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ORIGINAL PAPER

Long-term alteration in the expression of keratins 6 and 16 in the epidermis of mice after chronic UVB exposure

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Abstract The influences of chronic UVB exposure on epidermal differentiation have been poorly studied compared to dermal photo-aging although those effects are very important in terms of photo-damage to the skin. The purpose of this study was to investigate the effects of chronic UVB exposure on keratin expression in the epidermis. The effects on murine skin of chronic exposure to weak UVB (below 1 MED) was examined by immunoblotting for keratins K10, K5, K6, and K16, by immunohistochemistry using antibodies to K6, K16, and Ki67 as well as by conventional HE staining of skin sections. Alterations of keratin expression induced by the chronic UVB exposure were distinct from those elicited by a single acute UVB exposure. The expression of keratins K6 and K16 was quite long-lasting, continuing for 7 weeks after 6 weeks of chronic UVB exposure and for 6 weeks after 9 weeks of chronic UVB exposure. In contrast, K6 and K16 expression induced by a single UVB exposure at 0.5 MED or 3 MED almost ceased within 2 weeks after that exposure. Furthermore, the expression of the constructive keratins, K5 and K10, remained almost unchanged by chronic UVB exposure. Epidermal thickness was increased significantly immediately after the 9 weeks of chronic UVB exposure; however, it had returned to normal level 6 weeks later. The alterations in keratin expression accompanied the marked disruption of the ordered ultrastructure of keratin intermediate filaments, which were observed by TEM. Thus, chronic exposure to UVB has a deep impact on the biosynthetic regulation of different keratins in the epidermis, thereby interfering with the ordered ultrastructure of keratin intermediate filaments. Those events could have relevance to the mechanism of photo-damage, such as fine wrinkles observed in chronically UV-exposed skin in addition to dermal photo-aging.

Keywords Chronic UVB exposure · Keratin 6 · Keratin 16 · Long-term alteration of keratins · Wrinkle

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Introduction

Keratins are the major structural proteins of vertebrate epidermis, playing critical roles in shaping the functions of the skin [5]. Keratins form a superstructure, termed keratin intermediate filaments (KIFs), which are composed of 10-nm-diameter fibrous substructures. In normal skin, KIFs show orderly patterns (the so-called keratin pattern) of linear fibers at the ultrastructural level [3]. KIFs are heteropolymers comprised of two types of keratin species, type I keratins (K9–K23) and type II keratins (K1–K8). Specific pairwise combinations of keratin species from each of the two types form KIFs.

The expression of type I and type II keratins is strictly regulated in a keratinocyte proliferation/differentiation-specific manner. In the case of normal epidermis, K5 (type II) and K14 (type I) are coordinately expressed in the basal layer, whereas K1 (type II) and K10 (type I) are expressed in the suprabasal layer. K6 (type II) and its type I partner K16 (or K17) are usually expressed in the outer root sheath of hair follicles, in nail beds, in oral mucosa, and in several other tissues. K6 and K16 are not detectable in the

interfollicular epidermis under normal conditions, but are markedly induced in stratified epithelia undergoing hyperproliferation and/or aberrant differentiation, such from psoriasis or cancer, and after ultraviolet (UV) exposure.

Many studies have revealed the altered expression of epidermal keratins induced by UV radiation. UVB exposure induces abundant expression of K6 and K16, with or without reduction of K1 and K10 expression. Such alterations of keratin expression have usually been investigated after a single acute UVB exposure [4, 6, 11, 14, 28], and there are few studies on keratin expression patterns following chronic UVB exposure. Kambayashi et al. reported that chronic exposure to weak doses of UVA and UVB caused wrinkle formation, which was accompanied by changes in epidermal keratin expression detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [9, 10].

In exerting their cytoskeletal functions, the ultrastructural organization of KIFs is also of major importance. At the periphery of keratinocytes, KIFs are organized in a pancytoplasmic network connecting nuclei and adhesion complexes such as desmosomes. Ma et al. suggested that a network of KIF cross-bridges is crucial to maintain the structural resilience of the epidermis, which depends in turn on the intracellular organization of KIFs [15]. We previously demonstrated that chronic exposure of mouse skin to weak UVB disrupts the KIF ultrastructure and induces, in parallel, the formation of fine wrinkles [22].

In this study, we investigated the alteration of keratin expression induced by chronic UVB exposure of the skin of hairless mice, comparing it to changes induced by a single acute UVB exposure. We also assessed the relationship among altered keratin expression, the disruption of KIF ultrastructure and changes of skin morphology.

Materials and methods

Animals

HR/ICR albino hairless mice were used for all experiments in this study. This strain was established by crossing hairless mice (HR/HR) (originally obtained from Nisseiken Corp, Tokyo, Japan) with an albino strain, HaM/ICR. These mice have been maintained in our laboratory by breeding hairless males and phenotypically haired females under conventional conditions.

Antibodies

For western blot analyses, rabbit polyclonal antibodies against mouse keratin 10 (MK10), and MK5 were from Berkeley Antibody Company (BabCO, Richmond, CA, USA). Rabbit polyclonal antibodies against mouse MK6 and MK16 were kindly provided by Prof. P. A. Coulombe (Johns Hopkins University, Baltimore, MD, USA).

