA, USA) diluted 1:200. After several time washes, peroxidase-conjugated anti-goat antibody was added (final dilution 1:2000) for 1 h at room temperature. Signals was detected using the ECL Plus Western blotting Detection System (Amersharm, Buckinghamshire, UK).

## 8. Statistical analysis

Data are presented as mean ± SEM. The unpaired Student's t-test was used for analysis of differences between two groups. $^{*}p < 0.05$ was considered statistically significant.

# III. Results

## 1. Low-dose UVB irradiation for 3 days improves barrier recovery.

We first delineated the effects of suberythemal (low-dose) UVB exposure on epidermal permeability barrier homeostasis. Hairless mice (n=6 in each group) were exposed to 40 mJ/cm$^2$ UVB (about 1/2 MED) once daily for either 1 or 3 days. Control mice were sham-irradiated with fluorescent light for same exposure time. There were no significant differences in basal transepidermal water loss (TEWL) rates among the 3-day or 1-day UVB and control groups (Fig 1a). However, after prior UVB irradiation for 3 days, tape-stripped skin showed significantly accelerated barrier recovery in comparison to control mouse skin 3 hours after tape-stripping ($p$=0.011; Fig 1b). In contrast, there were no significant differences in barrier recovery rates following a single UVB dose versus sham light-exposed mice ($p$=0.090, $p$=0.052, respectively; Fig 1b). Thus, repeated, short-term exposure to low-dose UVB significantly accelerates the kinetics of barrier recovery after acute insults.

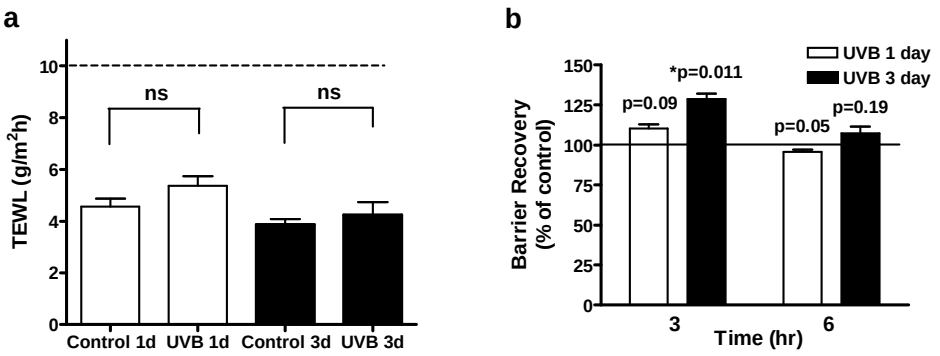


**Figure 1. Low-dose UVB irradiation for 3 days increases barrier recovery rate after acute permeability barrier disruption by tape stripping compared to sham light, without affecting basal TEWL.** Hairless mice were exposed to 40 mJ/cm$^2$ UVB once daily for one or three days. Sham light was exposed on control group. Basal TEWL rates (a) and barrier recovery rates following tape stripping (b) were measured. Statistical analysis was performed using an unpaired Student's t test. Results are expressed as mean ± SEM; n=6 in each group. (Dotted horizontal line: upper limit of normal range; solid horizontal line: control level)

## 2. Low-dose UVB irradiation augments AMP expression at protein and mRNA level.

Since the expression of mBD3 and CRAMP change in parallel with permeability barrier status[6], we next assessed whether the expression of mBD2, mBD3 and CRAMP protein increase in murine epidermis after low-dose UVB exposure. Epidermis from 3-day UVB-exposed mice demonstrated a marked increase in immunostaining for mBD2, mBD3 and CRAMP than either control or 1-day UVB-exposed animals. Moreover, the UVB-induced increase in AMP expression was largely localized to the outer epidermis (Fig 2). These immunohistochemical data were confirmed by Western blot analysis, which showed that levels of mBD2, mBD3 and CRAMP expression increased after 3-days of UVB exposure in comparison to either control or 1-day exposure (Fig 3). These results show that repeated suberythemal doses of UVB stimulate expression of AMP homologous of hBD2 and LL-37 in the outer epidermis of murine skin.

We next examined whether the changes in mBD2, mBD3 and CRAMP protein reflect increased expression at the transcriptional level, by assessing changes in epidermal RNA expression. Total RNA was extracted from epidermis, reverse transcribed by standard methods, and expression was quantified by real-time reverse transcription-PCR. Increased mRNA expression of mBD2, mBD3 and CRAMP was observed in 3-day UVB irradiated mice compared to sham-light irradiated controls (Fig 4a). Expression of mBD2 and CRAMP mRNA was more than two-fold higher (216% and 248%, respectively) in 3-day UVB-irradiated epidermis than in controls, with the up-regulation of mBD2 even more significant than for CRAMP (Fig 4b). In contrast, one-day UVB exposure did not up-regulate either of these AMP (Fig 4a). These results confirm that the UVB-induced increase in protein levels is reflected by a prior or concurrent increase in mRNA expression.

