Alterations in Epidermal Barrier Induced by Long Term Exposure of Suberythemal Dose Ultraviolet Light in Hairless Mice

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Directed by Professor Sung Ku Ahn

The Doctoral Dissertation Submitted to the Department of Medicine and the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

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(Directed by Professor Sung Ku Ahn)

Background Exposure of ultraviolet (UV) radiation induces various cutaneous changes including erythema and pigmentation as well as thickening of the epidermis. UV radiation on mammalian skin produces dose- and wavelength dependent changes of skin barrier including compromised integrity of the epidermal permeability barrier with an increased TEWL (transepidermal water loss). Although several studies have reported on the biological effects of UV radiation, there have been no reports on the changes in epidermal structure, skin barrier function and epidermal lipids of murine skin following long term UV irradiation at suberythemal doses.

Objectives To investigate the changes of functions, morphological structures and epidermal lipid properties of the skin barrier after long term UV radiation at suberythemal dose (SED).

Methods Hairless mice were irradiated 3 times weekly for 15 weeks at a SED of UV (UVB: 20 mJ/cm^2; UVA:14 J/cm^2). Every three weeks, visible skin changes and epidermal barrier function were assessed and skin specimens were taken, and then hematoxylin & eosin stain, immunohistochemical stain and calcium ion capture cytochemistry were done. The morphological alterations of stratum corneum (SC) lipid lamellae were examined by electron microscopy (EM) using ruthenium tetroxide postfixation. Activities of three key enzymes for mRNA of serine palmitoyl transferase, fatty acid synthase and HMG Co A reductase were analyzed with real time RT-PCR. I analyzed the activity of three key enzymes with real time RT-PCR for mRNA of serine palmitoyl transferase, fatty acid synthase and HMG Co A reductase. I also measured the amount of ceramide, cholesterol sulfate and free fatty acid in the SC by high performance thin layer chromatography (HPTLC) with exposed time.

Results Visible fine wrinkles were found since 3 weeks to irradiation, and progressively worsened in proportion to the duration of irradiation. There were significant increases in epidermal and dermal thickness and the epidermal differentiation markers including involucrin, loricrin, filaggrin and K5/10/16. These changes were accompanied with alteration of epidermal calcium gradient and SC intercellular lamellae in EM findings. TEWL was increased up to 12 weeks. SC hydration was gradually decreased in proportion to exposure time. mRNA of three key lipid synthesis rate limiting enzymes were increased until 6 weeks of UV-irradiation and decreased thereafter. However three major lipids gradually decreased throughout exposed period with a notable decrease in ceramide.

Conclusions In conclusion, long term UV irradiation even with SED to which people are usually exposed during their life time influences skin barrier function and structure. Notable ceramide decreases in SC intercellular lipid

after long term UV irradiation can be implicated in the treatment or prevention of photoaging.

Key words: ultraviolet light, suberythemal dose, skin barrier, cornified envelope, stratum corneum lipid, ceramide

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Introduction

A main function of the skin is to provide a protective barrier at the interface between the hazardous external environment and the organism[1]. Although the skin has many functions, the formation of a permeability barrier that impedes transepidermal water loss (TEWL) is of major importance, because it is required for life in a dry environment. This permeability barrier is localized to the outermost, the stratum corneum (SC), and it is mediated primarily by extracellular, nonpolar, lipid-enriched lamellar membranes that are impermeable to water[1]. A variety of insults, including mechanical trauma, produced by tape stripping, or contact with either solvents or detergents, can injure the SC, resulting in acute perturbations of cutaneous permeability barrier function. Disruption of the permeability barrier stimulates a vigorous homeostatic repair response in the underlying viable epidermis that leads to the rapid restoration of permeability barrier function[2].

Epidermal differentiation leads to the formation of the SC, heterogeneous tissue composed of lipid-depleted corneocytes embedded in a lipid-enriched extracellular matrix, which subserves the barrier. These lipids derive from a highly active, lipid-synthetic factory, operative in all of the nucleated cell layers of the epidermis, which generates a unique lipid and hydrolase-enriched, secretory organelle, the epidermal lamellar body (LB)[3-5]. Following secretion of their contents at the stratum granulosum (SG) –SC interface, LB contents are processed from a polar lipid mixture into a hydrophobic mixture of ceramides, free fatty acid (FFA), and cholesterol, organized into the lamellar membranes that form the hydrophobic matrix within which corneocytes are embedded[6]. Although corneocyte proteins have been studied intensively as markers of epidermal differentiation, their role in the permeability barrier is less clear[7]. Yet, these SC is well known to perform other critical epidermal functions. The corneocytes influence the permeability barrier through their function as ‘‘spacers’’, they force water and xenobiotes to traverse a tortuous, extracellular hydrophobic pathway, and by serving as a scaffold for lamellar membrane organization[8].

Proteins of the cornified envelope (CE), and its external, ceramide-enriched, cornified-bound lipid envelope together provide a stable, mechanically and chemically resistant scaffold for the deposition and organization of the extracellular matrix[9]. The CE, a uniform, 15 nm thick, peripheral envelope that encloses the corneocyte cytosol, consists of several highly cross-linked, cytosolic proteins, including involucrin, loricrin, elafin, desmoplakin, envoplakin, cytostatins, and pancornulins/cornifins (small proline-rich proteins)[9-12]. Involucrin, a 68 kDa rod-shaped molecule with a series of highly conserved 10 amino acid repeats, containing 3 glutamine residues each as potential cross-linking sites, accounts for 5–15% of the CE expressed in the late spinous and SG layer, and it appears to be the first envelope precursor that is cross-linked by transglutaminase 1 (TG1), and therefore localizes to the

outer SG[12]. Loricrin is a cysteine (7%), serine (22%), and glycine (55%) enriched, 38 kDa, highly insoluble peptide, comprising one component of keratohyalin granules, and accounts for up to 80% of CE mass. Loricrin is cross-linked into the CE late in differentiation, immediately after LB secretion[11,13].

CE-associated proteins, while being necessary for the steady-state maintenance of normal barrier homeostasis, are transiently downregulated following acute barrier perturbations, apparently as a form of metabolic conservation, but subsequently upregulated during later stages of barrier recovery[14,15]. Keratins are the most abundant structural proteins of the epidermis and its appendages, contributing to the mechanical properties of these epithelium[16]. Keratins are of two types, type l or acidic (K9–20) and type II or basic (K1–8), which are co-expressed in pairs, and all keratins display a similar secondary structure, with a central, rod domain comprising four a helices, and distinctive, non-helical, head and tail sequences[17]. Whereas K5 and 14 are expressed in the basal layer, K1, 2e, and 10 are expressed in suprabasal layers, eventually accounting for 80% of the mass of the corneocyte[7,16,18]. Profilaggrin is a large, histidine-rich, highly cationic phosphoprotein, consisting of 37 kDa filaggrin repeats, connected by peptide segments enriched in hydrophobic amino acids[19]. Profilaggrin is concentrated within keratohyalin granules, where it may sequester loricrin, which also localizes to keratohyalin. During terminal differentiation, profilaggrin is both dephosphorylated and proteolytically processed by a Ca2þ-dependent protein convertase, furin, at the N-terminus to yield filaggrin, which ionically binds to Kl/l0, inducing the formation of macrofibrils in the corneocyte cytosol [20,21].

The processing of secreted LB contents leads to the progressive generation of a mixture of relatively non-polar lipids, which is enriched in ceramides, cholesterol, and FFA, present in an approximately equimolar ratio [22]. The

‘‘mortar’’ which means endogenous synthetic lipids in the keratinocytes also contain abundant cholesterol, which is secreted unchanged from LB[23]. Although a variety of studies have shown that cholesterol is critical for permeability barrier function, cholesterol derived from cholesterol sulfate is not required[24,25]. The key lipid constituent of the mortar is a family of nine ceramide species[26]. Ceramides 1, 4, and 7 are the principal repositories for the essential fatty acid (EFA), linoleic acid, a critical structural ingredient in the barrier[27,28].