For immunohistological staining, primary antibodies used were against MK6 (BAbCO, Richmond, CA, USA), MK16 (kindly provided by Prof. P. A. Coulombe, Johns Hopkins University, Baltimore, MD, USA) or the cell proliferation marker Ki67 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). Secondary antibodies used for immunolabeling were rhodamine-labeled goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA; for K6 immunostaining) or FITC-labeled goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology, Inc.; for Ki67 immunostaining).

UVB radiation source

In all experiments, Toshiba SE20 lamps were used as the UVB source without any filtering. The energy output of the lamps was 0.66 mW cm^{-2} as determined using a UVB radiometer 305/365DII (Topcon, Tokyo, Japan). The peak emission was near 312 nm, and the irradiance between 290 and 320 nm accounted for approximately 55% of the total energy output of UVB [25].

Chronic UVB-exposure protocol

An incremental UVB exposure regimen was employed to avoid erythema due to the repetitive exposure. Mice at 6 weeks of age were irradiated once a day, 5 days a week for a total of either 6 or 9 weeks, after which the mice were sacrificed for examination as shown in Fig. 1. For the irradiations, the following progressive UVB exposure regimen was used; starting at approximately 47 mJ cm^{-2} per exposure during week 1, increased step-wise by 6–7 mJ cm^{-2} per week until week 4, and then kept at a constant dose of 67 mJ cm^{-2} for the remaining exposure period. The dose of 67 mJ cm^{-2} is slightly below the minimal erythemal dose (MED) for these mice.

Single UVB-exposure protocol

Mice were randomly divided into three groups from the same stock at 6 weeks of age; a non-irradiated control and two UVB-irradiated groups at either 0.5 MED or 3 MED. UVB exposure doses were approximately 34 and 201 mJ cm^{-2} for 0.5, and 3 MED, respectively.

Western blot analyses

The dorsal skin was excised from euthanized mice. Sheets of epidermis were physically peeled from the skin after soaking in deionized water at 60°C for 1 min. Proteins were extracted from the separated epidermis in 50 mM Tris–HCl

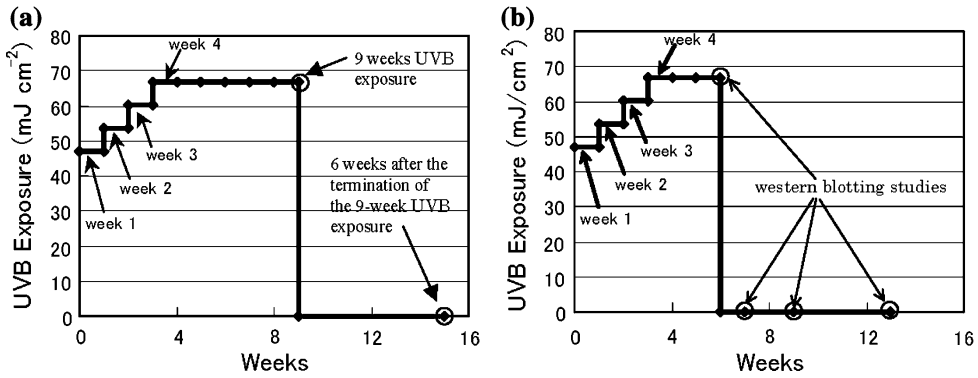

Fig. 1 Schematic diagrams of the UVB exposure regimens used in this study. An incremental UVB exposure regimen was used in order to avoid erythema due to repetitive UVB exposure. UVB-irradiations were done once a day, 5 days a week. **a** Western blotting, histological, immunological staining, wrinkle grading, and TEM measurements were performed at 6 weeks after the 9 week chronic UVB exposure. **b** Western blotting of various keratins was performed at 0, 1, 3, and 7 weeks after the 6 week chronic UVB exposure

buffer, pH 7.6 containing 8 M urea, 100 mM 2-mercaptoethanol, 1 mM dithiothreitol, and protease inhibitors [21]. For immunoblotting, equal amounts of proteins were separated by SDS-PAGE on 12.5% gels, on 5 − 15% gradient gels or on 7–15% gradient gels, and then were transferred to nitrocellulose membranes for 1.5 h at 250 mA. After blocking with 2% nonfat dried milk in PBS, the nitrocellulose membranes were incubated with primary antibodies against mouse keratins diluted in 2% nonfat dried milk in PBS. Antibodies were diluted as follows: MK10 (BAbCO, Richmond, CA, USA), 1:2000; MK5 (BAbCO, Richmond, CA, USA), 1:2,000; MK6 (kindly provided by Prof. P. A. Coulombe) 1:2,000; MK16 (kindly provided by Prof. P. A. Coulombe), 1:4,000. β-actin rabbit polyclonal antibody (ab8227 Abcam, Cambridge, UK) 1:1,000.

Immunoreactive proteins were visualized with peroxidase-labeled secondary antibodies against rabbit immunoglobulins using enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) and were developed on X-ray films (Hyperfilm MP, Amersham Pharmacia).

Loading controls were performed for western blotting using an antibody to β-actin. Protein concentrations were determined using a RC DC Protein Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). To quantitate the expression of proteins, the intensities of protein bands were measured by densitometry and are expressed as relative intensities.