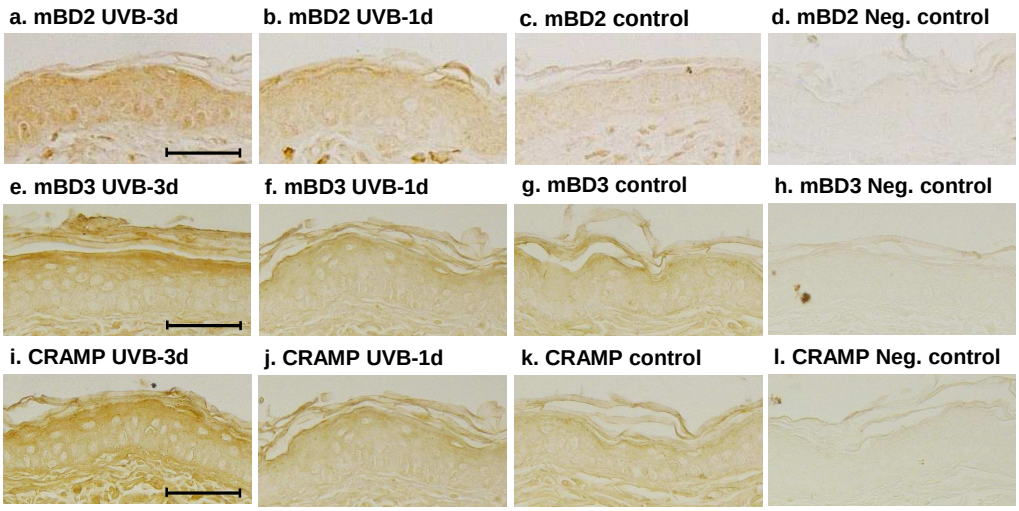


**Figure 2. Low-dose UVB exposure increases expression of anti-microbial peptides in murine epidermis.** Hairless mice (n=6 in each group) were exposed to 40 mJ/cm$^2$ UVB once daily for either three (a, e, i) or one days (b, f, j). Sham light was exposed on control group(c, g, k). Negative controls were the skin samples stained without primary antibodies for mBD2, mBD3 and CRAMP (d, h, l). At 24 h after the last irradiation, skin biopsies were taken and prepared for paraffin blocks. Immunohistochemical stains with DAB were used for mBD2 (a-d), mBD3 (e-h) and CRAMP (i-l). Scale bar = 40 μm

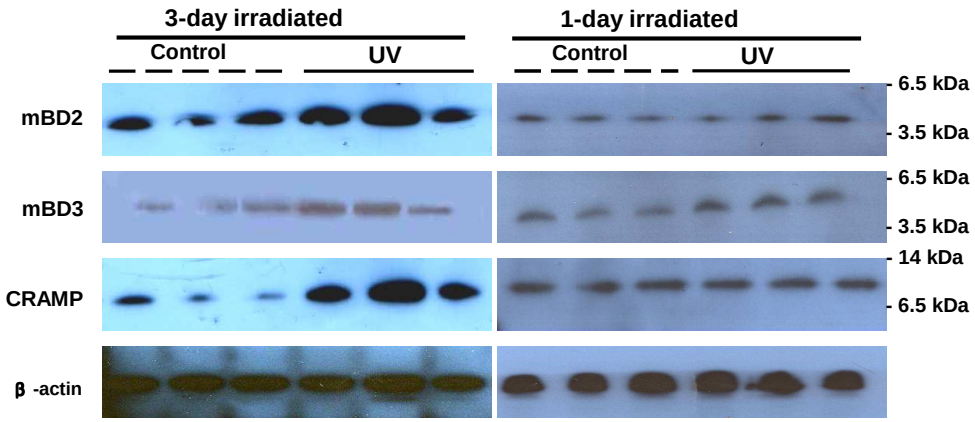


**Figure 3. Western immunoblotting reveals increased bands of mBD2, mBD3 and CRAMP in 3-day UVB irradiated murine epidermis.** Immunoblotting by SDS-PAGE electrophoresis with anti-mBD2, anti-mBD3, and anti-CRAMP antibodies revealed a distinct band with molecular weights of approximately 4.3 kDa, 5 kDa, and 8 kDa, respectively in murine epidermis (n=3 in each group) after 3-day UVB exposure.