A 10 nm, tightly apposed, electron-lucent, plasma membrane-like structure replaces the plasma membrane on the external aspect of corneocytes[29,30]. The cornified bound lipid envelope comprises o-hydroxyceramides , with very long-chain, Nacyl fatty acids, covalently bound to the CE[29,30]. These o-hydroxy derive from insertion of the b-glucosyl ohydroxyceramide-enriched limiting membrane of the LB into the apical plasma membrane of the outermost granular cells[31,32]. The cornified bound lipid envelope could restrict water movement to extracellular domains, while limiting both water uptake into the corneocyte, and egress of water-soluble, hygroscopic amino acids out of the corneocyte cytosol[33].

These significant advances in our understanding of the homeostasis mechanisms that regulate epidermal barrier function have been achieved over the past two decades[6]. For example, an acute perturbation stimulates a well coordinated repair response within the epidermis, including the immediate secretion and regeneration of LB, the generation of key barrier lipids, and increased epidermal proliferation[34-38]. Since occlusion with a vapor impermeable membarane blocks all of these responses, permeability barrier integrity is linked to epidermal lipid and DNA synthesis. As hazards, Ultraviolet (UV) irradiation of mammalian skin produces responses including not only inflammation, erythema, hyperproliferation, and desquamation but

also changes in biophysical properties of skin barrier.

UV irradiation induces various cutaneous responses including physiologic and morphologic changes in the skin. These include inflammatory responses such as erythema, epidermal proliferation, apoptosis, hyperpigmentation and immunosuppression[39-42]. Moreover, UV irradiation of mammalian skin produces dose- and wavelength- dependent responses including compromised integrity of the epidermal permeability barrier[43-45]. The researches about UV exposure to the skin have commonly were performend with high minimal erythemal dose (MED), that is to mean, more than 1 MED UV light which could implicated in sunburn[44,46,47]. A single exposure of human skin to 2MED ultraviolet B (UVB) irradiation induces a delayed increase in TEWL[48]. Likewise, increased TEWL also occurs after exposure of hairless mice skin to a single high dose of UVB, a combination of UVA and UVB or UVC exposure[43,49,50]. Furthermore, UV irradiation of human or rat skin also results in increased transepidermal delivery of xenobiotics[51]. Haratake et al. demonstrated that the UVB-induced barrier abrogation (1.5-7.5 MED) is dose dependent and delayed by at least 48 hours after exposure in young hairless mice[44]. A single UVB irradiation with high MED (7.5 MED) caused not only a significant increase in TEWL but also marked morphological abnormalities in the intercellular domains, including abnormal profile of LB and its contents at the interface between SC and SG[52]. Adult hairless mice irradiated for 7 days with high MED (1.5-7.5 MED) UVB showed delayed alteration in barrier function and both an epidermal proliferative response and thymocyte-mediated events appear to contribute to UVB induced abrogation of the permeability barrier[46].

People are commonly exposed to low dose, that is to mean, suberythemal dose (SED) of ultraviolet A (UVA) and UVB during their lifetime. Photoaging results from accumulation of responses of these SED UV exposure. Narbutt et

al. demonstrated that short term UVB irradiation with SED (0.7 MED) protected to a limited extent against the effects of an erythemal UVB dose on cytokine expression and thymine dimer formation[47]. Hairless mice irradiated with 1/3 MED of UVB and UVA for 10 weeks showed alteration of the physical properties of the skin and increases in keratin content of SC[53]. These results showed the early events of wrinkle formation only with limited data. Wherease we can easily expect that suberythemal UV exposure have important role of photoaging, relatively few functional and structural abnormalities in the epidermis irradiated with long term UV with SED was reported. The aim of this study was to evaluate the effects of long term UV irradiation with with SED on function and morphology of skin barrier.

II. Materials and Methods

1. Animals and UV irradiation

All animal experiments were performed with 7–8-week-old female hairless (hr-hr) mice (n=40), which are purchased from the animal laboratory of Yonsei University, in an ambient environment of 23°C, 50% relative humidity. The MED which means the minimum dose necessary to cause a discernable erythema after 24 hours measured on randomly selected mice with a UV-dosimeter (Villingen-Schwenningen, Germany), was UVB 80±5 mJ/cm^2 and UVA 20±5 J/cm^2. Treatment group was irradiated with SED of UVB (20 mJ/cm^2) and UVA (14 J/cm^2) three times a week for 15 weeks. Hairless mice in the control group were sham-irradiated during the same minutes. UVB irradiation was delivered with Philips TL20W/12 fluorescent lamps (Philips, Eindhoven, Netherlands). As a UVA source, Philips, UVA sunlamp 40W (Philips, Eindhoven, Netherlands) was used. Irradiance was measured with a UV-dosimeter (Villingen-Schwenningen, Germany). Under general anesthesia with 4% chloral hydrate by intraperitoneal injection, the dorsal skin of each mouse was irradiated with a suberythemal dose of UV. Skin biopsies and the measurements were performed on the dorsal skin of the hairless mice at every three weeks after the first UV irradiation.

2. Visual score / Assessment of epidermal barrier function and stratum corneum hydration

Prior to the measurements hairless mice were acclimatized for at least 15 minutes to standardized laboratory environment. Visual scoring was performed every week by a trained investigator using a grading system developed by Bisset et al[50]. The scale ranged from 0 for normal skin to 3 for heavily wrinkled skin. The minimum difference of the scale was decided as 0.5.

TEWL was measured on the dorsal surface of hairless mice by using a Tewameter TM210 (Courage and Khazaka, Klon, Germary) once a week. Each value used for the final calculations represented the arithmetic mean of two single measurements. SC hydration was measured using a Corneometer CM 825 (Courage and Khazaka, Klon, Germary) once a week.

3. Assessment of epidermal/dermal thickness, epidermal prolifera -tion and differentiation

Whole skin sections were fixed in formalin and stained with hematolxilin and eosin. The thickness of epidermis considered as the distance from the basal layer to the stratum granulosum/stratum corneum (SC) junction, was randomly measured on 10 points on each photograph using Photoshop 3.0 (Adobe Systems, Mountain View, CA). The dermal thickness considered as the distance from the basal layer to the lower dermis/subcutaneous fat junction, was measured in the same way to the epidermal thickness measurement. For the light microscopic examination, the specimen was fixed in 10 % formalin solution and was embedded in paraffin. Sections of 5μm thickness were cut and stained with differentiation markers such as involucrin, loricrin, filaagrin, Keratin 5 (K5) (BabCo, Richmond, CA), Keratin 10 (K10) (BabCo, Richmond, CA), Keratin 16 (K16) (BabCo, Richmond, CA). Briefly, after deparaffinization, the sections were treated sequentially with 100%, 90% and 70% ethanol solution for rehydration. Then the samples were treated to inactivate endogenous peroxidases by incubation for 5min in Tris-buffered saline (TBS). Samples were then blocked for 10 min with blocking serum solution (DAKO, Carpinteria, CA, USA) and incubated overnight at 4℃ with the primary antibody. After several washes in TBS, they were incubated for 30 min with a secondary biotinylated antibody. The 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 (Sigma Chemical Co., St Louis, MO, USA), as the

substrate. Then they were examined under a light microscope.

4. Electron microscopic studies

1) Calcium ion-capture cytochemistry electron microscopy

At every 3 weeks after UV irradiation, the skin samples were taken and fixed for ultrastructural examination. Calcium ion-capture cytochemistry was performed in order to investigate the epidermal calcium ion distribution. Briefly, biopsized samples were finely minced and were immersed in an ice-cold fixative which contained 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate and 1.4% sucrose (pH 7.4); they were left overnight in the ice-cold fixative in the dark.

2) RuO_4 postfixation

To see the SC intercellular lipid lamellae, RuO_4 postfixation was done. Briefly, biopsized samples were fixed in modified Karnovsky's fixative overnight, washed in 0.1 M cacodylate buffer, and postfixed in 0.25% ruthenium tetroxide (RuO_4) (Polysciense Inc., Warrington, PA, .S.A.) in 0.1 M cacodylate buffer for 45 min in the dark at room temperature, or processed routinely. After rinsing in a buffer, samples were dehydrated in graded ethanol solutions, and embedded in an Epon-epoxy mixture. Ultrathin sections were examined using an electron microscope (Joel, Tokyo, Japan) after further contrasting with uranyl-lead citrate.