Histological examination and morphological analysis

Excised tissue samples were frozen in tissue-Tek O·C.T compound (Sakura Finetechnical Co. Ltd.; Tokyo, Japan) in liquid nitrogen/isopentane cooling-medium. Cryosections of 3 μm thickness were prepared and fixed in 4% paraformaldehyde for 10 minutes at room temperature, followed by staining with hematoxylin and eosin (HE).

For immunostaining, 6 μm cryosections were processed according to the following protocol: Sections were fixed either in methanol for 20 s at 4°C for K6 and K16 immunostaining, or in 4% paraformaldehyde for 10 min at room temperature for Ki67 immunostaining. Sections were blocked with 1% BSA in PBS and were incubated for 20 min at room temperature with the diluted primary antibody against MK6, MK16 or the cell proliferation marker Ki67. Antibodies were diluted as follows: K6 antibody (BAbCO, Richmond, CA, USA) 1:200; MK16 (kindly provided by Prof. P. A. Coulombe), 1:500; and Ki67 antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) 1:500. Sections were then washed at 4°C with PBS five times, and then were incubated for 20 min at room temperature with the diluted secondary antibody. For secondary antibodies, goat anti-rabbit-rhodamine was used for K6 and K16 staining or goat anti-rabbit-FITC for Ki67 staining. After mounting on glass slides with Fluoromount G (Southern Biotechnology Associates; Birmingham, AL, USA), the sections were observed and photographed using a LEICA DMR/Q550FW fluorescent microscope system (Leica Microsystems, Wetzlar, Germany).

Epidermal thickness was determined by image analysis of microphotographs of HE-stained sections on the basis of rectangular approximation. The average thickness of the epidermis (D) was calculated using the following equation; $D = A/L$, where A is the area of an arbitrarily specified epidermal area in a microphotograph (green area in Fig. 6d) and L is the lateral length along the epidermis at its half-depth level (red line in Fig. 6d). Images were analyzed using Adobe Photoshop and free image-analysis software (Data Picker).

Grading of visible wrinkles

The grade of wrinkles in mouse skin was assessed at 6 weeks after the 9-week chronic UVB exposure according

to the method described by Bissett et al. [2]. Briefly, the severity of wrinkling was graded from 1 to 4: grade 1, no wrinkles; grade 2, a few shallow coarse wrinkles; grade 3, some coarse wrinkles; grade 4, several deep coarse wrinkles. Quarter-intermediate grades (1.25, 1.5, 1.75, 2.25, 2.5, 2.75, 3.25, 3.5, 3.75) were also used in this evaluation. Unlike human skin, hairless mice have no wrinkles without UVB irradiation, so the grades of wrinkles in control mice are all grade 1.

Transmission electron microscopy

Full-thickness mouse skin was minced into 1 mm^3 pieces, and was fixed overnight at 4°C in freshly prepared 2% glutaraldehyde. The tissue specimens were then soaked in 2% osmium tetraoxide solution (pH 7.4, phosphate buffer) for 1 h. After rinsing in 0.25 M sucrose–0.1 M phosphate buffer (pH 7.4), the specimens were dehydrated in a graded ethanol series and were subsequently embedded in epoxy resin. Following double-staining with uranyl acetate and lead citrate, ultrathin sections were examined by TEM (Hitachi High-Technologies Corp., Tokyo Japan), operated at 70 KV.

Statistical analysis

Data from western blot analysis, epidermal thickness measurements and gradings of visible wrinkles are reported as means ± SD using Student's *t* test**.** The grading of visible wrinkles is also reported as means ± SD using Mann–Whitney's *U* test. Differences are considered significant at $P \leq 0.05$.

Results

Alterations of keratin expression after chronic UVB exposure

During the observation of murine skin chronically exposed to UVB, we noticed that striking changes, such as skin morphological changes, changes of elastic properties of the epidermis, and disruption of KIFs, occurred in the skin long after the completion of the UVB exposure protocol [22]. Thus we focused on studying alterations in the composition of keratins, the major components of epidermal proteins, in the UVB-exposed epidermis. Keratin expression was characterized by immunoblotting using keratin species-specific antibodies in epidermal extracts prepared from excised skin specimens. Keratin species examined included K5 and K10, as well as K6 and K16. Epidermal extracts were examined at 6 weeks after the completion of the 9-week chronic UVB exposure protocol as shown in Fig. 1a. Apparent and significant increases of K6 and K16 expression ($P < 0.01$) occurred compared with the non-irradiated controls, whereas K5 and K10 expressions were almost unchanged (Fig. 2a, b).

In order to confirm the induction of K6 and K16, we next investigated the time course expression of keratins after shorter series of chronic UVB exposures as shown in Fig. 1b. Six weeks of UVB exposure again induced the expression of K6 and K16 (Fig. 3). The expressions of K6 and K16 were consistently greater than in non-irradiated controls over a period of 7 weeks after completion of the chronic UVB exposure protocol. In contrast, the expression of K10 was unchanged from the controls throughout this set of experiments. Although K5 expression appeared to be weaker than the control immediately after the chronic UVB exposure, its expression was almost unchanged from the controls at 1, 3, and 7 weeks after the chronic UVB exposure.

It should be noted that K6 and K16 are not expressed in normal interfollicular epidermis, but they are usually expressed exclusively in follicular tissue. The expression of K6 and K16 continued for 6 or 7 weeks after the 9-week chronic UVB exposure. In contrast to K6 and K16, K5, and K10, which are normally expressed in the interfollicular epidermis, were almost the same as the controls. The expression of K6 and K16 did not appear to affect the expression of K10.

