Cell Injury, Repair, Aging and Apoptosis

Mechanistic Effects of Long-Term Ultraviolet B Irradiation Induce Epidermal and Dermal Changes in Human Skin Xenografts

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UVB irradiation has been reported to induce photoaging and suppress systemic immune function that could lead to photocarcinogenesis. However, because of the paucity of an UVB-induced photodamaged skin model, precise and temporal mechanism(s) underlying the deleterious effects of long-term UVB exposure on human skin have yet to be delineated. In this study, we established a model using human skin xenografted onto severe combined immunodeficient mice, which were subsequently challenged by repeated UVB irradiation for 6 weeks. Three-dimensional optical image analysis of skin replicas and non-invasive biophysical measurements illustrated a significant increase in skin surface roughness, similar to premature photoaging, and a significant loss of skin elasticity after long-term UVB exposure. Resembling authentically aged skin, UVB-exposed samples exhibited significant increases in epithelial keratins (K6, K16, K17), elastins, and matrix metalloproteinases (MMP-1, MMP-9, MMP-12) as well as degradation of collagens (I, IV, VII). The UVB-induced deterioration of fibrous keratin intermediate filaments was also observed in the stratum corneum. Additionally, similarities in gene expression patterns between our model and chronologically aged skin substantiated the plausible relationship between photodamage and chronological age. Furthermore, severe skin photodamage was observed when neutralizing an-

tibodies against TIMP-1, an endogenous inhibitor of MMPs, were administered during the UVB exposure regimen. Taken together, these findings suggest that our skin xenograft model recapitulates premature photoaged skin and provides a comprehensive tool with which to assess the deleterious effects of UVB irradiation. (*Am J Pathol* 2009, 174:401–413; DOI: 10.2353/ajpath.2009.070500)

Morphological changes in the skin, such as wrinkling and sagging, are often found in photoaged skin caused by chronic UV irradiation.^{1–3} Skin photodamage is physiologically correlated with several alterations including the increase and disorganization of elastic fibers and the reduction of collagens in the dermal extracellular matrix^{4–11} and the increase of keratin contents and the deterioration of keratin intermediate filaments (KIFs) in the epidermis.^{12–14}

Elastic fibers are age-dependent-decreasing dermal extracellular matrix that respond primarily to the fibrous mechanism controlling cutaneous elasticity.^{4,5,15,16} The accumulation of dystrophic elastotic material in the reticular dermis, referred to as solar elastosis, is commonly observed in photoaged skin.^{17–19} Reported in both *in vivo* and *in vitro*, UVB irradiation induces tropoelastin gene and protein expression in fibroblasts and keratinocytes, resulting in an aberrant accumulation of dermal elastic fibers and elastin content.^{5,8,10,11,20} On the other hand, it has been reported that the degeneration of these fibers in chronologically and/or photoaged skin stems from an

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increase in matrix metalloproteinase-12 (MMP-12) and/or elastase, an elastin-degrading enzyme that is produced and secreted by dermal fibroblasts.^{21–24} To date, the mechanisms underlying the alteration of elastin fibers in photoaged skin including their production, accumulation, and degradation are not fully understood.

As the main constituent of the dermal matrix, collagen fibers are responsible in regulating the mechanical properties of the skin (ie, skin resilience and skin elasticity). In photoaged skin, the concomitant reductions of collagen I and III occur as procollagen synthesis subsides and the existed dermal collagen degrades because of the disproportionate expression of MMP-1.^{25–28} The reduction of collagens VII and IV at the dermo-epidermal junction has been also reported in the pronounced wrinkling skin.²⁹ In hairless mouse, repetitive UVB exposure has been reported to escalate the degradation of collagens VII and IV by up-regulating MMP-2 and MMP-9 expression.^{30,31}

In addition to the alterations in the dermal compartment, the changes in several epithelial keratins have been well documented in photoaged skin. In healthy skin, keratins compose up to 85% of a fully differentiated keratinocyte and orderly form the epithelial-specific intermediate filament composed of acidic type I (K9 to K20)-basic type II (K1 to K8) coiled-coil heterodimer in healthy skin.^{32–37} The existence of keratin fibers strengthening the viscoelastic properties in the stratum corneum (SC) provides a semi-impermeable membrane that is tough and resilient.^{38–41} In hairless mice, a repeated low-dose UV exposure has been reported to deteriorate KIFs and induce the expression of epithelial keratins (K1, K5, and K10), resulting in the loss of skin elasticity and eventually initiating wrinkled skin, consistent with previous reports demonstrating no causal relationship between wrinkle formation and dermal changes.^{12–14,42,43}

Many studies of skin photoaging have been conducted using animal models and human skin substitute. The results may be misleading because of inferior architecture, such as the relative thin epidermal layer and compromised barrier function.⁴⁴ The use of actual human skin or human skin xenografts to study skin photoaging is more appropriate.^{45–47} The reversible hyperproliferation and differentiation of human epidermis after acute UVB exposure has been successfully demonstrated using human skin grafted onto congenitally athymic nude mice.⁴⁸ Although the lack of inflammatory cells, T and B cells, in athymic and severe combined immunodeficient (SCID) mice allows the implantation of human skin without rejection, it may pose a problematic interpretation of immune function and UV response. A result from a clinical study suggested that T lymphocytes are involved in collagen degradation by the activation of MMP-1.⁴⁹ To date, the detail mechanisms of premature photoaging because of long-term UVB exposure on actual human skin had not been assessed. Therefore, it is of particular interest to establish a chimeric model with human skin xenograft on SCID mice to examine the mechanism(s) of photo-damaged skin after long-term UVB irradiation. Here we report for the first time that repeated UVB irradiation in combination with the repetitive motion of the human skin xenograft represents a photodamaged skin model

reflecting the documented changes in both the epidermis and the dermis. In addition, we also suggest new insights for the involvement of K6 and its partners in this process.