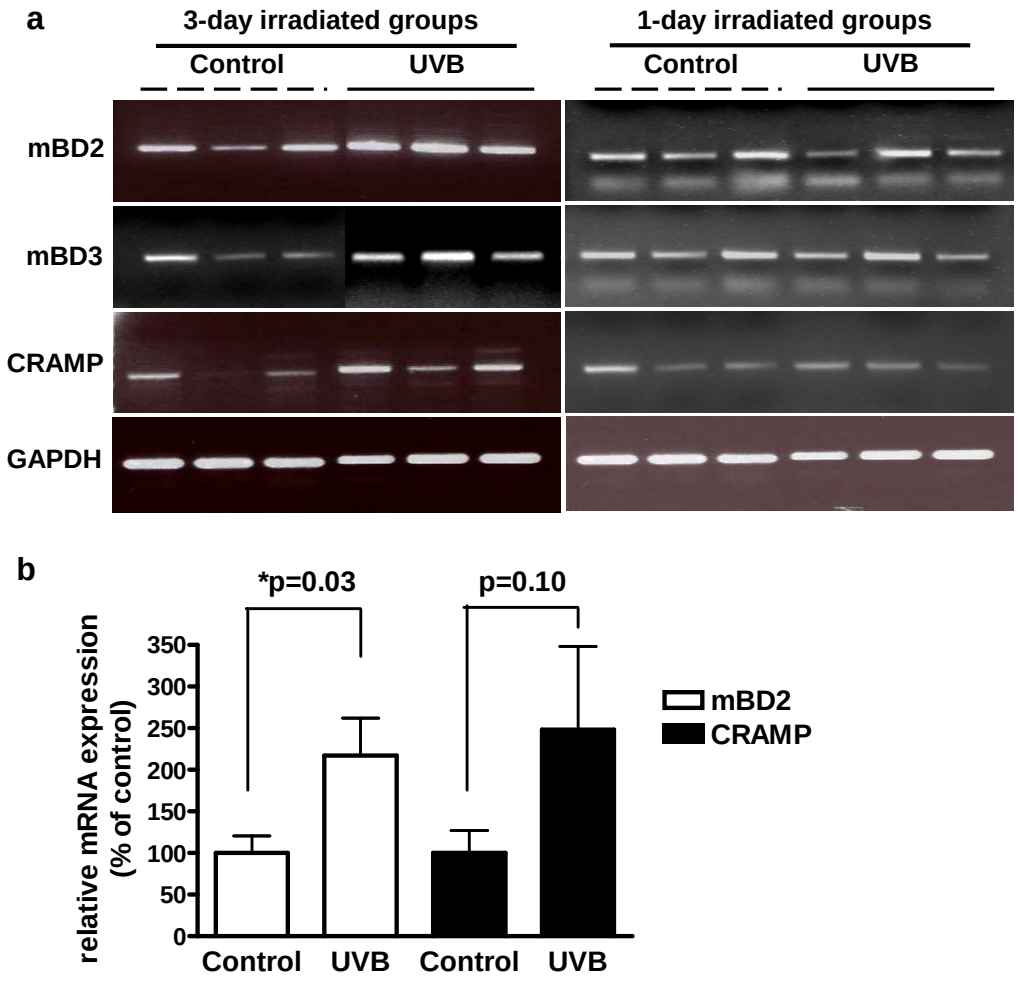


**Figure 4. mRNA expression of mBD2, mBD3 and CRAMP are increased after 3-day UVB exposure.** Total RNA was extracted from murine epidermis (n=3 in each group), reverse-transcribed, and analyzed by RT-PCR and quantitative real-time RT-PCR using GAPDH as an endogenous control. Real time quantitative RT-PCR was performed using a Lightcycler™ (Roche Diagnostics) (see Methods). After 3-day exposure, mBD2, mBD3 and CRAMP mRNA expression was increased compared to either control (a). In contrast, one-day UVB exposure did not induce up-regulation of these AMP. Results of 3-day UVB exposure groups are shown relative to the control mRNA levels (b).

## 3. Low-dose UVB irradiation stimulates the cutaneous vitamin $D_3$ system.

In order to ascertain whether 3-day exposure to low-dose UVB modulates expression of the cutaneous vitamin $D_3$ system, which is known to regulate LL-37 expression, epidermal mRNA levels of the vitamin D receptor (VDR) and 1α-hydroxylase, were quantified with real-time PCR. Sixteen hours after the last UV-B exposure, there was a significant increase in mRNA for both of these genes, though the increase in 1α-hydroxylase was less marked than for the VDR (mean levels of vitamin VDR and 1α-hydroxylase mRNA increased by 604% and 270%, respectively, Fig 5a). Six hours after the last exposure, no significant differences in these genes were found in comparison with control groups (Fig 5a). Interestingly, mRNA levels of the vitamin $D_3$ system were much more increased after 16 h than after 6 h. These results indicated that low-dose UVB exposure results in up-regulation of the cutaneous vitamin $D_3$ system.