5. Real-time RT-PCR for epidermal lipid synthesis rate limiting enzymes

Serine palmitoyl transferase (SPT), HMG-CoA reductase (HCR) and fatty acid synthase (FAS) mRNA were quantified with real-time PCR by using a

LightCycler FastStart Master Hybridization Probe kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, primers and two labelled probes were designed for SPT, FAS, HMG-CoA reductase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. In all experiments, primer concentrations were first optimized to avoid unspecific binding of primers, and after running the PCR products, a dissociation curve analysis was performed to verify the specificity of the amplification products. For each gene, a specific PCR primer pair based on the cDNA sequence published as indicated: GADPH 5'-AATGGTGAAGGTCGGTGTGA-3'/5'-CTGGAAGATGGTGATGGGC-3'; SPT 5'-CTGCTGAAGTCCTCAAGGAGTA-3'/ 5'-GGTTCAGCTCATCA CTCAGAATC-3'; HCR 5'-GATCCAGGAGCGAACCAA-3'/ 5'-GSGAATAGACACACCACGTT-3'; FAS 5'-CCTCACTGCCATCCA GATTG-3'/5'-CTGTTTACATTCCTCCCAGGAC -3'. PCR mixture contained 2 µl of DNA template, 2 µl of LightCycler FastStart Master Hybridization probes (Roche Diagnostics GmbH, Mannheim, Germany), 2 mM $MgCl_2$ and 0.5 uM forward primer, 0.5 uM reverse primer, 0.2 uM FL-probe and 0.2 uM LC-probe, respectively. The PCR reaction was performed in 20 µl (final volume) and the conditions for thermal cycling were as follows: initial denaturation for 10 min, followed by 55 amplification cycles at 95°C for 10 s, 56°C (SPT)/ 57°C (HCR,FAS)for 15 s and 72°C for 8 s, cooling at 40°C for 1min.

6. High performance thin layer chromatography (HPTLC) for SC lipid analysis

Epidermal lipids were extracted using the method of Bligh and Dyer, dissolved in chloroform/methanol (2:1, vol/vol) and stored in liquid nitrogen until used[54]. Lipids were separated by one-dimensional HPTLC on 10 x 20 cm glass plates coated with Kiesel gel 60 (Merk, Darmstadt, Germany). For quantification, lipid standards consisting of cholesterol sulfate, free fatty acid

and ceramide were run in parallel. The quantification was performed using scanning densitometry (Camag, Muttenz, Switzerland)) after charring of the lipids.

7. Statistics

The results are expressed as the mean ± SD. Statistical differences among groups were determined by unpaired Student's t-test using SPSS version 10. $P < 0.05$ was considered statistically significant.

III. Results

1. Visible scores of wrinkle increases in proportion to UV exposed time.

Long term UV radiation even with low dose induced wrinkle formation. The skin originally showed no wrinkles and smooth surface features (Fig.1). Wrinkles became deeper and wider following 15-week UV irradiation. The degree of wrinkling is summarized in Figure 2. The first significant sign of wrinkle, in comparison to the control group, was detected at 3 weeks of irradiation ($p < 0.01$). The skin wrinkles and surface features worsened progressively over the 15-week irradiation period.

2. Long term SED UV irradiation affects on epidermal barrier function and SC hydration in proportion to exposure time.

Epidermal barrier function was determined by measurement of TEWL (Fig. 4). Basal TEWL was not different until 9 week exposure, but increased significantly, relative to control group, after 12 weeks of UV irradiation. Figure 3 indicates SC hydration values. SC hydration decreased since 3 weeks of irradiation and progressively more decreased until 15 week post irradiation.

3. Long term SED UV irradiation induce alterations in both epidermal and dermal thickness.

Histopathologic changes including increases in epidermal and dermal thickness after long term UV exposure was most prominent over 15 weeks of UV irradiation (Fig. 5). The first significant increase in epidermal thickness was found at 9 weeks, while significant increase in dermal thickness started at 12 weeks post irradiation (Fig. 6,7). The change that epidermal thickness increased earlier than dermal thickness would be an interesting manifestation asking a further interpretation.

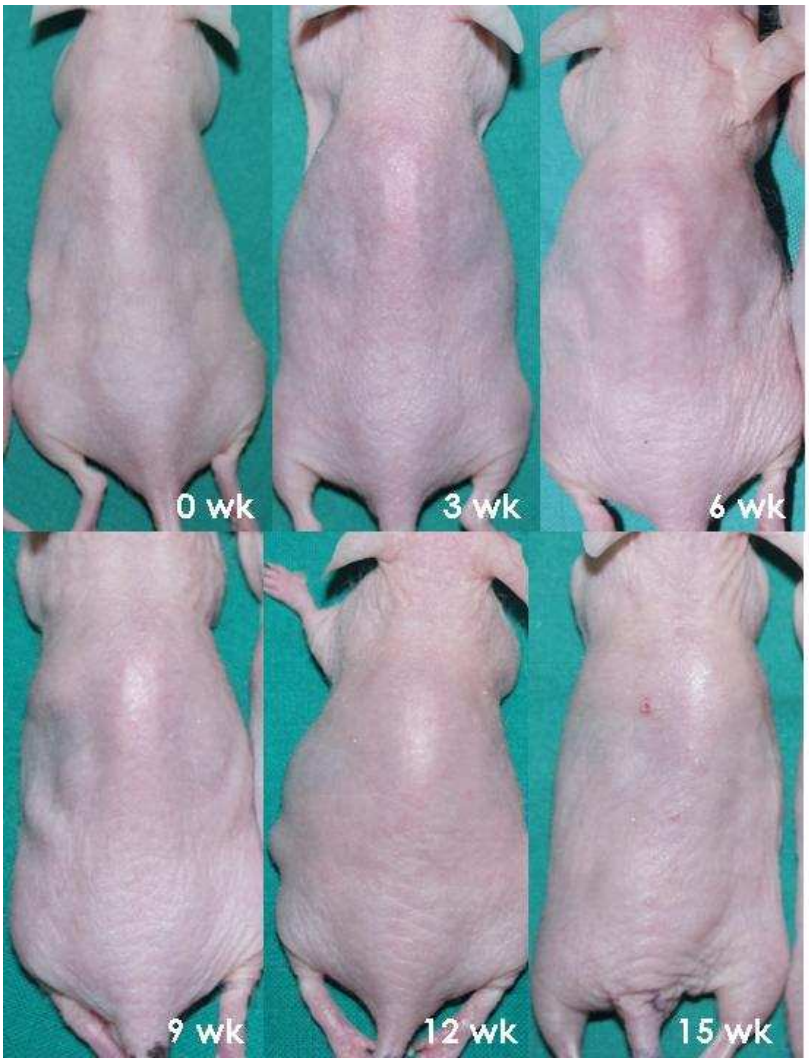

Figure 1. Gross appearance of hairless mouse skin. Control group showed normal looking appearance while UV treated group of 0, 3, 6, 9, 12 and 15 weeks exposure increased rough wrinkles.