Materials and Methods

Materials

RNAlater and RNeasy micro kits were purchased from Qiagen (Valencia, CA). The ABI Prism 7300 sequence detection system and the TaqMan gene expression assays were obtained from Applied Biosystems (Foster City, CA). Other chemicals were of reagent grade.

Human Skin

Full-thickness abdominal skin from a healthy 41-year-old Caucasian female with type II skin undergoing abdominoplasty, facial skin from a healthy 58-year-old African descent female from face-lifting (University Pointe, Cincinnati, OH), and three punch-biopsy skins at upper arm from healthy 26- to 28-year-old and 50- to 52-year-old Caucasian females with type II skin (Stephens and Associates, Carrollton, TX) were used in this study. Collection of the skins was approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center and informed consent was obtained from the volunteers before the procedure.

Animal Grafting

Animals were handled according to the guidelines of Institutional Animal Care and Use Committee at Cincinnati Children's Hospital. Twenty female ICR-SCID mice, 4 to 6 weeks old, (Taconic Farms, Germantown, NY) were kept under pathogen-free conditions throughout the experiments and maintained on a standard laboratory diet and water *ad libitum*. After 1 week of acclimation, animals were shaved with an electric clipper to remove the dorsal hair. Mice were anesthetized by isofluorane/oxygen (3%/0.8 L), and maintained using isofluorane/oxygen (2%/0.7 L) throughout the surgery. The shaved dorsal skin was treated with a nonstaining surgical scrub on confirmation of anesthesia. The dorsal site was cut to produce a wound bed of ~2.0 to 3.0 cm in diameter. Full-thickness abdominal skin was sutured in place with a reverse cutting precision monofilament PS-3, 6–0. The edges of the graft bed were treated with sensorcaine to provide analgesia. After the surgery, the mice were kept in an incubator at 37°C either for an hour or until recovered from anesthesia. The grafted skin was observed using a Charm View fitted with or without a polarizing lens (Moritex Corp., Tokyo, Japan).

UVB Irradiation

UVB irradiation of the human skin xenograft on the SCID mice was performed ~10 weeks after the grafting when healing was complete. UVB irradiation was performed as

described by Takema and colleagues⁵⁰ except for the source of UVB. Briefly, a bank of two UVB lamps (34-0044-01 lamps; UVB, Upland, CA) with a filter yielding an emission peak near 302 nm was used, and the energy output was measured with a UV light meter, UV-340 MSR7000 (Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan). The distance from the filters to the surfaces of skin was ~30 cm. A progressive UVB exposure regimen was used starting at ~40 mJ/cm² that equals 1 minimal erythema dose (MED) of grafted Caucasian abdominal skin (data not shown) and was increased by 10 mJ/cm² per week until week 3. The irradiation dose of 60 mJ/cm² was kept constant for the remaining period of exposure. The grafted abdominal skin was irradiated five times weekly for 6 weeks yielding the total UVB dose of ~1.65 J/cm². During the period of exposure, mice with human skin xenografts could move around freely in the ~100-cm² clear container. Before each UVB exposure, a 30-second mechanical stretching was performed to simulate the physiological movement of human skin (eg, facial expression).

Administration of TIMP-1-Neutralizing Antibody

In addition to UVB irradiation, human skin xenografts were injected subepidermally with 20 µg/ml of TIMP-1-neutralizing antibody (MorphoSys AG, Planegg, Germany) in another experiment.⁵¹ This treatment was performed three times per week (every other day) for 6 weeks. The same concentration of purified mouse IgG (Vector Laboratories, Inc., Burlingame, CA) was used as a control.

Skin Profilometric Measurement

A replica of the abdominal skin xenograft was produced by the use of a replica agent, SILFLO (Flexico Developments, Potters Bar, UK). Skin replicas were prepared before irradiation, and in the 3rd and the 6th weeks after initial exposure. In brief, skin replicas were compressed using GC exafine (GC Corporation, Tokyo, Japan). The three-dimensional optical measurement of each replica was depicted using PRIMOS Compact (GF Messtechnik GmbH, Berlin, Germany), as previously reported.^{52–56} After measurement, moiré fringes were eliminated, and an analytical straight line (7 to 9 mm) was drawn on each replica. Along this line, linear roughness parameter values were assessed. In a plane analysis, a rectangular area (4 to 5 mm × 7 to 9 mm) was chosen in the central part of each replica. Four parameters, Ra and Sa (arithmetical mean roughness) and Rz and Sz (mean peak to valley height) were used as a degree of photodamaging (wrinkle) indicator. Ra and Rz are one-dimensional and Sa and Sz are two-dimensional parameters. Ra and Sa are arithmetical mean roughness and Rz and Sz are arithmetical average of the maximum peak to valley height of the roughness values of five consecutive sampling sections over the filtered profile.