Alterations of keratin expression after a single UVB exposure

We next asked whether chronic UVB exposure was required for the induction of K6 and K16. We investigated

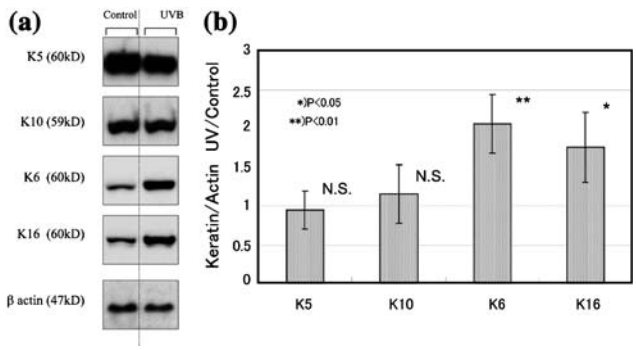

Fig. 2 Western blot analyses of keratin expression in the dorsal skin of unirradiated control mice and of mice at 6 weeks after the 9-week UVB exposure (see protocol Fig. 1a and "Materials and methods"). **a** Images of western blots using antibodies to K5, K10, K6, K16, and β-actin as a loading control. **b** Densitometric analysis of western blots using antibodies to K5, K10, K6, and K16 (amounts are reported as percentage of control, normalized against β-actin as a loading control); error bars represent SD, $N = 6$. *Asterisks* depict statistically significant differences between UVB-exposed mice and control mice. There are highly significant changes in the expression of K6 and K16

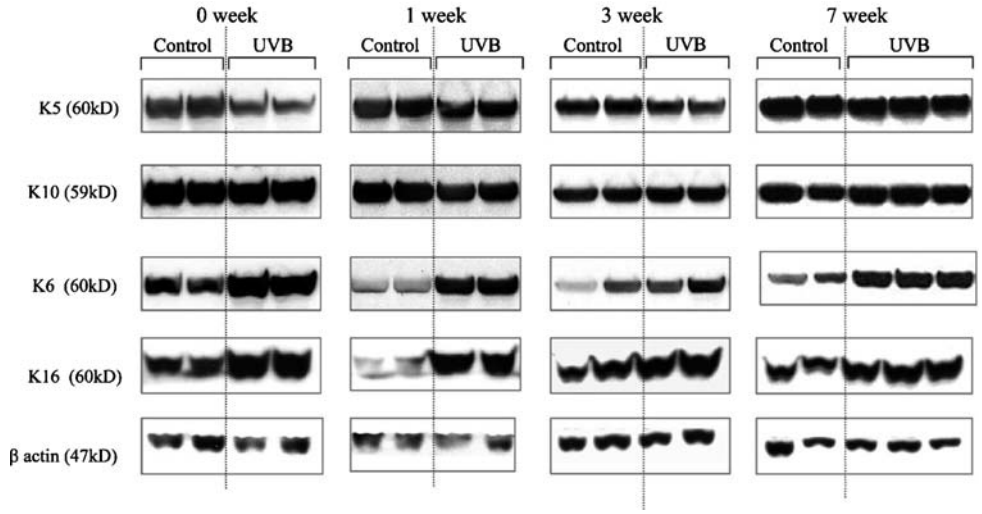

Fig. 3 Images of western blots using antibodies to K5, K10, K6, and β-actin in the dorsal skin of mice at 0, 1, 3, and 7 weeks after the 6-week chronic UVB exposure (see protocol Fig. 1b and "Materials and methods"). $N = 2$ at 0, 1, and 3 week(s) after the exposure. At 7 weeks after the exposure $N = 3$ for samples and $N = 2$ for control. It is difficult to distinguish by statistical differences at 1, 2, 3 or 7 weeks due to the small numbers of mice; however, the expression of K6 and K16 begins increasing immediately after the exposure and then increases further until 7 weeks. On the other hand, the expression of K5 and K10 remains virtually constant during the experiment

the expression of K5, K10, K6, and K16 after a single, rather than chronic, acute UVB exposure. The expression of K10, K5, K6, and K16 at 24 h, 48 h, and 2 weeks after two different doses of a single UVB exposure are shown in Fig. 4. The induction of K6 and K16 were relatively stronger than K5 and K10 after the single acute UVB exposure. The expression of K6 and K16 started increasing at 24 h after the single UVB exposure at both irradiation doses, 0.5 and 3 MED. The prominent increase of both K6 and K16 were observed after 3 MED exposure ($P < 0.01$ for both K6 and K16 at 24 and 48 h after the 3-MED exposure, $P < 0.01$ for K16 at 24 and 48 h after the 0.5 MED exposure, $P < 0.05$ for K6 at 24 and 48 h after 0.5 MED exposure). K16 expression seemed to be more sensitive to UVB than K6 expression. After the 0.5 MED exposure, the expressions of K10 and K5 were almost the same as the control at 24 and 48 h, but they were slightly increased by the 3-MED exposure ($P < 0.05$ for K5 at 24 h after the 3-MED exposure, $P < 0.05$ for K10 at 48 h after the 3-MED exposure).