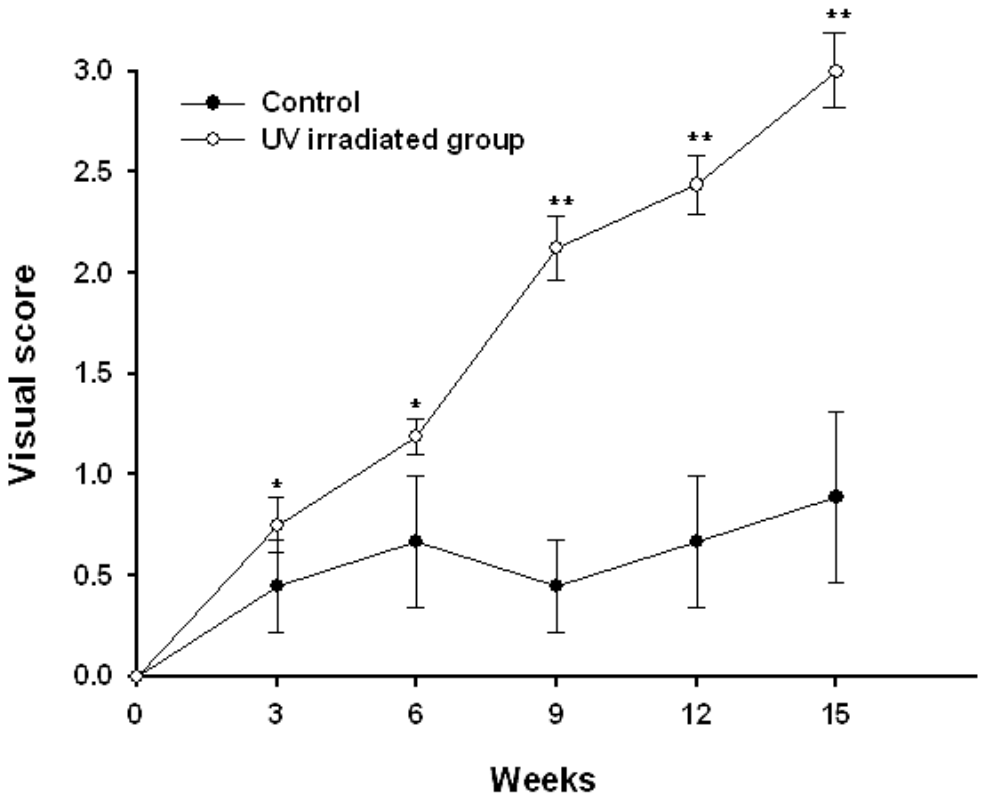

Figure 2. Changes of wrinkle grade in long term SED UV irradiated mice (O) and non-irradiated mice (●). The values of wrinkle grade were expressed as mean ± SD. Visible scores increased following irradiation. *$p < 0.05$, **$p < 0.01$, vs. non-irradiated mice

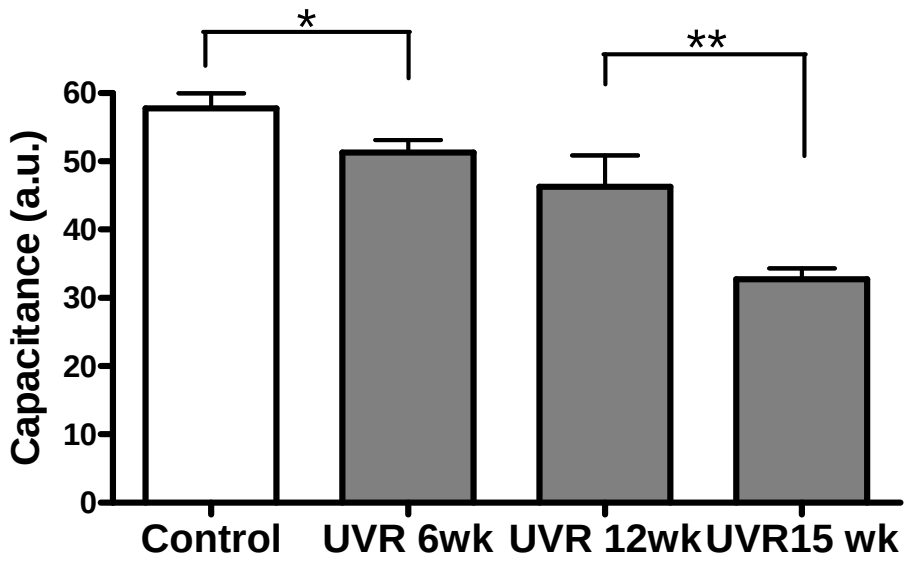

Figure 3. Changes in SC hydration after long term SED UV irradiation. The water content of the epidermis decreased in proportion to the irradiation time. (*$p < 0.05$, **$p < 0.01$)

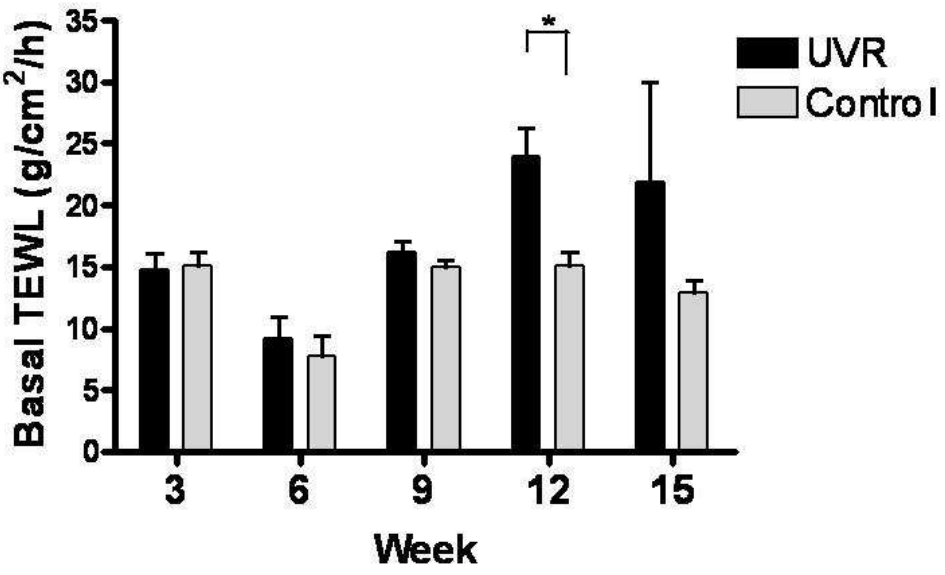

Figure 4. Changes in TEWL after long term SED UV irradiation. Basal TEWL increased significantly from 12 weeks of UV irradiation. (*$p < 0.05$) time. (*$p < 0.05$, **$p < 0.01$)

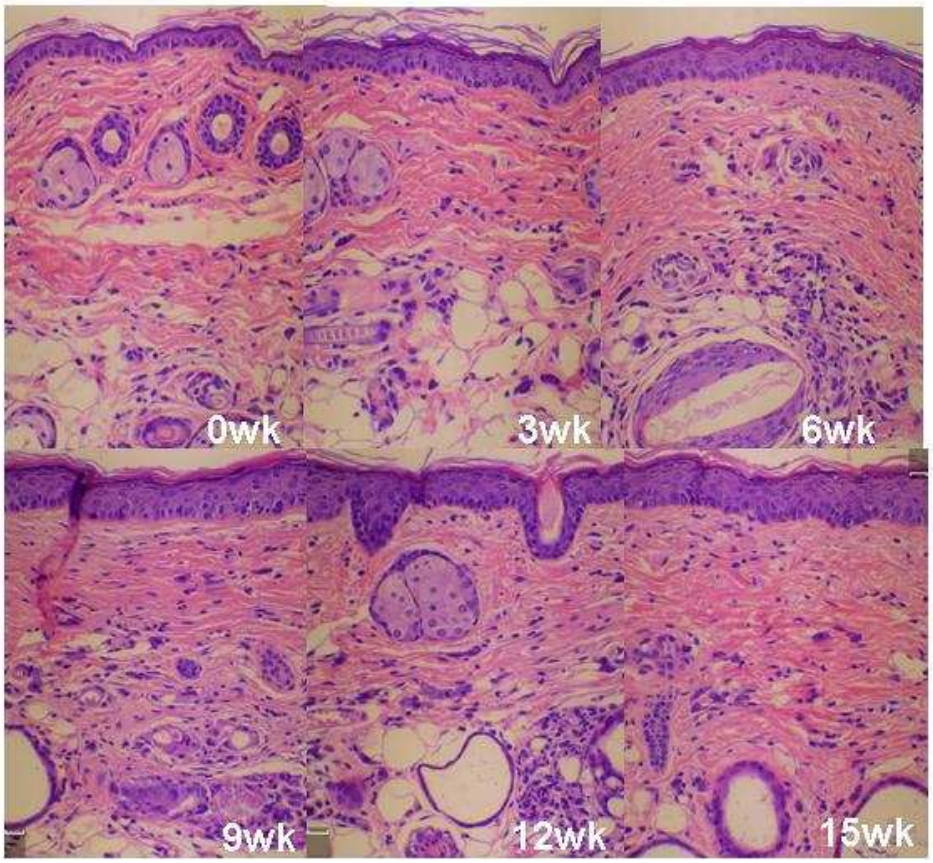

Figure 5. Histopathologic changes after long term SED UV irradiation. Epidermal hyperplasia and an increase in dermal thickness were more prominent in UV irradiated groups (9, 12, 15 weeks) compared to control. (H&E, X 200)