Elasticity Measurement

Skin elasticity was measured using the Cutometer SEM575 (Courage and Khazaka Electronic, Köln, Germany) as described elsewhere.⁵⁷ The time/strain mode was used with 5-second application loads of 200 hPa, followed by a 2-second relaxation period. The center of the skin xenograft was used for evaluation. Four parameters, Ue (the immediate deformation), Uv (the delayed distention), Ur (the immediate refraction), and Uf (the final distention) were used as a degree of elasticity indicator.

Ur/Ue = pure elasticity ignoring creep.

Uv/Ue = viscoelastic/elastic extension.

Ur/Uf = elastic recovery/total deformation.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Twenty-four hours after repeated UVB irradiation, the transcript expressions of keratins (K1, K5, K6, K16, and K17), collagen IV α1, and tropoelastin in human skin xenografts were examined using real-time quantitative RT-PCR normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Skin specimens were collected in RNeasy lysis buffer (Qiagen), and total RNA from each sample was prepared using a RNeasy micro kit (Qiagen). cDNA was synthesized by reverse transcription of total RNA using oligo dT and Moloney murine leukemia virus reverse transcriptase. Real-time quantitative RT-PCRs with TaqMan gene expression assays were performed using an ABI Prism 7300 sequence detection system (Applied Biosystems).

Western Blotting

To investigate the changes in keratin levels, epidermis was separated from skin xenografts 24 hours and 4 weeks after final repetitive UVB exposure and soaked in 2 mol/L NaBr.⁵⁸ Samples were lysed in the buffer containing 8 mol/L urea, 50 mmol/L Tris-HCl (pH 7.6), 100 mmol/L mercaptoethanol, 1 mmol/L dithiothreitol, and a protease inhibitor cocktail (Boehringer-Mannheim, Mannheim, Germany), and separated on 10% sodium dodecyl sulfate gels (Bio-Rad Laboratories, Hercules, CA).

For the investigation of collagens I, IV, and VII, MMP-1, -9, and -12 and tropoelastin, the whole skin was solubilized in cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing 1 mmol/L phenylmethyl sulfonyl fluoride (Sigma, St. Louis, MO). The 20 µg of tissue extracts were separated on 7.5% sodium dodecyl sulfate gels (Bio-Rad). Samples were transferred to polyvinylidene difluoride membranes (Bio-Rad) and incubated with one of the following antibodies: keratin K1 (Covance, Princeton, NJ), keratin K6 and K17 (Acris Antibodies GmbH, Hiddenhausen, Germany), collagen I (United States Biological, Swampscott, MA), collagen IV and VII (Abcam, Cambridge, MA), MMP-1 and -9 (BIOMOL, Plymouth Meeting, PA), MMP-12 (R&D Systems, Minneapolis, MN), tropoelastin (Cosmo Bio, Tokyo,

Figure 1. Skin profilometric measurement: a progressive deterioration of skin surface roughness after long-term UVB irradiation. **A:** *En face* images of skin were captured at 0, 3, 6, and 10 weeks using a hand-held microscope, Charm View. Skin profilometry of replicas was used to evaluate the severity of furrow formation, Ra (**B**), Rz (**C**), Sa (**D**), and Sz (**E**). Gray squares and circles represent control and UVB-irradiated skin, respectively. The values reported represent mean $\pm$ SD from 10 independent xenografts per group. * $P < 0.05$, ** $P < 0.01$ (compared to the values at 0 weeks). Scale bars = 3 mm.

Japan). Subsequent visualization was performed and normalized against a control protein, β -actin (Sigma) as described elsewhere.⁵⁹

Hematoxylin and Eosin (H&E) Staining

The 5- μm -thick sections of the wrinkle and photodamaged skins perpendicularly cut to the principal axis were stained by conventional H&E staining.

Immunohistochemistry

Abdominal skin xenografts described above were fixed in 10% buffered formalin and embedded in paraffin. The immunoreactivities of keratins K1, K6, K16, K17, and collagens I and IV were assessed using one of the following primary antibodies: monoclonal mouse anti-

human cytokeratin 6, 16, and 17 (Acris Antibodies GmbH), rabbit polyclonal anti-human keratin 1 (Covance), and collagen I (United States Biological) and IV (Calbiochem, San Diego, CA). Normal rabbit or mouse IgG (Vector Laboratories) in place of the primary antibodies were used as negative control. After 20 minutes of heat pretreatment in either 10 mmol/L citrate buffer (pH 6.0) or ethylenediaminetetraacetic acid buffer (pH 8.0) for keratin and collagen detection, respectively, the paraffin sections were enzymatically digested in a warm proteinase K solution (DAKO, Carpinteria, CA) for 5 minutes (except for K6, K16, and K17). The immunoreactivities of K1 and collagen I were visualized using the DAKO Envisionplus kit, and the immunoreactivities of K6, K16, K17, and collagens I and IV were detected using a CSAII biotin-free catalyzed signal amplification system (DAKO) with aminoethylcarbazole.