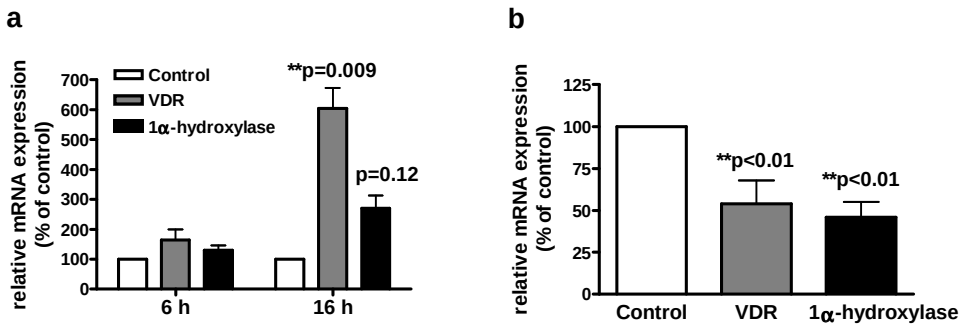


**Figure 5. Low-dose UVB irradiation stimulates the cutaneous vitamin $D_3$ system, but this effect is inhibited by ketoconazole.** Hairless mice (n=3 in each group) were exposed to 40 mJ/cm$^2$ UVB once daily for three days. Sham light was exposed on control group. (a) At 6 h or 16 h after last UVB exposure, expression of mRNA was quantified using a Rotor-Gene™ 3000 instrument system (Corbett Life Science) from epidermis of dorsal skin. Relative mRNA expression of vitamin D receptor (VDR) and 1α-hydroxylase represent the activity of cutaneous vitamin $D_3$ system. (b) Immediately after each exposure, ketoconazole 2% cream or vehicle cream was applied to irradiated dorsal skin. At 16 h after the last exposure, expression of mRNA was quantified. Results are shown relative to the control mRNA levels.

## 4. Blockade of the cutaneous vitamin D system reverses the benefits of low-dose UVB on epidermal permeability barrier function and AMP expression.

To elucidate whether the beneficial effects of low-dose UVB are mediated by UVB-induced activation of the cutaneous vitamin $D_3$ system, we applied ketoconazole cream (2%) immediately after UVB exposure to block epidermal synthesis of active vitamin $D_3$, $1,25(OH)_2D_3$. There was a significant decrease in mRNA for VDR and 1α-hydroxylase (54% and 46%, respectively) in the ketoconazole- vs. vehicle-treated epidermis, which indicated suppression of intracutaneous vitamin $D_3$ activity (Fig 5b). The mean basal TEWL in ketoconazole-treated mice was 14.4 $g/m^2h$ (significant increase over the hydrobase-treated group - 10.3 $g/m^2h$) (Fig 6a). The kinetics of barrier recovery also was slightly delayed in the ketoconazole-treated vs. vehicle-treated mice at 3 h ($p$=0.09) and 6 h ($p$=0.09) (Fig 6b). In control, epidermal immunostaining of mBD2, mBD3 and CRAMP was less intense in ketoconazole treated than in vehicle-treated mice (Fig 7). Together, these results indicate that the benefits of suberythemal doses of UVB on the barrier and antimicrobial defense are mediated by the cutaneous vitamin $D_3$ system.

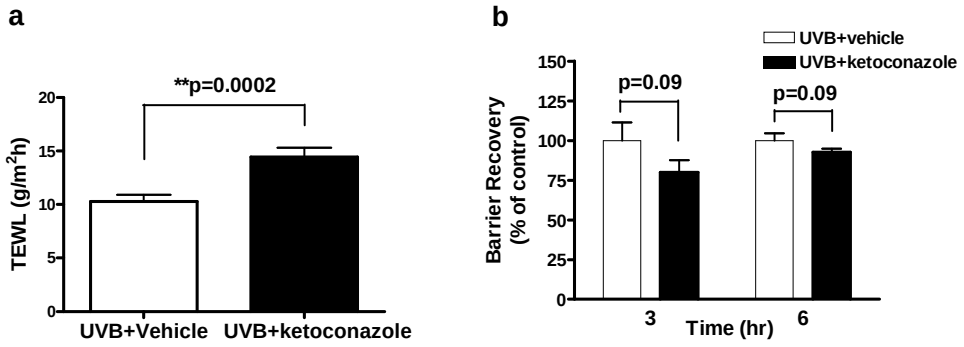


**Figure 6. Inhibition of active vitamin $D_3$ synthesis by ketoconazole weakened the effect of low-dose UVB on the permeability barrier function.** Hairless mice were exposed to 40 mJ/cm$^2$ UVB once daily for three days. Sham light was exposed on control group. Immediately after each UVB irradiation, 2% ketoconazole cream (Nizoral®, Janssen) or vehicle cream (hydrobase) were topically applied about 4 mg/cm$^2$ to irradiated dorsal skin. Basal TEWL (a) and barrier recovery rates after acute barrier perturbation with tape stripping (b) were measured at 16 h after the last irradiation. Statistical analysis was performed using an unpaired Student's t test. Results are expressed as mean ± SEM; n=5 in each group.