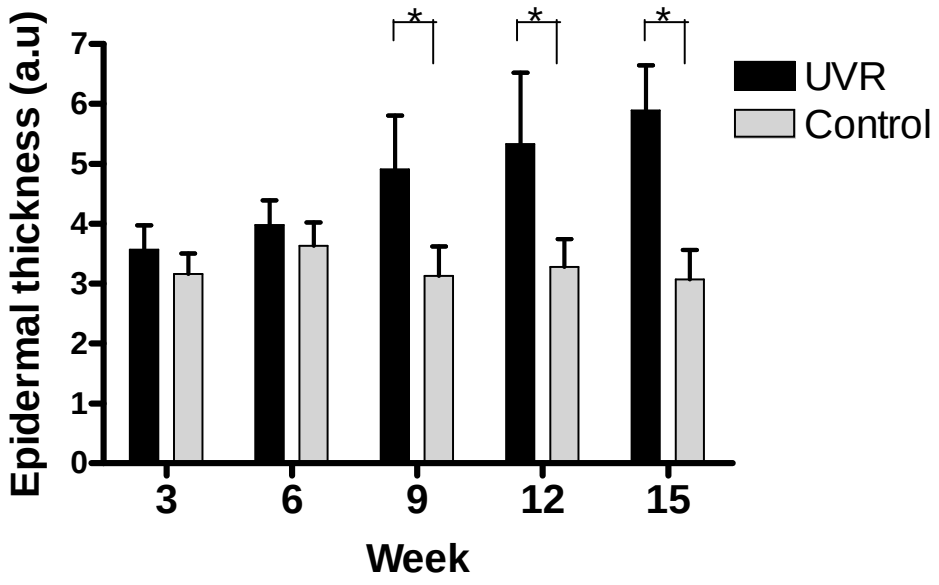

Figure 6. The changes in epidermal thickness after long-term SED UV irradiation. The first significant increase in epidermal thickness was found at 9 weeks (*$p < 0.05$, **$p < 0.01$)

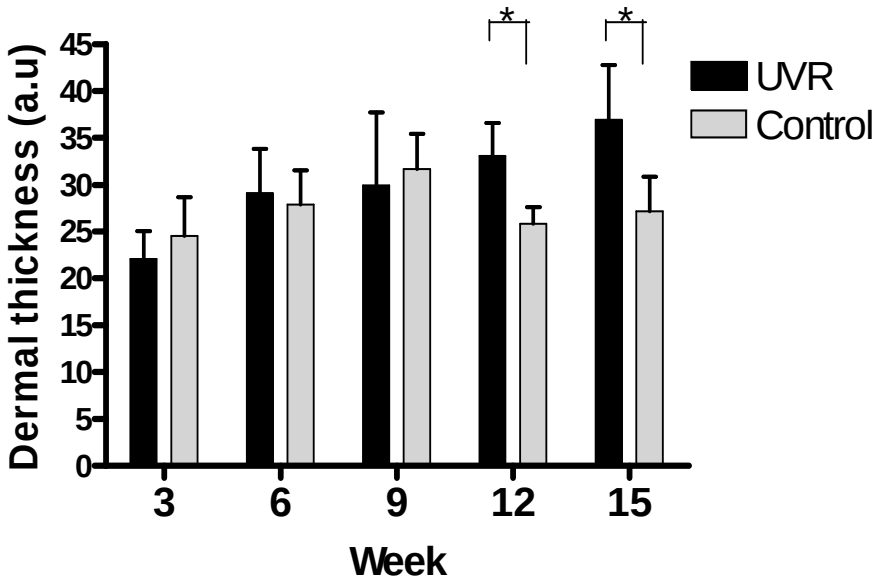

Figure 7. The changes in dermal thickness after long-term SED UV irradiation . The first significant increase in epidermal thickness was found at 12 weeks (*$p < 0.05$, **$p < 0.01$)

4. Long term SED UV irradiation affects epidermal differentiation.

To determine whether structural changes of the epidermis were induced by long term UVB and UVA at low doses, we examined the expression of the epidermal differentiation related proteins such as involucrin, loricrin, filaggrin, K5, K10, and K16 (Fig. 8). After long term SED UV irradiation, involucrin, loricrin and filaggrn are overexpressed from the stratum spinosum (SS) to the SG, compared to the control. Wherease K5 are normaly expressed in the basal layer, K10 and K16 are overexpressed from the SS to the SG in the epidermis of UV irradiated mice with epidermal hyperplasia. Following the irradiation period, these findings were more prominent compared to the control group. These results suggest that long term UV irradiation even at low doses influences epidermal differentiation as well as epidermal proliferation.

5. Long term SED UV irradiation induces changes in epidermal calcium gradient.

The epidermis in the control group displayed a characteristic calcium gradient in which calcium is sparse in the basal and spinous layers, increasing to the highest levels in the granular layer, and declining again in the stratum corneum. The epidermal calcium gradient began to change at 3 week post UV irradiation. The epidermis after 6 week UV irradiation displays evenly dispersed calcium precipitates in all epidermal layers (Fig. 9) These perturbed calicium gradient was persisted to 15 weeks post UV irradiation.

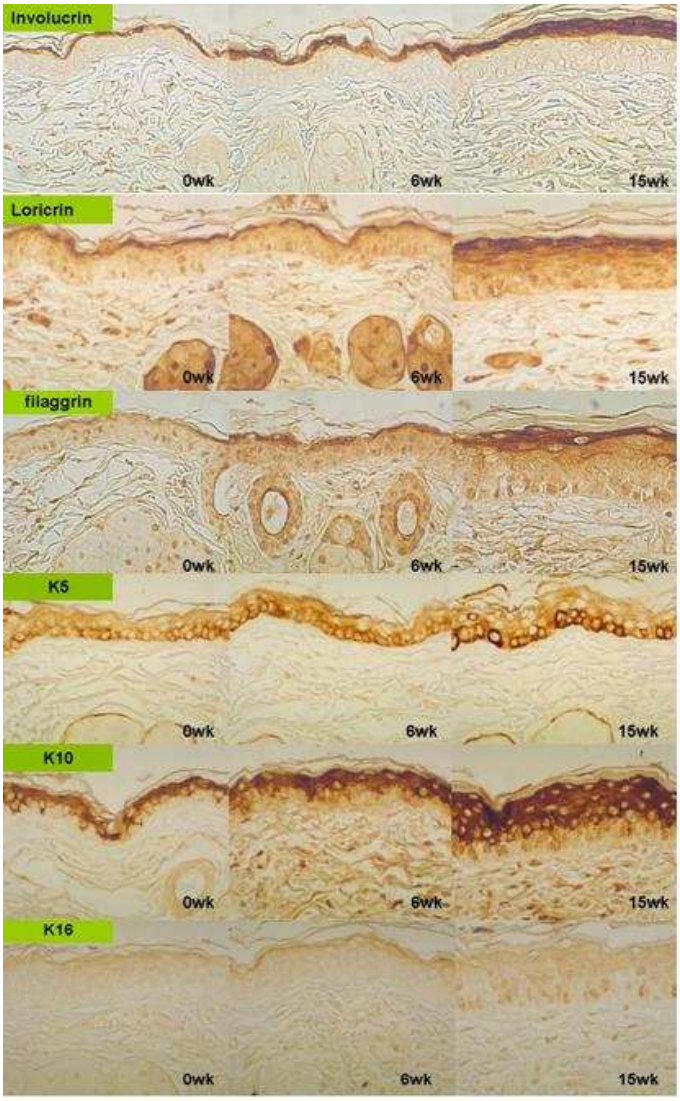

Figure 8. The expression of the epidermal differentiation related proteins such as involucrin, loricrin, filaggrin, K5, K10, and K16. Immunohistochemical staining for each of these proteins except K5 were overexpressed in the epidermis of UV irradiated mice with epidermal hyperplasia. (X 200)