Table 1. Mechanical Parameter Changes after Chronic UVB Irradiation in Human Skin Xenografts

	Mechanical parameter $\pm$ SD		
	Ur/Uf	Uv/Ue	Ur/Ue
Control (0 weeks)	0.402 $\pm$ 0.085	0.537 $\pm$ 0.105	0.571 $\pm$ 0.089
Control (6 weeks)	0.341 $\pm$ 0.037	0.518 $\pm$ 0.090	0.500 $\pm$ 0.048
UVB (0 weeks)	0.423 $\pm$ 0.114	0.574 $\pm$ 0.216	0.645 $\pm$ 0.124
UVB (6 weeks)	0.328 $\pm$ 0.104*	0.649 $\pm$ 0.236	0.452 $\pm$ 0.166*

* $P < 0.05$, significantly different from the values at 0 weeks.

Figure 2. Morphological changes of skin xenografts after repetitive UVB exposure recapitulated authentic wrinkle skin. H&E staining of skin section from nonexposed skin (**A, B**), repeated UVB-exposed skin collected 24 hours (**C, D**) and 4 weeks after the final irradiation (**E, F**), genuine wrinkle skin from face-lift surgery (**G, H**), and young healthy skin (**I, J**). **Inset** to **E** demonstrates compact cell in spinous layer. **Asterisks** indicate punctuate collagen aggregates. Scale bars: 100 μ m (**left**); 50 μ m (**right**). **Inset** to figure 2E, **arrowhead** indicates compact cells in the spinous layer. Magnification = 100 $\times$.

Elastica Van Gieson Staining

Elastic fibers were visualized using Accustain elastic stain kit (Sigma) according to the manufacturer's instructions.

Microarray Analysis

Isolated RNA from abdominal grafts in control and UVB groups as well as RNA from the upper arm of young (20's) and old (50's) donors were purified using RNeasy columns (Qiagen). mRNA was converted to cDNA and biotinylated complementary RNAs were synthesized using T7 RNA polymerase and hybridized in Hg-U133Plus v.2 (Affymetrix, Santa Clara, CA). Raw data were processed using GC-RMA algorithm and normal-

Figure 3. Transcript mRNA expression of keratins, collagen IV, and tropoelastin in human skin xenografts after repeated UVB irradiation. Human skin xenografts on SCID mice were exposed to 1 to 2 MED UVB for 6 weeks, followed by real-time quantitative RT-PCR analysis of keratin, collagen IV, and tropoelastin mRNA transcript levels. Real-time quantitative RT-PCR was performed using an ABI Prism 7700 sequence detection system as detailed in the Materials and Methods. The expression of keratin, collagen IV, and tropoelastin transcripts in the UVB-exposed xenograft was normalized against the expression of GAPDH and expressed relative to that of nonirradiated control. The values reported represent mean $\pm$ SD from 5 to 11 independent xenografts per group. White and gray bars represent the values of controls and UVB-irradiated xenografts, respectively. * $P < 0.05$.

ized in GeneSpring GX 7.3 (Agilent, Palo Alto, CA). Differentially expressed genes were identified using Student's *t*-test ($P < 0.05$).

Transmission Electron Microscopic Analysis

To confirm that the deterioration and/or disorganization of KIFs in SC and of collagens in the dermis contribute to morphological changes, transmission electron microscopy was conducted using skin xenografts 24 hours after the final irradiation of repetitive UVB exposure as described elsewhere.⁶⁰

Statistics

The level of significance of the difference was calculated by the Student's *t*-test. In addition, a nonparametric one-way analysis of variance (Kruskal-Wallis test) was used to evaluate differences between before and after UVB irradiation of image analyses with replica. Where appropriate, a nonparametric post hoc multiple comparison test (Steel-Dwass test) was performed to evaluate differences between the groups. A *P* value < 0.05 was considered statistically significant, unless otherwise noted.

Results

A significant increase in skin surface roughness was observed on abdominal skin xenografts after long-term UVB irradiation, accompanied by a significant decrease in elasticity. To investigate whether premature

Table 2. Similarity of Gene Expression Profiles Between Prematurely Photoaged Skin and Chronologically Aged Skin from Microarray Data Analysis