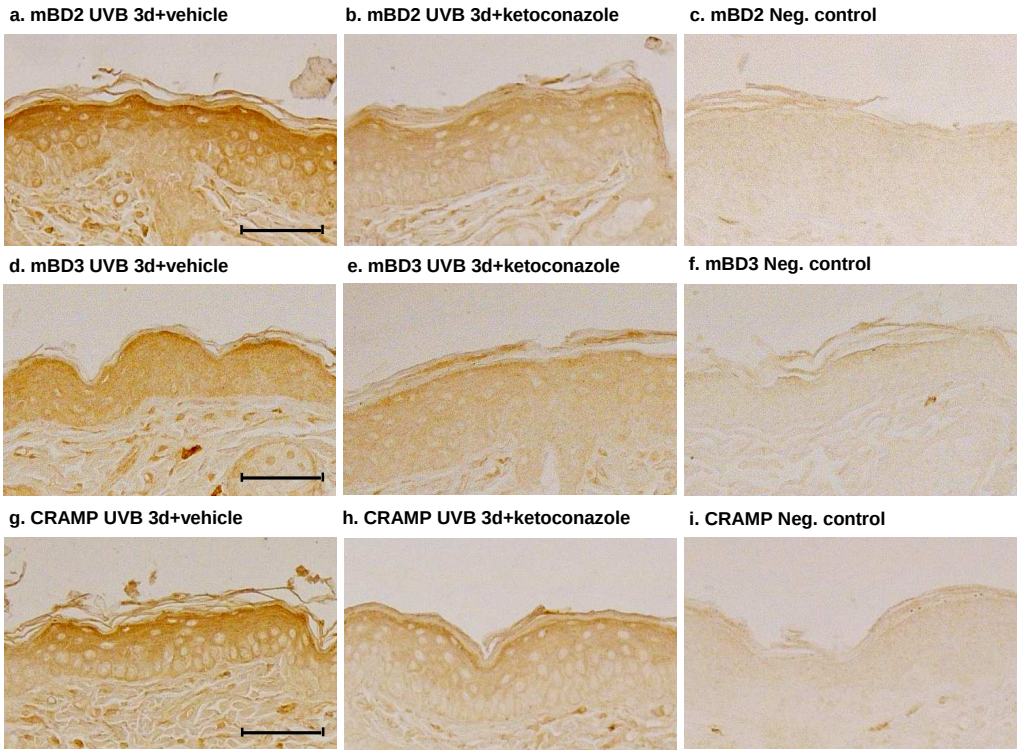


**Figure 7. Inhibition of active vitamin $D_3$ synthesis by ketoconazole diminished the effect of low-dose UVB on the epidermal expression of anti-microbial peptides.** Hairless mice (n=3 in each group) were exposed to 40 mJ/cm$^2$ UVB once daily for three days. Immediately after every exposure, ketoconazole 2% cream (b, e, h) or vehicle cream (a, d, g) was applied to irradiated dorsal skin. At 16 h after the last irradiation, skin biopsies were taken and prepared for paraffin blocks. Immunohistochemical analysis was performed using DAB to stain mBD2 (a-c), mBD3 (d-f) and CRAMP (g-i). Skin immunostained for mBD2, mBD3 and CRAMP without primary antibody (c, f, i). Scale bar = 40 μm

## 5. Low-dose UVB irradiation increases both epidermal lipid synthetic enzymes and epidermal differentiation markers.

We next assessed the basis for the UVB-induced improvement in barrier function. The changes in lipid synthetic enzymes, HMG-CoA reductase (HMG-CoA), fatty acid synthase (FAS), and serine-palmitoyl transferase (SPT), paralleled changes in the vitamin $D_3$ system (Fig. 5a). The mRNA levels of the rate-limiting enzymes for epidermal lipid synthesis such as FAS, SPT and HMG-CoA increased by 340%, 420% and 341%, respectively at 16 h (Fig 8a). Yet, by six hours, there was no significant increase in any of these enzymes. mRNA levels of the rate-limiting enzymes for the epidermal lipid synthetic enzymes, including FAS, SPT and HMG-CoA, also significantly decreased in ketoconazole-treated epidermis (by 49.7%, 70.4%, and 52.2%, respectively, Fig 8b).