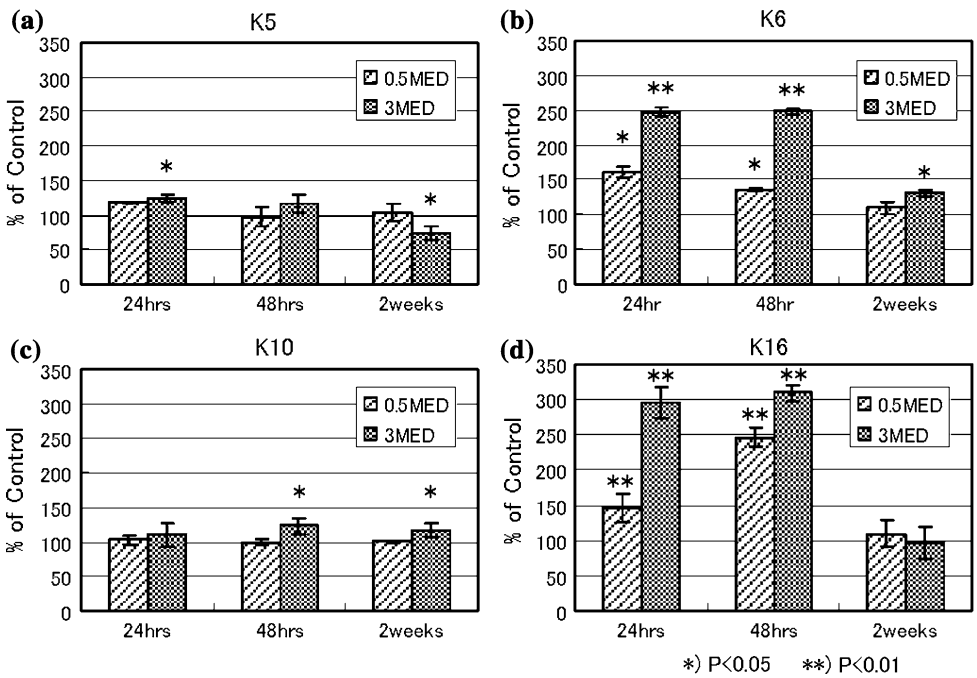

Fig. 4 Quantitative analyses of Western blots of keratin expression in the dorsal skin of unirradiated control mice and of mice exposed to two different doses of UVB at 24 h, 48 h, and 2 weeks after the single UVB exposure. Antibodies to K5, K10, K6, and K16 were used and amounts are reported as percentage of control; error bars represent SD, $N = 4$ for control and 3 MED, $N = 3$ for 0.5 MED at all measurement times (24 h, 48 h, and 2 weeks). Asterisks depict statistically significant differences between UVB-exposed mice and unirradiated control mice

The increased expression of K16 induced by the 0.5 and the 3-MED exposures and that of K6 by the 0.5 MED exposure returned to baseline in 2 weeks. Although considerably diminished, the expression of K6 induced by the 3-MED exposure remained at 2 weeks after the exposure ($P < 0.05$). Two weeks after the 3-MED exposure, the expression of K5 was notably diminished although the expression of K5 slightly increased at 24 and 48 h. The expression of K10 after the 3-MED exposure had slightly increased during this experiment ($P < 0.05$, K10 at 2 weeks after the 3-MED exposure).

Immunochemical and histochemical studies of epidermis after 9 weeks of chronic UVB exposure

In order to determine the expression site of the newly induced K6 and K16, we used immunohistochemistry to examine the epidermal tissue with antibodies specific to K6 and K16 immediately after and at 6 weeks after the completion of the 9-week chronic UVB exposure protocol (Fig. 5). The distributions of K6 and K16 in the epidermis were almost identical. Immediately after completion of the UVB exposure, the immunostainings of K6 and K16 were much stronger than in the non-irradiated control. K6 and K16-positive cells were spread over the entire epidermis (Fig 5b, f). At 6 weeks after the 9-week chronic UVB exposure, the immunostaining of K6 and K16 were still strong compared to the control and K6- and K16-positive cells were still found over the entire epidermis. However, the distribution of K6 and K16-positive cells at 6 weeks after the 9-week chronic UVB exposure was slightly sporadic compared with that immediately after the 9-week chronic UVB exposure (Fig. 5c, g).

The results of immunostaining of K6 and K16 at 6 weeks after the 9-week chronic UVB exposure are in accordance with the results of western blotting of K6 and K16 (Figs. 2, 3) The 9-week chronic UVB exposure seemed to thicken the epidermis prominently compared with the control (Fig. 6). Determining the mean epidermal thickness confirmed that the increase was significant ($P < 0.0001$, Fig. 7). Epidermal hyperplasia and an expanding spinous compartment were obvious in HE-stained