Biological function	Gene symbol	Gene name	Fold changes	
			UVB/control	Old/young
1. Apoptosis	<i>SH3GLB1</i>	SH3-domain GRB2-like endophilin B1	1.86	3.16
	<i>SIVA</i>	CD27-binding (Siva) protein	1.94	5.38
	<i>PDCD5</i>	Programmed cell death 5	2.02	2.41
	<i>CASP1</i>	Caspase 1	2.68	2.40
2. Ion transport	<i>CCL11</i>	Chemokine (C-C motif) ligand 11	8.29	1.48
	<i>CACNA1A</i>	Calcium channel, P/Q type, α 1A subunit	1.62	2.49
	<i>KCNK1</i>	Potassium channel, subfamily K, member 1	2.57	2.24
	<i>KCTD12</i>	Potassium channel tetramerization domain containing 12	0.47	0.51
3. Cytokines and immune response	<i>LILRB1</i>	Leukocyte immunoglobulin-like receptor	1.97	0.50
	<i>TNFRSF4</i>	Tumor necrosis factor receptor superfamily, member 4	0.79	0.17
	<i>FCER1A</i>	Fc fragment of IgE, receptor for α polypeptide	0.52	0.46
	<i>AIF1</i>	Allograft inflammatory factor 1	0.43	0.18
4. Collagens and MMPs	<i>COL1A1</i>	Collagen 1, α 1	0.59	0.03
	<i>COL3A1</i>	Collagen 3, α 1	0.73	1.55
	<i>MMP1</i>	Matrix metalloproteinase 1	11.62	1.22
	<i>MMP9</i>	Matrix metalloproteinase 9	2.74	2.59
	<i>MMP13</i>	Matrix metalloproteinase 13	71.38	1.26
5. DNA metabolism and repair	<i>PTTG2</i>	Pituitary tumor-transforming 3	1.59	3.62
	<i>MSH6</i>	mutS homolog 6 (<i>E. coli</i>)	3.48	4.97
	<i>DNMT1</i>	DNA (cytosine-5-)-methyltransferase 1	2.30	3.96
	<i>POLE2</i>	Polymerase (DNA directed), ϵ 2 (p59 subunit)	3.80	1.69
	<i>POLRMT</i>	Polymerase (RNA) mitochondrial (DNA directed)	2.37	1.51
6. Cell proliferation, differentiation, and desquamation	<i>KRT1</i>	Keratin 1	1.40	2.31
	<i>KRT6B</i>	Keratin 6B	1.78	3.63
	<i>KRT10</i>	Keratin 10	1.35	2.32
	<i>KRT16</i>	Keratin 16	3.42	1.70
	<i>KRT17</i>	Keratin 17	1.28	1.25
	<i>CTNBP1</i>	Catenin, β interacting protein 1	2.84	4.15
	<i>GATA3</i>	GATA-binding protein 3	2.50	1.86
	<i>S100A14</i>	S100 calcium-binding protein A14	2.48	1.82
	<i>FLG</i>	Filaggrin	1.65	2.69

The *t*-test *P* value is less than 0.05 unless otherwise indicated.

photoaging/photodamaging is induced on human abdominal skin xenografts after repetitive UVB irradiation, the roughness of the skin surface and skin elasticity were assessed at baseline and at 3, 6, and 10 weeks. The visible furrows were observed by the end of UVB exposure regimen and maintained at least 4 weeks after the final irradiation. No macroscopic changes were observed in the nonirradiated control (Figure 1A). Quantitation of fine lines on the replicas obtained 6 weeks after continuous UVB exposure demonstrated a significant increase ($P < 0.05$ or $P < 0.01$) in all parameters (Ra, Sa, Rz, and Sz) with no change in the nonirradiated control (Figure 1, B–E). Skin elasticity as expressed by the elastic recovery (Ur/Ue) was significantly ($P < 0.05$) decreased after repeated UVB irradiation (Table 1), consistent with a previous report.⁶¹ In addition, the ratio between the viscoelastic properties and intermediate distention (Uv/Ue) increased moderately, whereas the ability of the skin to regain the shape after deformation (Ur/Uf) decreased significantly ($P < 0.05$).

Morphological Characterization of Premature Photoaged Skin Xenografts Demonstrated Similarities in Epidermal and Dermal Alteration Compared to Authentic Aged Skin

H&E staining illustrated that long-term UVB exposure in combination with a repetitive motion dramatically altered epidermal and dermal morphology compared to nonirradiated control or biopsy obtained from a young donor (Figure 2, A and B). Epidermal hyperproliferation and the breakage of collagen fibrils were documented in the samples collected within 24 hours after final irradiation (Figure 2, C and D). Compact cells in the spinous layer (Figure 2E, inset arrows) and punctate collagen remodeling were noted in samples collected 4 weeks later (Figure 2F, asterisks). Likewise, the marked difference in skin morphology was observed between genuine wrinkled skin (Figure 2, G and H) and young healthy skin (Figure 2, I and J). At the bottom of authentic wrinkled skin, epithelial cells were tightly

Figure 4. The decrease in collagen I expression after continuous UVB irradiation was irreversible. Western blotting analysis of collagen I, MMP-1, and β -actin (**A**) and immunohistochemistry of collagen I and rabbit IgG (**B**) confirmed that collagen I protein expression was significantly decreased 24 hours after the final irradiation and did not return to normal levels 4 weeks after the cease exposure. Densitometric analysis was performed to evaluate the intensity of each band. * $P < 0.05$, ** $P < 0.01$. The values reported represent the mean $\pm$ SD from five to eight independent xenografts per group. Scale bars = 100 μ m.

compressed compared to the surrounding skin, whereas the dermal compartment was loosely packed with anomalous collagen arrangements.

Alteration in Collagen and Elastin Fibers Were Related to the Loss of Elasticity and Furrow Formation after Repetitive UVB Exposure

Real-time quantitative RT-PCR analysis of samples collected 24 hours after the final UVB irradiation demonstrated a significant ($P < 0.05$) decrease and increase in expression of collagen IV α 1 and tropoelastin mRNA expression, respectively, consistent with previous reports (Figure 3).^{8,10} In addition, microarray analysis of samples from young and old Caucasian skin compared to human skin xenograft with or without continuous UVB exposure demonstrated many similarities (Table 2). Consistently,

Figure 5. Long-term UVB irradiation diminished dermal collagens and stimulated MMP production. **A:** Western blotting analysis of collagens (IV and VII) and MMP-9 was conducted to evaluate the effects of UVB on their expressions. The band of β -actin was used to normalize the expression level. Each band shows a representative result of Western blotting analyses, which were repeated three to six times at 24 hours after the final UVB irradiation, with similar results. Densitometric analysis was also performed to evaluate the intensity of each band. * $P < 0.05$, ** $P < 0.01$. The values reported represent means $\pm$ SD. **B:** Immunohistochemistry of collagen IV and rabbit IgG also illustrated the substantial reduction of collagen IV protein expression along the dermo-epidermal junction and blood vessels 24 hours after chronic UVB exposure. Scale bars = 100 μ m.