We next determined whether low-dose UVB induces the changes in the expression of two epidermal differentiation-related proteins, involucrin and filaggrin. Immunohistochemical staining for both of these proteins increased in the epidermis of suberythemal UVB-exposed mice without the concurrent development of epidermal hyperplasia (Fig 9). The 3-day irradiated group showed more prominent up-regulation of involucrin and filaggrin than did 1-day irradiated mice. These results indicate that UVB regulates not only epidermal lipid synthesis, but also epidermal differentiation, which provide a further mechanism for improved epidermal permeability and antimicrobial barrier function.

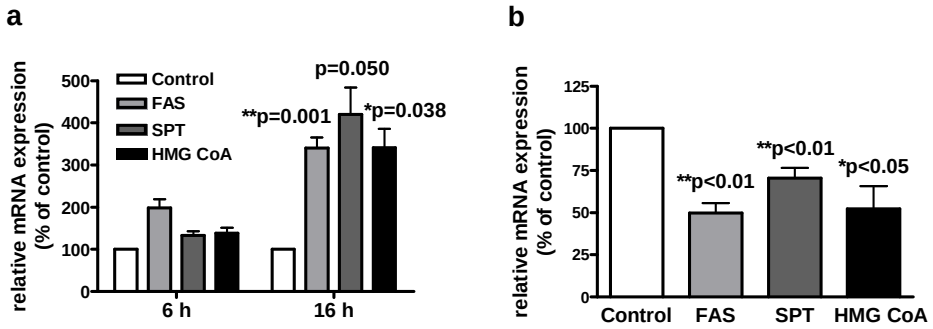


**Figure 8. Low-dose UVB irradiation increases both epidermal lipid synthetic enzymes but this effect is inhibited by ketoconazole, too.** (a) At 6 h or 16 h after last UVB exposure, mRNA expression of rate-limiting enzymes of epidermal lipid synthesis including fatty acid synthase (FAS), serine-palmitoyl transferase (SPT) and HMG-CoA reductase (HMG-CoA) (b) was quantified using a Rotor-Gene™ 3000 instrument system (Corbett Life Science) from epidermis of dorsal skin. (b) Immediately after each exposure, ketoconazole 2% cream or vehicle cream was applied to irradiated dorsal skin. At 16 h after the last exposure, expression of mRNA was quantified. Results are shown relative to the control mRNA levels.

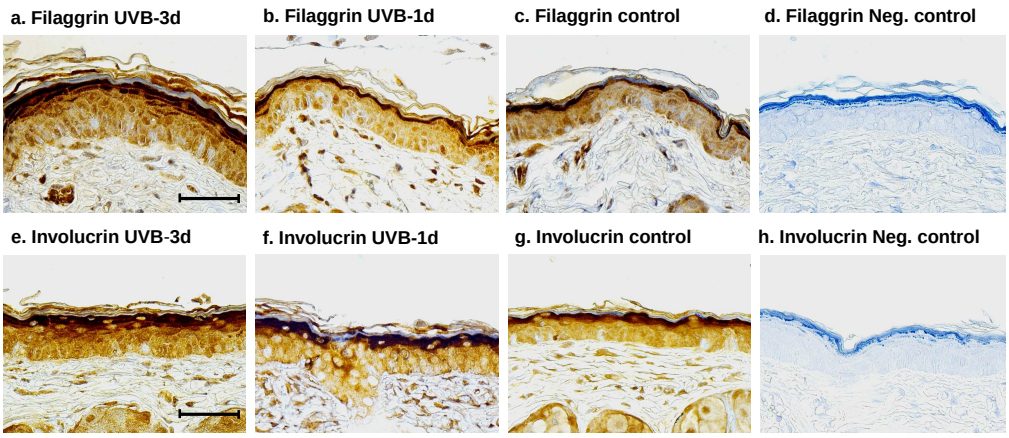


**Figure 9. UVB exposure increases expression of differentiation markers in murine epidermis.** Filaggrin (a-d) and involucrin (e-h) were immunostained using DAB (n=3 in each group). 3-day UVB irradiated group (a, d) showed more prominent expression compared to either 3-day sham-light irradiated control (c, g) or 1-day UVB irradiated group (b, f). Negative controls were the skin samples stained without primary antibodies for filaggrin (d) and involucrin (h). Scale bar = 40 μm

# IV. Discussion

Because of the apparent importance of barrier dysfunction and impaired antimicrobial defense in the pathogenesis of atopic dermatitis, we assessed here whether barrier function and AMP expression are inducible by repeated low-dose UVB exposure in vivo. Our results from real-time RT PCR, Western blot and immunohistochemical stain show that the expression of mBD2, mBD3 and CRAMP are markedly up-regulated by 1/2 MED (40 mJ/cm$^2$) of UVB irradiation daily over 3 consecutive days. Prior studies reported that a single dose of UVB up-regulates expression of both hBD1 and hBD2 in human keratinocytes[21], and a single MED dose of UVB up-regulates hCAP18 mRNA in human skin[22]. In contrast, a 6-week course of narrowband UVB phototherapy with dose increment led to a decrease in cutaneous levels of hBD2 in patients with atopic dermatitis, while hBD3 did not change[23]. However, these results cannot be compared with ours, because Gambichler et al. evaluated AMP levels in full-thickness skin.