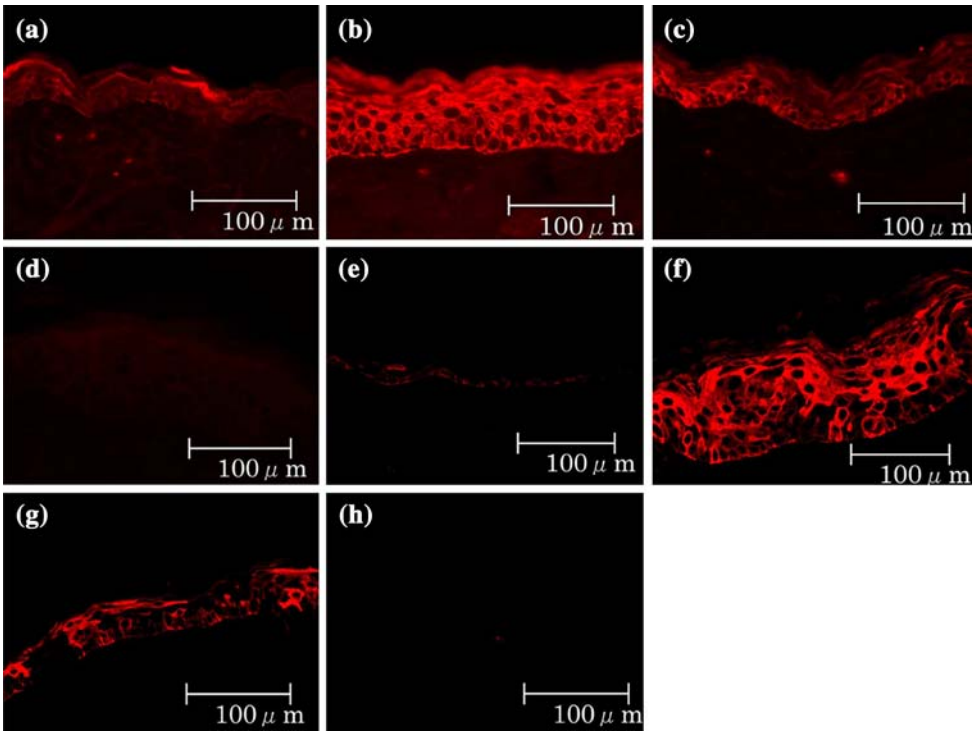

Fig. 5 Representative micrographs of immunostained sections using the K6 and K16 antibodies. **a** Non-UVB exposed control for K6. **b** The immunostaining of K6 just after the 9-week chronic UVB exposure. **c** The immunostaining of K6 at 6 weeks after the 9-week chronic UVB exposure. **d** Negative control (IgG) for K6. **e** Non-UVB exposed control for K16. **f** The immunostaining of K16 just after the 9-week chronic UVB exposure. **g** The immunostaining of K16 at 6 weeks after the 9-week chronic UVB exposure. **h** Negative control (IgG) for K16. The distributions of K6 and K16 in the epidermis were almost identical. Immediately after completion of the 9-week chronic UVB exposure, the immunostaining of K6 and K16 were much stronger than in the non-irradiated control. K6 and K16-positive cells were spread over the entire epidermis (**a** and **b** for K6, **e** and **f** for K16). At 6 weeks after the 9-week chronic UVB exposure, the immunostaining of K6 and K16 was still strong and K6- and K16-positive cells were found over the entire epidermis. However, the distribution of K6- and K16-positive cells at 6 weeks after the 9-week chronic UVB exposure was slightly sporadic compared with that immediately after the 9-week chronic UVB exposure (c)