Western blotting analysis of samples collected 24 hours after final irradiation showed the significant ($P < 0.01$ or $P < 0.05$) decreased expression of collagens I, IV, and VII and a significant ($P < 0.05$) increase in the MMP-1 (data not shown), MMP-9 and MMP-12 and tropoelastin protein expression (Figures 4A and 5A). The significantly ($P < 0.05$) decreased expression of collagen I and the significant ($P < 0.05$) increase in MMP-1 were demonstrated 4 weeks after exposure. Immunohistological analyses confirmed that collagen I expression was remarkably degraded throughout the dermis and irreversible 4 weeks after the final exposure (Figure 4B). In addition, collagen IV expression along the dermo-epidermal junction was substantially decreased in UVB-exposed skin in the papillary dermis (Figure 5B). Furthermore, Elastica Van Gieson staining indicated the accumulation of elastotic material in the reticular dermis after continuous UVB exposure, but devoid in the upper papillary dermis (Figure 6A). On the other hand, fine elastic fibers (elaunin and oxytalan fibers) were observed in the upper papillary dermis in the nonexposed skin compared to the UVB-exposed skin (Figure 6A). In authentic aged skin from face-lift surgery, a lesser amount of elastic fibers were observed in both papillary dermis and reticular

Figure 6. Depletion of papillary dermal elastin and the irregular deposition of elastin in reticular dermis after repetitive UVB irradiation. **A:** Elastica Van Gieson staining was performed to evaluate elastic fibers in control, premature photoaging model, and aged skin from face lifts. **B:** Western blot analysis was also conducted to confirm the effect of UVB irradiation on tropoelastin and MMP-12 levels. The values reported represent mean $\pm$ SD from five to eight independent xenografts per group. Scale bars = 100 μ m. * P < 0.05, ** P < 0.01.

dermis (Figure 6A). The paucity of robust elastic fibers in aged skin may be a primary basis of skin sagging, which was not clearly observed in our premature aging model. The significant increase in tropoelastin and MMP-12 as a result of repeated UVB irradiation was also confirmed (Figure 6B).

The Aberrant Expression of Keratins K6, K16, and K17 Was Involved in Both Photoaging and Chronologically Aged Skin

By using a "find similar sample" tool in GeneSpring, we observed a close correlation between our photodamaged skin model and chronologically aged skin in keratin expression profile (Table 2). We further investigated the mRNA expression of keratins and found that keratin K6, K16, and K17 expression was significantly (P < 0.05) increased 24

hours after the final UVB irradiation, but not K1 and K5 (Figure 3). Data from Western blotting analysis demonstrated similar results with a significant (P < 0.01) increase of K6 protein expression that was maintained 4 weeks after the last exposure (Figure 7A). In addition, immunohistochemistry analysis showed the aberrant expression of K6 and K16 in UVB-exposed skin when compared with their absence in control (Figure 7B). This augmentation of K6 and K16 protein expression in our photodamaging/photoaging model was sustained 4 weeks after final exposure, comparable with genuine wrinkled skin obtained from face-lift surgery (Figure 7B).

Ultrastructural Changes of KIFs and Collagen Fibers Were Observed after Long-Term UVB Irradiation

To examine the ultrastructural changes after repetitive UV exposure, transmission electron microscopic analysis was performed. Compared to SC from a nonirradiated control skin where KIFs were organized in large fibrous bundles, KIFs in UVB-exposed skin exhibited an irregular network with the large intrafibril spaces (Figure 8). In the stratum spinosum and basale, cell polarity and distinctively large intracytoplasmic spaces within the keratinocytes were observed after repeated UVB irradiation (data not shown). The disorganization of the dermal collagen was also demonstrated. In the repetitive UVB-irradiated xenograft, collagen fibers were less orderly in their arrangement with focal compaction, whereas fine fibrous bundles were observed in the control (data not shown).

The Administration of TIMP-1-Neutralizing Antibody Significantly Accelerated Photodamaging on the Abdominal Skin Xenografts after Long-Term UVB Irradiation

TIMP-1 has been reported to intrinsically inhibit the activities of all known MMPs that are responsible for extracellular matrix degradation.⁶² To further clarify the critical roles of MMPs in induction of premature photoaging skin, an intradermal injection of TIMP-1-neutralizing antibody was used to accelerate the deleterious effects of repetitive UV exposure on skin surface roughness. Skin profilometric measurement was examined at baseline and 6 weeks. The specificity of TIMP-1 neutralization was confirmed by investigating the protein expression level of collagen I, which is primarily digested by MMP-1, by Western blotting analysis (Figure 9A). Quantitation of the photodamaging revealed a significant increase (P < 0.05 or P < 0.01) in all of the parameters (Ra, Sa, Rz, and Sz) at 6 weeks of the continuous UVB-irradiated grafts when TIMP-1 antibody was administered compared to the irradiated skin with nonspecific IgG injection (Figure 9, B–E). No significant change occurred in the nonspecific IgG-administrated control without any exposure to UVB irradiation (Figure 9, B–E).