The molecular signaling mechanisms by which expression of AMP could be up-regulated by UVB exposure remain poorly understood. Beta-defensins are regulated by a variety of cytokines, while recent studies have shown 1α,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] up-regulates cathelicidin expression[8, 22, 26, 28]. It is well-known that keratinocytes possess the capacity to synthesize a biologically-active form of vitamin $D_3$, 1,25$(OH)_2D_3$, through their own 1α-hydroxylase[24, 29]. But this pathway of 1,25$(OH)_2D_3$ production can be blocked by topical applications of ketoconazole, which inhibits cytochrome P450-dependent enzymes, such as the 1α-hydroxylase[27]. Maximum cutaneous vitamin $D_3$ production after UVB exposure occurs at approximately 10% to 20% of the original epidermal 7-dehydrocholesterol concentration, a limit achieved with suberythemogenic UV exposures[30]. As a result, a small amount of UVB exposure should suffice to generate synthesis sufficient vitamin $D_3$ to impact

downstream events in epidermis.

Vitamin D action in peripheral tissues, such as the epidermis, is mediated by the VDR. Accordingly, the up-regulation of epidermal VDR levels likely predicts enhanced effects of either exogenous/topical or intracutaneously-generated $1,25(OH)_2D_3$ in epidermis[22, 31]. Our study showed that mRNA levels for both the VDR and 1α-hydroxylase are up-regulated by 3-day UVB exposure with suberythemal dose, and conversely, down-regulated by blockade of vitamin D generation by ketoconazole. Hence, it is likely that considerable active $1,25(OH)_2D_3$ is produced in response to suberythemal UVB, but that ketoconazole likely lowered vitamin $D_3$ levels. Therefore, it is plausible that AMP up-regulation induced by low-dose UVB is mediated through cutaneous production of $1,25(OH)_2D_3$, the most active form of vitamin $D_3$.

We also showed that suberythemal doses of UVB irradiation improved permeability barrier homeostasis. In prior studies, permeability barrier was disrupted in murine epidermis with high-doses of UVB irradiation[13, 14], which was followed by a delayed increase in epidermal lipid synthesis as well as accelerated production and secretion of LB, which eventually restore permeability barrier homeostasis[14]. In our study, only 1/2 MED of UVB was employed daily. Although this dose provokes neither clinically evident inflammation nor barrier disruption, an increase in mRNA levels for HMG-CoA, FAS and SPT as key synthetic enzymes of cholesterol, fatty acid and sphingolipid resulted, which could account for the ability of low-dose UVB to improve permeability barrier homeostasis.

Downstream effects of increased endogenous vitamin $D_3$ production could also explain, in part, the benefits of low-dose UVB for barrier function. Previous studies have shown that $1,25(OH)_2D_3$ likely plays an important role in the generation and maintenance of the permeability barrier, and in epidermal differentiation[24, 25]. And, $1,25(OH)_2D_3$ increases expression of major epidermal differentiation proteins, such as involucrin, loricrin, filaggrin, and transglutaminase as well as stimulating cornified envelope formation, which are required for optimal epidermal differentiation, and therefore, permeability barrier homeostasis[24]. Also,

topical 1.25$(OH)_2$ vitamin $D_3$, calcitriol increases the lipid content of murine epidermis and counteracts the permeability barrier abnormality induced by topical steroids[32]. Our immunohistochemical assessment of murine epidermis after UVB irradiation for 3 days revealed an augmentation in involucrin and filaggrin expression, suggesting that suberythemal doses of UVB may not only stimulate lipid synthesis, but also corneocyte formation. Thus, low-dose UVB irradiation may result in an overall enhancement of barrier function through a variety of mechanisms.

Acute exposure to UV light induces a variety of cutaneous responses that can be either beneficial or harmful. This study clearly shows the beneficial effects of UVB, including increased epidermal AMP expression and permeability barrier reinforcement. Exposure dose is an important factor in determining the effects of UVB exposure. UV-induced DNA damage and barrier disruption increase linearly with increasing dose[33 33]. Indeed, previous studies have shown that a treatment regimen of repeated suberythemogenic doses is more effective for AD than conventional dose increments, in part because lower doses of UVB could reduce UV-induced adverse effects, such as erythema, xerosis, and inflammation, without increasing tanning and inflammation[12]. Our studies suggest that suberythemal doses of UVB not only have an acceptable side effect profile, but they also induce beneficial effects on barrier function and antimicrobial defense, presumably mediated by vitamin $D_3$ production, leading to both epidermal lipid/DNA synthesis and AMP expression. However, further studies are needed to determine whether the local tissue concentrations of UVB-induced AMP are within an effective antimicrobial range, and whether UVB exposure enhances barrier function and antimicrobial defense in atopic dermatitis, as well.