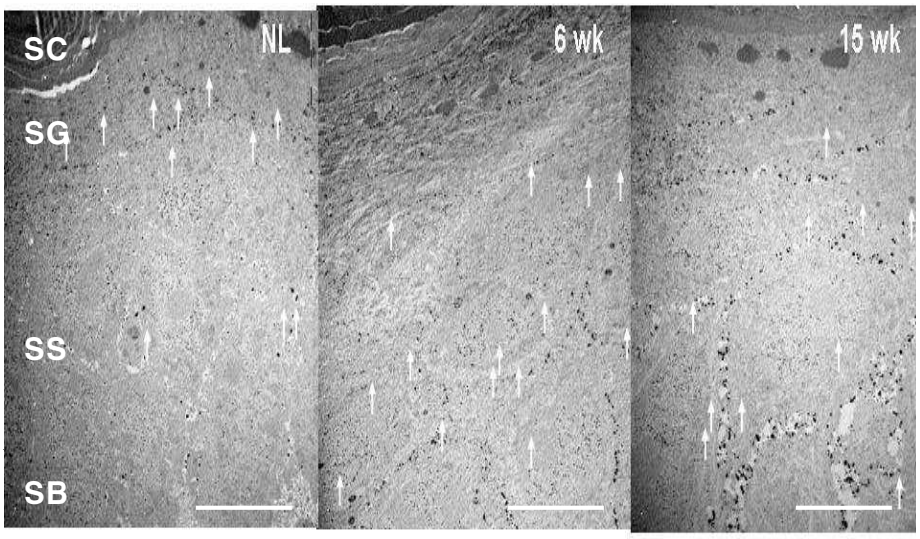

Figure 9. Changes in epidermal calcium gradient examined by calcium ion capture cytochemistry. The changes of epidermal calcium gradient in the control group (NL) showed normal calcium gradient. However, long term UV irradiated group after 6 weeks, and 15 weeks showed increased calcium ions within whole epidermis including stratum corneum (arrows). SC: stratum corneum, SG: stratum granulosum, SS: stratum spinosum, SB: stratum basale, Scale bar:0.2μm

6. Long term SED UV irradiation affects ultrastuctural changes of SC intercellular space.

To gain further insight into the mechanisms by which UV irradiation affects epidermal lipid structure, we examined the ultrastructure of the epidermis in the hairless mice. In normal epidermis, SC intercellular space contains multiple, alternating dense and lucent bands indicating the lipid lamellae (Fig. 10). In the SC intercellular space after the first 6 week after UV irradiation, incomplete SC lipid lamellae can be seen (Fig. 10). Following the irradiation period, more prominent abnormal findings including incomplete and separated lamellae with fragmentation could be seen as shown in Figure. 10.

7. The mRNA levels of epidermal lipid synthesis rate limiting enzymes were affected by long term suberythemal dose UV irradiation.

To determine whether long term UV irradiation with low doses affects epidermal lipid production including ceramide, fatty acid, and cholesterol, we measured mRNA levels of SPT, FAS, and HMG CoA reductase. This study demonstrated an increase in SPT, FAS, and HMG CoA reductase up to 6 weeks of UV irradiation (Fig. 11). But these increased mRNA levels for the three key enzymes decreased from 9 weeks to 15 weeks of UV irradiation (Fig. 12). Thus, barrier disruption by UV irradiation results in an increase in the mRNA levels for the three key enzymes, ceramide, fatty acid, and cholesterol synthesis until 9 weeks of the irradiation. Thereafter, even the levels of the enzymes represented as compensatory epidermal lipid synthesis were downregulated after 9 weeks of the irradiation.

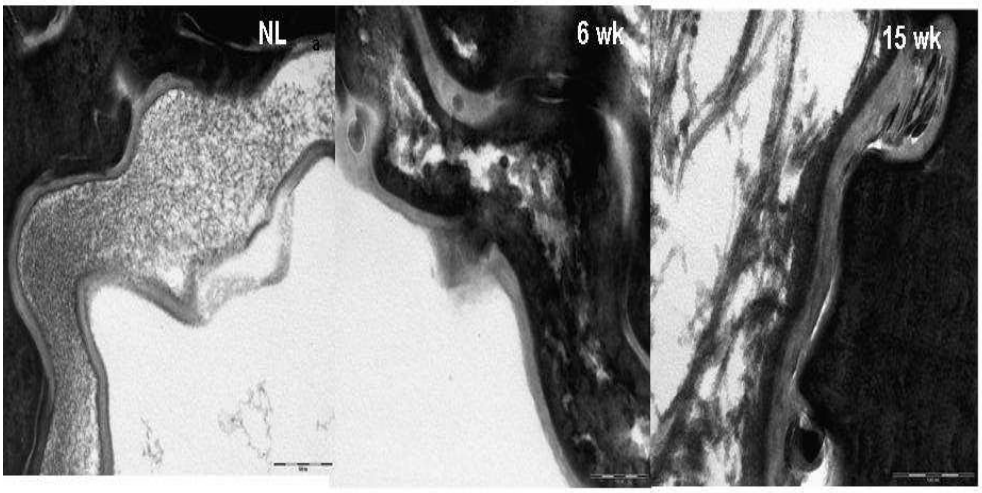

Figure 10. Changes in SC intercellular spaces. The SC intercelluar spaces including lipid lamellae of the control group (NL) showed normal in appearance. The UV irradiated group after 6 weeks and 15weeks showed remarkable changes including incomplete bilayer with fragmentation. (RuO4 postfixation) Scale bar: 100nm

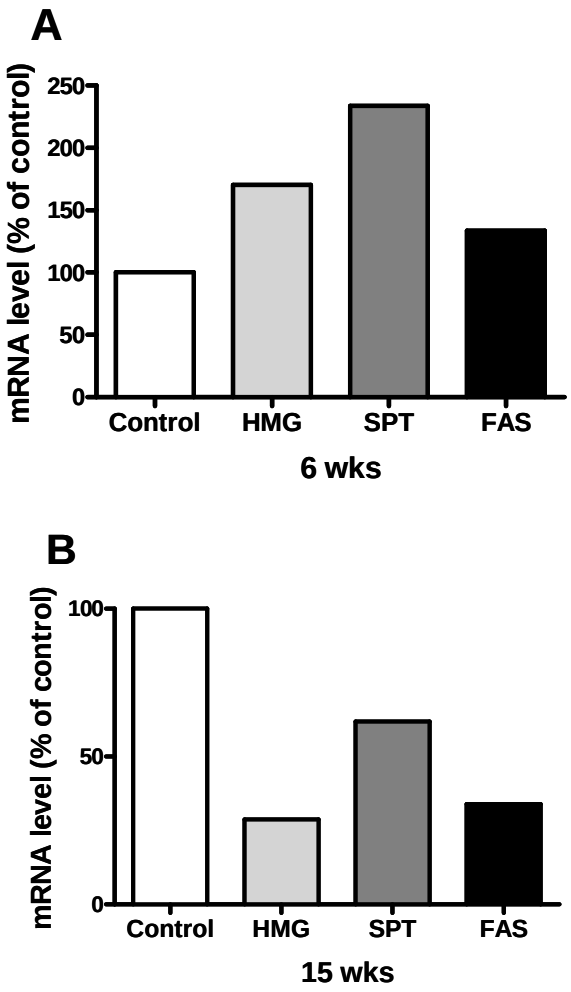

Figure 11. Relative mRNA expressions of three major key enzymes of cholesterol, ceramide, and fatty acid synthesis. The values were up-regulated in murine epidermis until 6 weeks of UV exposure (A), but were down-regulated at 15 weeks (B), suggesting the possibility that even compensatory epidermal lipid synthesis is disturbed by chronic UV irradiation. (HMG, HMG CoA reductase; SPT, serine palmitoyl transferase; FAS, fatty acid synthase)