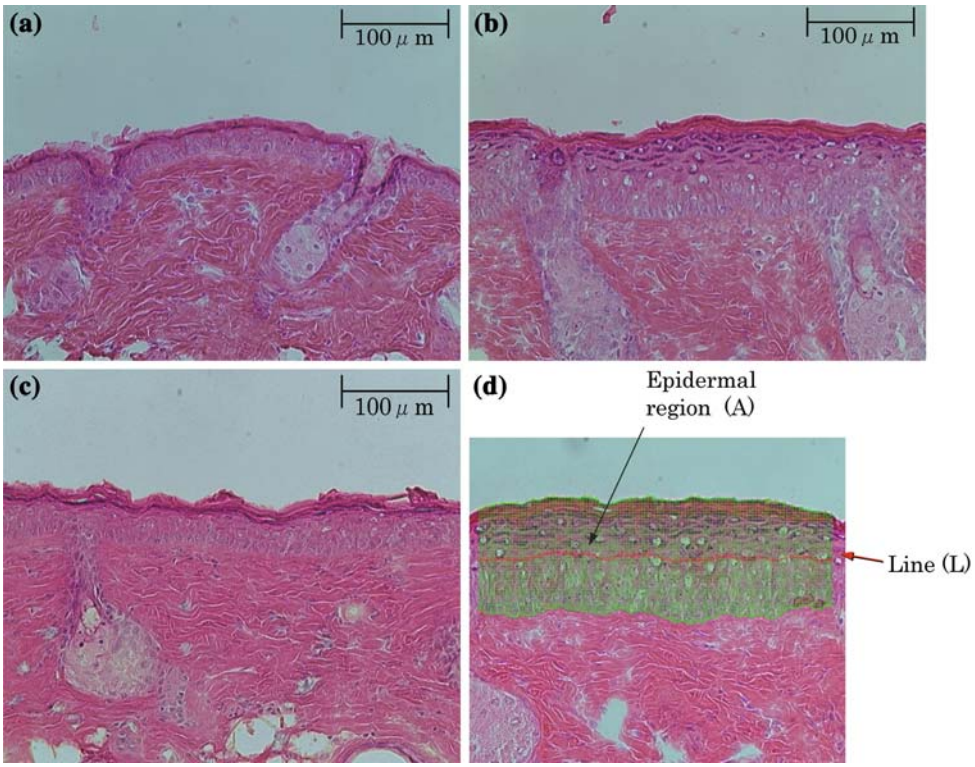

Fig. 6 Representative micrographs from HE-stained sections. **a** Non-UVB exposed control, **b** just after the 9-week chronic UVB exposure. **c** At 6 weeks after the 9-week chronic UVB exposure. **d** Graphic explanation of how epidermal thickness is measured. See the histological examination and morphological analysis in "Materials and methods" for detail. The 9-week chronic UVB exposure seemed to thicken the epidermis prominently compared with the control (**a**, **b**). Epidermal hyperplasia and an expanding spinous compartment were obvious in HE-stained sections of skin immediately after the 9-week chronic UVB exposure protocol (**b**). The HE-stained skin at 6 weeks after the 9-week chronic UVB exposure was not notably different from the unirradiated control (compare **a**, **c**). Note here that the stratum granulosum is evident in the epidermis at 6 weeks after the 9-week chronic UVB exposure, but is not seen immediately after the 9-week chronic UVB exposure (**b**, **c**). The disappearance of the stratum granulosum is a histological sign of a hyperproliferated epidermis in sunburned skin [23]

sections of skin immediately after the 9-week chronic UVB exposure (Fig. 6b). The epidermis was still significantly thicker at 6 weeks after the 9 week chronic UVB exposure ($P < 0.001$), although the difference from the non-irradiated controls was smaller (Fig. 7). The HE-stained skin at 6 weeks after the 9-week chronic UVB exposure was not notably different from the unirradiated control (Fig. 6c). We should point out here that the stratum granulosum was evident in the epidermis at 6 weeks after the 9-week chronic UVB exposure, but was not seen immediately after the UVB exposure (Fig. 6b, c). The disappearance of the stratum granulosum is a histological sign of a hyperproliferated epidermis in sunburned skin [23]. In this sense, the skin at 6 weeks after the 9-week chronic UVB exposure lacked any signs of hyperproliferation. These data therefore suggest that epidermal differentiation may be normal at 6 weeks after the 9-week chronic UVB exposure regimen.

To delineate the mechanism of long-term expression of K6 and K16 in relation to the epidermal thickening, the mitotic activities of keratinocytes were examined in the basal layer of the epidermis immediately after and at 6 weeks after the 9-week chronic UVB exposure. Ki-67 was used as a marker for mitotic activity of basal keratinocytes. The number of Ki67-positive cells in the basal layer immediately after the 9-week chronic UVB exposure was markedly increased compared with the non-irradiated controls (Fig. 8a, b). However, the number of Ki67-positive cells in the basal layer at 6 weeks after the 9-week chronic UVB exposure had returned to the level of the controls (Fig. 8a, c). Since the UVB-induced expression of K6 and K16 still continued at this time, the mitotic activities of keratinocytes are not correlated with the K6 and K16 expression.

Grading of wrinkles and analysis of fibrous ultrastructure in the stratum corneum

Sustained expression of aberrant keratins, K6 and K16, could affect skin morphology. Therefore, we evaluated wrinkle formation at 6 weeks after the 9-week chronic UVB exposure. The chronic UVB exposure elicited significant formation of wrinkles ($P < 0.01$) (Fig. 9). The skin

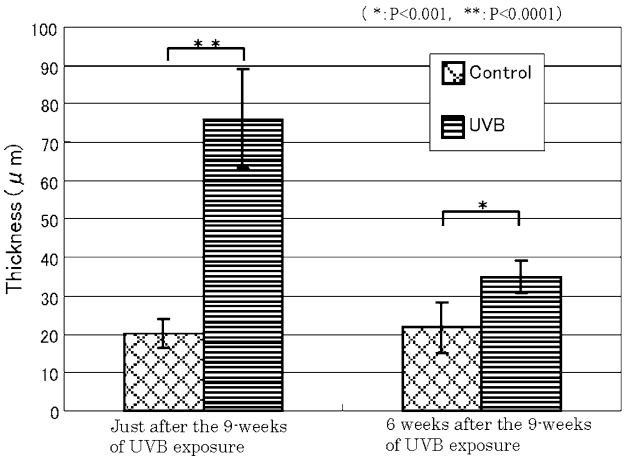

Fig. 7 Epidermal thickness measured in HE-stained sections of control (non-UVB exposed) skin and skin just after the 9 weeks of chronic UVB exposure and at 6 weeks after the 9 weeks of chronic UVB exposure (nine pictures per histological section were taken and three biopsies per condition were taken in this study). Epidermal thickness was determined by image analysis of microphotographs of HE-stained sections on the basis of rectangular approximation. The average thickness of the epidermis (*D*) was calculated using the following equation; $D = A/L$, where *A* is the area of an arbitrarily specified epidermal area in a microphotograph (*green area* in Fig. 6d) and *L* is the lateral length along the epidermis at its half-depth level (*red line* in Fig. 6d). Determining the mean epidermal thickness confirmed that the increase was significant ($P < 0.0001$). The epidermis was still significantly thicker at 6 weeks after the 9-week chronic UVB exposure ($P < 0.001$), although the difference from the controls (non-UVB exposed) was smaller

wrinkling scores of all unirradiated control mice were grade 1; i.e., no wrinkles. The score of the UVB-irradiated mice was significantly larger than that of the control mice. The impact of the induction of K6 and K16 on the fibrous ultrastructure of the stratum corneum was also studied by TEM. Typical TEM images of the stratum corneum at 6 weeks after the 9-week chronic UVB exposure are shown in Fig. 10. In TEM images of unirradiated control skin, microfibrils with an ultrastructure similar to the keratin pattern consisting of KIFs were evident in the corneocytes (Fig. 10a). In contrast, the images of KIFs in the stratum corneum at 6 weeks after the 9-week chronic UVB exposure are quite different from the controls (Fig. 10a, b). The fibrous ultrastructure, which is apparent in the control stratum corneum, had completely disappeared in the skin at 6 weeks after the 9-week chronic UVB exposure, where the orientation of KIFs became distorted and random.