# V. Conclusion

In conclusion, our results demonstrate beneficial effects of low-dose UV irradiation on the skin, and provide indirect evidence for a possible connection between the epidermal permeability barrier and antimicrobial defense, which at least in part, is mediated by cutaneous vitamin $D_3$ activation. The positive effects of low-dose UVB radiation on AMP expression and permeability barrier homeostasis can explain why UVB could be a useful therapeutic strategy for atopic dermatitis.

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# Abstract in Korean (국문요약)

## 저용량 자외선-B 조사가 표피투과장벽 기능의 강화와 항균펩티드 발현에 미치는 긍정적 영향


< 지도교수 안 성 구 >

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홍 승 필



고용량의 자외선 B 가 피부에 조사되면, 표피 투과장벽 기능이 손상 받게 되는데 반하여, 저용량 자외선 B 광선치료는 피부장벽 기능의 이상이 관찰되는 아토피 피부염과 건선 같은 피부질환의 치료에 효과적으로 사용되고 있다. 이를 통해 자외선이 피부에 이로운 작용을 할 수 있다는 해석이 가능하나 그 정확한 기전은 아직 밝혀지지 않았다. 또한, 최근 표피 투과장벽과 항균펩티드의 발현이 서로 유기적으로 연결되어 있다고 밝혀지고 있다. 그러나, 피부 질환 치료에 사용되는 저용량 자외선 B 조사에 의해 이러한 표피 장벽기능과 항균장벽이 어떻게 변하는지에 대해 구체적으로 밝혀진바 없다.

이에 저용량 자외선 B 가 피부에 긍정적으로 항균펩티드 발현을 유도함과 동시에 피부장벽 기능을 강화시켜 아토피 피부염의 치료효과를 나타낼 수 있을 것이다라는 가설 하에 이를 확인하고자 연구를 시행하였다. 또한 이러한 기전이

작용하는데 자외선 B 에 의해 피부에서 합성되는 비타민 D3 가 중요한 매개체로 작용하는지 여부를 확인하고자 하였다.

무모생쥐의 피부에 하루 한번 40 mJ/cm2 (최소홍반량의 1/2 용량)의 자외선 B 를 3 일간 조사한 후, 마지막 조사 24 시간 후 표피 투과장벽 기능을 측정하고 피부를 채취하여 표피분화의 표지 단백의 발현을 면역형광검사로 확인하였다. 또한, 대표적인 항균펩티드인 mouse beta-defensin 2 와 3, cathelin-related antimicrobial peptide(CRAMP)의 발현을 면역형광검사와 western blot, RT-PCR 을 통해 확인하였다. 또한 자외선이 조사된 피부에서 비타민 D3 시스템의 활성을 확인하기 위해 비타민 D 수용체(VDR)와 1a-hydroxylase 의 발현을, 표피지질 합성의 변화를 확인하기 위해 지질 합성의 주요 효소인 fatty acid synthase, HMG-Co A reductase, serine palmitoyl transferase 의 발현도 real-time RT-PCR 을 통해 확인하였다. 자외선 B 를 저용량으로 3 일간 조사하였을 때, 장벽 기능의 항상성 회복 능력이 향상되었고 이때 표피 분화의 표지자와 항균펩티드 발현도 동시에 증가하였다. 또한 비타민 D3 시스템과 지질 합성 효소들의 발현도 항진되었다.

비타민 D 의 역할을 알아보기 위해 비타민 D 의 합성을 강력히 억제하는 ketoconazole 을 자외선 조사 직후에 피부에 도포하였을 때에는 표피 지질 합성 효소들의 발현이 의미 있게 감소되었고, 이때 장벽기능의 저하와 항균펩티드 발현의 감소가 관찰되었다.

결론적으로 저용량의 자외선 B 는 피부장벽 기능의 항상성을 유지를 돕고 동시에 항균펩티드 발현을 증가시키는 긍정적인 효과가 있었으며, 이러한 효과는 표피에서 생성되는 비타민 D3 가 매개하는 것을 확인할 수 있었다. 본 실험을 통해 자외선 B 와 비타민 D3, 표피 투과장벽 기능, 항균펩티드의 발현이

유기적으로 서로 연결되어 조절된다는 사실을 알 수 있으며, 이를 통해 아직까지 잘 알려지지 않았던 아토피 피부염에서 광선치료의 기전을 밝히는데도 기여할 수 있을 것으로 생각한다.

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핵심 되는 말: 항균펩티드, 베타-디펜신, cathelicidins, 표피 투과장벽, 비타민 D, 자외선 B

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