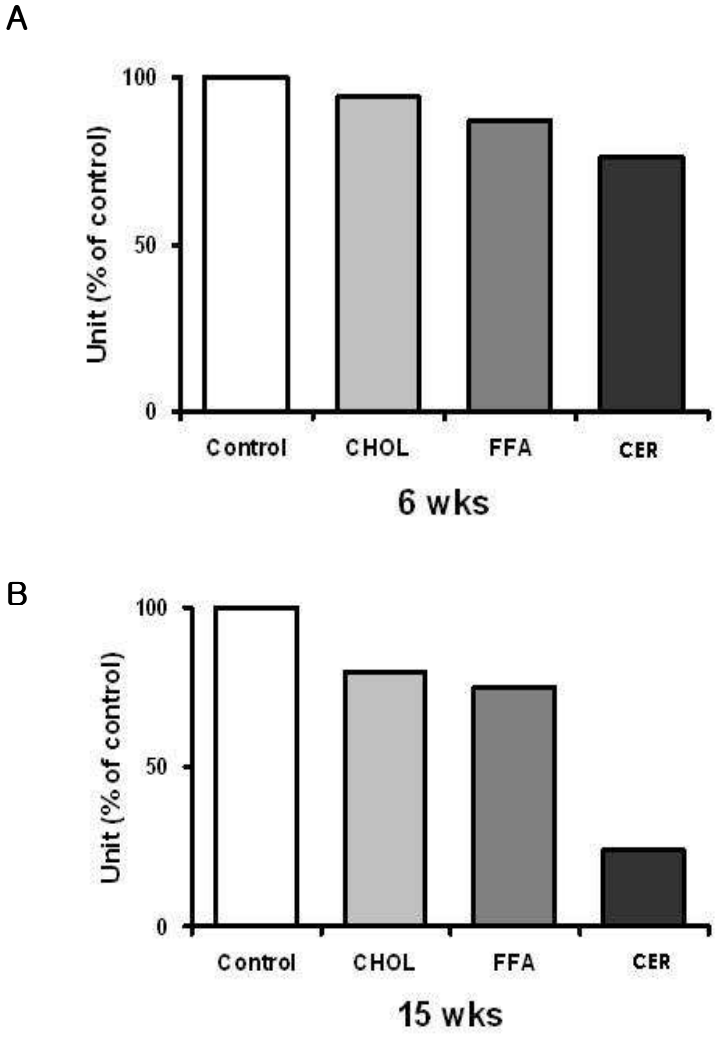

Figure 12. SC lipids analysis by HPTLC. Lipids including cholesterol, free fatty acid and ceramide were slightly reuced in murine epidermis at 6 weeks of UV exposure (A), but were more reduced at 15 weeks (B). This result implicates the possibility that epidermal lipid synthesis is disturbed by long term UV irradiation. (CHOL, Cholesterol; FFA, Free fatty acid; CER, Ceramide) * Arbitrary unit

8. SC lipids were decreased in proportion to UV exposure time.

To analyze the amounts of ceramide, fatty acid, and cholesterol, we used the HPTLC. Figure 12 shows the level of ceramide, fatty acid, and cholesterol obtained. The results reveal that the levels of these three main lipids started to decrease from 6 weeks of UV irradiation. It could be a meaningful finding that, following UV irradiation, the ceramides had more marked decreased rate than fatty acid and cholesterol.

IV. Discussion

Chronic sun exposure causes various changes in the skin that are recognized as photoaging, immunosuppression and photocarcinogenesis. Long term exposure to solar UV irradiation leads to alterations in human skin, a process referred to as photoaging. Individuals who have outdoor lifestyles, live in sunny climates, and are lightly pigmented will experience the greatest degree of photoaging.

UV irradiation of mammalian skin induces a variety of well-documented acute responses, including erythema, hyperproliferation, desquamation, and permeability barrier alterations[52]. Diminished permeability barrier fuction also has been reported in response to UVB, combined UVA and UVB or UVC[43,48,50,51]. UV irradiation stimulates the generation of a number of effector molecules immediately after exposure including a family of epidermal cytokines and prostaglandins initiating a complex cutaneous response[55-59]. UV light has a distinct effect on the production and secretion of cytokines from KC, depending upon its wavelength[60]. These mediators initiate both an inflammatory response, including erythema and epidermal hyperplasia[61]. The delayed UV light induced barrier abnormality occurs within this setting of epidermal hyperplasia and cutaneous inflammation[55].

The researches about UV exposure to the skin have commonly performed with high MED which means more than 1 MED UV light implicated in sunburn[44,52,46]. A single exposure of UVB irradiation with high MED perturbed skin barrier function and triggered cutaneous inflammatory responses by directly inducing epidermal keratinocytes to elaborate specific pro-inflammatory cytokines, such as IL-1a and TNF-a[62]. A single UVB irradiation with high MED caused not only a significant increase in TEWL but also marked morphological changes in the SC intercellular space[52].

But commonly exposed sun light to people is usually UV with SED. Photoaging process might result from accumulation of UV irradiation with SED. But because of experimental limits, there were few reports on the effects of long term UV irradiation with SED[50]. In this study, performed on the hairless mice, I have shown that long term UV irradiation even at a SED influence not only biophysical properties but also morphologic changes of the epidermis and SC intercellular spaces.

Wrinkles and sagging are characteristically observed in photoaged skin[63-65]. Earlier studies have reported decreased collagen, disappearance of reticular structure of elastic fibers, accumulation of abnormal elastic fibers, and deposition of glycosaminoglycans in the dermal extracellular matrix of photoaged skin[66-68]. Kambayashi et al. reported that chronic low dose of UV irradiation induced wrinkle formation in hairless mice and the increased keratin content of the stratum corneum may result in changes to the physical properties of the skin[53]. In this study, long term UV irradiation with SED induced wrinkle formation. These might result from the above causes in those researches. Also, the reason why fine wrinkle formation developed early at 3 week post irradiation might result from decreases in SC hydration shown in figure 2 and 3.

To elucidate the meanings of the results, we assembled and newly arranged the data obtained from the study. First, to assess the impact of long term UV irradiation on permeability barrier function, we compared the TEWL in each group. Although SC hydration decreased as seen in Figure 4, the TEWL had no significant change until 12 weeks of UV irradiation when the only significant increase found at. In this study, these result before 12 weeks of the treatment is coincident with that of Choi as regard to the skin barrier protective responses after short term UV irradiation with SED (in press). Kambayashi et al. demonstrated that low dose UV irradiation induces wrinkle

formation in hairless mice and TEWL increased significantly after 10 weeks of irradiation and this increase persisted 24 weeks without irradiation. In this study, the TEWL increased significantly after 12 weeks of irradiation and conversely, decreased at 15 weeks of treatment[53]. Up to this point, I suggest that the cumulative hazardous responses of UV irradiation with SED take approximately 12 weeks considering the result of skin barrier disruption represented as TEWL at 12 weeks of the treatment. The TEWL decreased at 15 weeks post irradiation. That might means that there could also have been some compensatory mechanisms during UV irradiation considering decrease in TEWL at 15 weeks of the treatment. Moreover light microscopy revealed a significant increase in both epidermal and dermal thickness (Fig. 6,7). Increases in epidermal thickness began to appear at 9 weeks of UV irradiation, while increases in dermal thickness began at 12 weeks of the treatment. I suggest that epidermal and dermal thickness, might partially contribute to the recovery of TEWL at 15 weeks of UV irradiation even though SC hydration decreased following UV irradiation period and lipid synthesis decreased from 9 weeks after UV irradiation, as shown previously. Alterations in epidermal proliferation are known consequences of acute UV irradiation[61]. Haratake et al. reported that hyperproliferative response is required for the UVB induced barrier defect and inhibition of epidermal proliferation diminished the effects of acute UVB exposure on the barrier[44].

Earlier researches demonstrated that low dose UV irradiation with SED for 10 weeks induced wrinkle formation with primary altering the epidermal components of the skin, rather than the dermis[53]. But, changes of dermal components might involved in photoaging process after some period of UV irradiation considering increased dermal thickness after 12 weeks of UV irradiation in this study. In addition, Werner et al. reported that keratinocytes stimulate fibroblasts to synthesize growth factors, which in turn will stimulate keratinocyte proliferation in a double paracrine manner[70]. Up to this reason, I

found that epidermal thickness increased earlier than dermal thickness after UV irradiation in this study.