Figure 7. Aberrant keratin expression in human skin xenografts during premature photoaging. Quantitative and qualitative analyses of keratins protein (K6, K16, K17, and K1) were evaluated 24 hours and/or 4 weeks after the final exposure and actual wrinkle skin from face-lifting. **A:** Western blotting and densitometric analyses were performed to evaluate the intensity of each keratin normalized to β -actin. * $P < 0.05$, ** $P < 0.01$. The values reported represent means $\pm$ SD from five to seven independent xenografts per group. **B:** Immunohistological analysis demonstrated that both K6 and K16 were abundantly expressed after UVB irradiation and in actual wrinkle skin, and that K6 expression was not returned to normal level 4 weeks after the final exposure. Scale bars = 100 μ m.

Discussion

Skin, the largest organ of the body, plays the most important role in protecting the internal organs against such environmental assaults as well as trauma and pathogens.

UV exposure has been well documented to cause harmful damage to human skin including suntan, sunburn, immunosuppression, photoaging, and skin carcinogenesis.^{3,63,64} To date, several mechanisms underlying UV-

Figure 8. Ultrastructural appearance of SC in nonexposed control and repetitive UVB-irradiated abdominal skin. Ultrastructural analysis of KIFs in SC of nonirradiated control (A) and long-term UVB exposure (B) skin was performed by transmission electron microscopic analysis in the 6th week after the first exposure as detailed in the Materials and Methods. **Arrows** demonstrate an apparent increase in intrafibril spaces after UVB exposure. Scale bars = 0.5 μ m.

induced damage have been proposed based on the evidence from studies using animals (eg, mice, rats), whose skins differ from human skin particularly in the epidermal thickness and the rate of terminal differentiation. Recent studies have demonstrated the advantages of using human skin xenografts onto SCID mice as a chimeric model in studying human skin diseases.^{65–68} Similar gene expression patterns in xenografts between before and after grafting onto SCID mice revealed that this chimeric model maintains much of its original tissue function.⁶⁷ As an alternative to animal studies, an *in vitro* human skin substitute model has been developed to investigate mechanisms underlying UV-induced damages.^{43,69,70} Regardless of its resemblance to human skin architecture, human skin substitute is susceptible to artificial alterations by culture conditions. Comprehensive microarray analysis between human skin substitute and native human skin demonstrate a clear physiological and molecular difference, especially in keratin genes that are associated in hyperproliferative keratinocytes.⁷¹ In this study, we revealed that human skin xenografted onto SCID mouse could recapitulate photodamaged skin, which may subsequently result in photoaged skin when rigorous UVB regimen and repetitive mechanical manipulation were used. Without complete cellular components of the skin immune systems, we clearly demonstrated that skin immunity may be functioning in part, but is not required, to induce skin photodamage. The increased expression of epidermal keratins K6, K16, and K17 and anomaly dermal rearrangements because of the disproportional MMP expression were observed in both UVB-exposed xenograft model and genuine aged skin.

The purpose of this study is to demonstrate that photodamage can be emulated in a human skin xenograft model when mechanical manipulation was introduced during the escalating UVB exposure regimen. Results from the preliminary study demonstrated that the energy required to induce 1 MED on the xenograft was gradually increased with time. This may be attributable to effects of UVB to induce keratinocyte hyperproliferation. In this current study, an escalating UVB irradiation regimen was conducted until several fine lines were observed on the skin surface, then the exposure was halted for 4 weeks. H&E staining of UVB-exposed skin (4 weeks) illustrated similar epidermal architecture compared to authentic

Figure 9. Premature photoaging was found to be significantly accelerated by the administration of TIMP-1-neutralizing antibody during long-term UVB exposure. After the confirmation of the specificity of TIMP-1 neutralization by investigating the protein expression level of collagen I in Western blotting analysis (A), skin profilometry of replicas treated with TIMP-1-neutralizing antibody combined with long-term UVB irradiation were used to evaluate the severity of furrow formation, δ Ra (B), δ Rz (C), δ Sa (D), and δ Sz (E) to justify our premature photoaging model. The values reported in Western blotting analysis and skin profilometry analysis represent mean $\pm$ SD from four and eight independent xenografts per group, respectively. * $P < 0.05$, ** $P < 0.01$ (compared to the changes of the samples treated with continuous UVB irradiation with nonspecific IgG administration at 6 weeks). Non-UVB, UVB, and TIMP-1UVB represent the skin xenografts treated by the administration of nonspecific IgG without any irradiation, with irradiation, and administered with TIMP-1-neutralizing antibody with repetitive UVB exposure, respectively.