UVB irradiation has also been reported to decrease DNA, RNA, and protein synthesis within the first few hours after exposure, with each of these parameters increasing subsequently, culminating with accelerated desquamation[71]. Exposure of normal adult human skin to mild erythema inducing doses of UV induces p53 and proliferating cell nuclear antigen expression, both of which, however, are associated with cell cycle progression but neither induce a mitotic response nor increased the replication-associated antigens, DNA polymerase or Ki 67[72]. Likewise, my study showed increased expression of keratinocyte differentiation represented by markers such as involucrin, loricrin, filaggrin and K10 and proliferation processes represented by K16 except K5 which commonly expressed in basal layer of the epidermis. Denda et al reported that exposure to changes in environmental humidity alone induces increased keratinocyte proliferation and markers of inflammation, and these changes are attributable to changes in stratum corneum moisture content[73]. Moreover It was well known that 1,25-dihydroxyvitamin D_3 which synthesized endogenously by a cascade of reactions including UVB-induced photochemical reaction and subsequent hydroxylations in the keratinocytes appear to play an important role in keratinocyte differentiation[74]. 25-dihydroxyvitamin D_3 increases expression of major epidermal differentiation proteins such as involucrin, loricrin, filaggrin, and transglutaminase and stimulates cornified envelope formation, which is required for optimal epidermal differentiation and permeability barrier homeostasis[75,76]. Vitamin D receptor knockout mice exhibit reduced epidermal differentiation marker expression[76]. Also, in this study, the expression of keratinocyte proliferation and differentiation markers increased and SC hydration decreased in proportion to the irradiation time. Then I suggest that UV irradiation induced keratinocyte differentiation and proliferation and

consequent dry skin represented as decreased SC hydration might accelerate these processes.

Calcium plays various roles including induction of terminal differentiation, formation of the cornified cell envelope, and also epidermal lipid synthesis[77,78]. Menon et al demonstrated that alterations of the calcium gradient affect exocytosis of the LB in SC-SG interstices[79]. My results show disruption of the calcium gradient after long term UV irradiation with SED. This disruption might be mediated by various mechanisms including alteration of epidermal differentiation and lipid synthesis.

In addition to lipid synthesis, barrier homeostasis requires the assembly and secretion of epidermal `lamellar bodies, followed by postsecretory, extracellular processing[6,80,81]. The epidermal lamellar body is a unique secretory organelle that delivers lipid precursors and hydrolytic enzymes to the SC interstices, leading to barrier formation[6]. Holleran et al. reported that the generating capacity of the lamellar body was diminished in epidermis with UVB irradiation and that T-cell dependent hyperproliferative response in the epidermis correlates with barrier abnormality[44,52,82]. In addition to potential reduction in the number of cells available for LB formation, incomplete stratum corneum lipid lamellae was found after UV irradiation and more prominent abnormal findings appeared including incomplete and separated lamellae with fragmentation could be seen following the irradiation period.

The SC intercellular lipid levels play critical roles in cutaneous barrier homeostasis. In addition, barrier homeostasis after damage to the barrier includes a series of biochemical phenomena such as an increase of lipid synthesis and lipid processing[6]. We examined changes in lipid synthesis abnormality after long term UV exposure with SED. It should be noticed that the three major enzymes for lipid synthesis were activated until 6 weeks of UV irradiation to compensate the unrecognizable microscopic changes and

conversely decreased from 9 weeks to 15 weeks of treatment. These result show that compensatory processes of lipid synthesis were sustained until 6 weeks and thereafter, downregulated from 9 weeks of UV irradiation. I hypothesized that some inefficient lipid formation processes including abnormal LB formation could be the reason for these results.

In lipids profile after UV irradiation, the levels of ceramides, fatty acid, and cholesterol began to decrease significantly at 9 weeks of UV irradiation. These results were accompanied with down-regulated activity of lipid synthetic enzymes as shown previously. Moreover, there was a particularly significant reduction of ceramide among the lipids, although Holleran et al. reported an increase of ceramide synthesis accompanied by increased SPT activity following high doses and short term UVB irradiation[83]. In chronologically aged mice, a deficiency in lipid synthesis, particularly in cholesterologenesis, accounts for barrier abnormality and lipid-induced acceleration of barrier recovery in aged epidermis correlates with repletion of the extracellular spaces with normal lamellar structures[84]. Although the total lipid content decreased in the stratum corneum of aged mice (approximately 30%), the distribution of ceramides (including ceramide 1), cholesterol, and free fatty acids was unchanged[85]. Compared to these previous report, it was the distinguished point that, in this study, a significant reduction of ceramide was found in the hairless mice treated with long term UV irradiation with SED. Although further study to elucidate the underlying mechanisms of diminished lipid profiles after long term UV irradiation is warranted to confirm the alteration and compensatory responses, it is supposed that reduced lipid profiles resulted from cumulative result of long term UV irradiation with SED. Also, although we need more research about recovery after application of ceramide containing moisturizer, we suggest that ceramidogenesis can be associated with barrier disruption and down regulated

in photoaged mice. These results could implicate phototherapy, photoaging, and photooncogenesis.

V. Conclusion

In conclusion, long term UV irradiation even with SED to which people are commonly exposed influences skin barrier function and structure. Marked ceramide decreases in SC intercellular lipid after long term UV irradiation can be implicated in the treatment or prevention of photoaging.

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무모생쥐에서 장기간의 자외선 조사가 피부장벽에 미치는 영향

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자외선 조사는 피부의 물리적, 생화학적 성상의 변화를 초래하여 홍반의 발생, 표피세포의 증식, 과색소 침착, DNA 손상 등을 일으킨다. 또한 이러한 반응은 자외선의 파장과 용량에 따라 차이가 있음이 여러 연구를 통해 밝혀졌다. 본 연구에서 무모 생쥐의 정상 피부에 홍반을 일으키지 않는 저용량(suberythemal dose)의 자외선 A와 B를 1주에 3회씩 15주간 반복 조사함으로써 지속적인 장기간의 자외선 조사가 피부장벽에 미치는 영향을 관찰하고자 하고자 3주 간격으로 피부 장벽의 기능과 구조적인 변화를 측정하였다.

자외선 조사 3주 경부터 육안적인 주름이 생기고 조사기간이 증가함에 따라 점점 심해지는 양상을 보였다. 경표피수분 손실량은 증가하는 경향을 보이고 각질층 피부 함유량은 조사기간에 비례하여 감소하였다. 조직병리학적 검사소견상 표피와 진피의 두께는 시간에 따라 증가하였고 각질형성세포 분화의 표지자에 해당하는 involucrin, loricrin, filaggrin, K5, K10, K16에 대한 면역조직화학 염색상 과발현 되거나 비정상적인 표현이 관찰되어 표피증식과 분화이상을 관찰하였다. 표피에서 칼슘 이온의 분포는

자외선 조사기간이 길어질 수록 칼슘 이온 농도의 기울기가 정상에서 벗어나 왜곡되어 있음이 관찰되었다. 전자현미경 소견에 의하면 각질층의 각질세포간 지질막구조가 불완전하고 열공이 확장되어 가는 것을 관찰하였다. 지질 생성의 주요 효소인 serine palmitoyl transferase, HMG CoA reductase, fatty acid synthase의 mRNA에 대한 real time RT-PCR상 6주까지 점차 증가되어 있다가 9주부터 감소하는 경향을 보였고 특히 세라마이드의 생성에 중요한 역할을 하는 serine palmitoyl transferase의 mRNA가 현저하게 감소하였다. 각질층 지질의 주요 성분인 세라마이드, 콜레스테롤, 지방산에 대한 지질분석상 6주부터 감소하는 경향을 보였으며 특히 세라마이드의 뚜렷한 감소가 관찰되었다.

결론적으로 일상 생활에서 흔히 접할 수 있는 저용량의 자외선일지라도 반복적으로 장기간 노출되면 피부장벽의 기능적이고 구조적인 변화를 초래하여 손상을 줄 수 있음을 알 수 있었다. 특히 장기간의 자외선 조사 후에 각질세포간 지질막 조성의 변화를 동반하는 세라마이드의 두드러진 감소의 발견은 향후 예방 및 치료제 개발에 이용할 수 있다.

핵심 되는 말 : 자외선, 홍반하 용량, 피부장벽, 각질세포 외막, 각질세포간 지질막, 세라마이드