aged skin obtained from face-lift surgery. It is important to note that the degradation of dermal collagens was more severe in chronological aged skin. These structural changes because of UVB exposure might be initiated by the severe disintegration of SC allowing UVB radiation to permeate through layers of the skin. Repetitive UVB exposure has been suggested to aggravate the disruption of KIFs resulting in elasticity loss and eventually cause skin surface wrinkles.¹⁴ In epidermis, hyperproliferation and the increase of flattened keratinocytes in stratum spinosum were observed, but declined 4 weeks after exposure. In dermis, UVB irradiation abolished papillary elastotic materials, triggering a self-correcting mecha-

nism to produce more elastins as shown in the abnormal elastic fiber accumulation in the reticular dermis, which might, in turn, induce more secretion of MMPs by fibroblasts. These results are in consistent with the induction of pro-MMP-1 by elastin peptides in skin fibroblasts.^{72,73} Throughout the dermis, collagen remodeling, including collagen disruptions and collagen production, was observed. The imbalance between MMPs and collagen production may contribute to the unusual punctate collagen bundles that were observed 4 weeks after halt irradiation. The proportion of collagen III relative to collagen I has been documented to be altered after cutaneous wound healing.^{74–76} The causal relationship between changes in collagen and elastin production and cutaneous photodamage was further confirmed when TIMP-1-neutralizing antibody was administered. Skin grafts that were subjected to treatment with UVB irradiation and TIMP-1-neutralizing antibody illustrated the significant skin surface deterioration compared to control skin grafts treated with UVB exposure and IgG injection.

Apart from histological observations, quantitative RT-PCR and Western blotting analyses confirmed similar changes in collagen, elastin, MMP, and keratin expression between our premature photoaging model and genuine aged skin, consistent with previous reports.^{4,6,7,9,11,21,22,24,29} Moreover, gene chip analysis indicated a lot of similarities in the comparisons between young versus old skins and our control versus UVB-exposed skins, corroborating the validity of our photodamage or premature photoaging model. Taken together, the present study discloses a novel photodamage model with human skin xenograft that recapitulates mechanisms underlying morphological changes such as wrinkling reported to date, and can be used for the further study of photoaging.

In addition to the known changes in premature photoaging, the present study suggested a plausible relationship between cutaneous aging and aberrant keratin expression. Previous studies in animal models indicate the cause effect relationship between repetitive UV irradiation and an increase of keratins K1, K5, and K10, with no changes in the collagen fibers and elastic fibers, resulting in loss of skin elasticity and the formation of skin wrinkles.^{12,13} Conversely, in our study, repeated UVB irradiation stimulated a stable expression of keratins K6 and its partners K16 and K17, but not K1, K5, and K10. Keratins K6, K16, and/or K17, which are not usually expressed in the innate state of normal epidermis, have been reported to initially induce in the suprabasal layers as early as 6 hours after injury or during the first 24 to 48 hours of UVB irradiation.^{48,77–81} The increase of these epidermal keratins has a direct impact on epidermal cytoarchitectural alteration and its migratory behavior during re-epithelization. The presence of these keratins has been documented in early hair germs and in the companion layer of the outer root sheath during embryonic development and the mature hair follicle anagen.⁸² In stratified epithelia, it has been suggested that K6 and K16 are up-regulated during chronic hyperproliferation, such as in psoriatic skin, or abnormal differentiation, such as a cancer, and that the ubiquitous expression of K6 and K16 is correlated with changes in keratinocyte differenti-

ation.^{77,83–86} In agreement with these documents, over-expression of the human K16 gene in transgenic mice has been reported to initiate re-organization of the intermediate filament network in keratinocytes in both outer root sheath and epidermis, resulting in aberrant keratinization and hyperproliferation as well as the rescue of the lethal skin blistering phenotype of K14-null mice.^{87,88} These observations indicate that K6 and its partners K16 and/or K17 play a direct role in eliciting the cutaneous morphological changes to regulate epidermal proliferation and differentiation. In this study, the expression of keratin K6 and its partners was found for the first time to be up-regulated in genuine facial wrinkle skin and in our premature photoaging model, in parallel with the previous report revealing that ectopic expression of K16 in epidermal basal layer lead to a wrinkled skin accompanied by severely thickened epidermis and delayed hair follicle morphogenesis.⁸⁹ Therefore, we speculated that apart from the alteration of collagens and elastin in dermal component, the epidermal remodeling, ie, the interactions between keratins K6 and K16 and/or K6 and K17, significantly contributes to the cutaneous photoaging process. In addition to UVB irradiation, the increase in K6 expression might be also explained by the mechanical stretching that imitates facial expression that has been reported to induce proliferation of human keratinocytes.⁹⁰ It is noteworthy to mention that protein expression of keratins K6 and K16 was sustained in UVB-exposed epidermis at least 4 weeks after the final irradiation. However, the precise mechanisms inducing K6, K16, and K17 expression in the cutaneous aging processes need to be addressed.

In conclusion, our results suggest that human skin xenograft on SCID mouse is a promising photodamaged skin model. In addition, the deterioration of SC and the increased keratins K6, K16, and K17 in the epidermis are suggested to be initial events that trigger morphological changes after repeated UVB irradiation. Subsequently, atypical dermal alterations deteriorate skin integrity. These findings provide a new insight into the complex network underlying human skin photoaging after repetitive UVB exposure. Most importantly, our results add to the fundamental understanding of regulatory mechanisms underlying photodamage in the skin as well as the cutaneous homeostasis in many skin disorders.

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