Discussion

Sustained alteration of K6 and K16 expression after chronic UVB exposure

In this study, we demonstrated that chronic UVB exposure of mice induces long-lasting expression of abnormal keratin species, K6 and K16, in the epidermis. The alterations

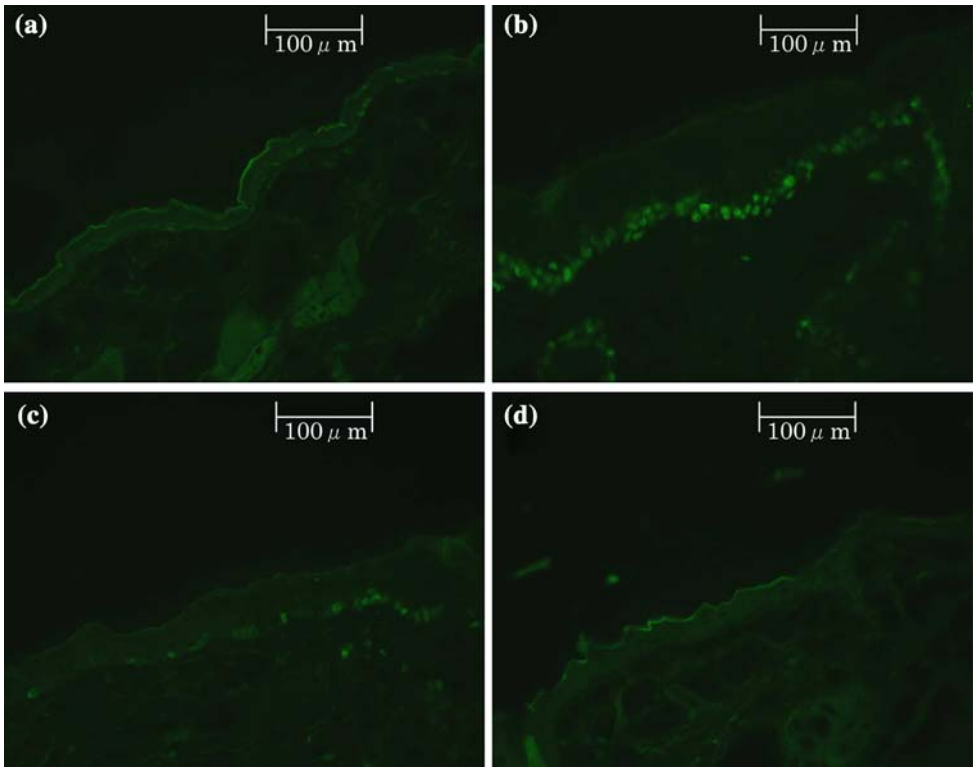

Fig. 8 Representative micrographs from sections immunostained with the Ki67 antibody. **a** Non-UVB exposed control. **b** Just after the 9 weeks of chronic UVB exposure. **c** At 6 weeks after the 9 weeks of chronic UVB exposure. **d** Negative control (IgG). Ki-67 was used as a marker for mitotic activity of basal keratinocytes. The number of Ki67-positive cells in the basal layer immediately after the UVB exposure was markedly increased compared with the controls (non-UVB exposed) (**a**, **b**). However, the number of Ki67-positive cells in the basal layer at 6 weeks after the UVB exposure had returned to the level of the controls (**a**, **c**)

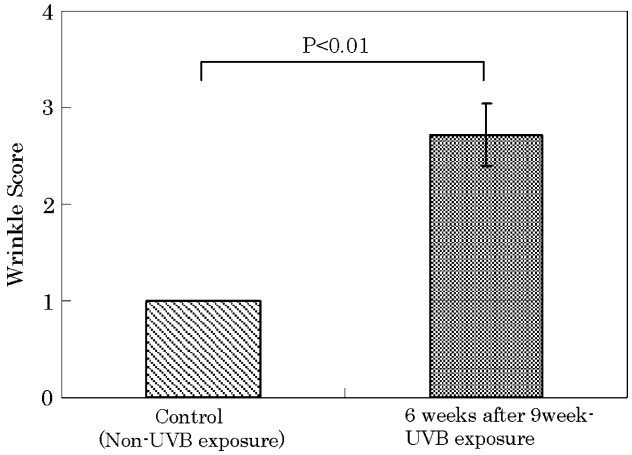

Fig. 9 Average skin wrinkling grades of control (non-UVB exposed) mice and mice at 6 weeks after the 9-week chronic UVB exposure. The error bars represent SE. The score of the mice at 6 weeks after the 9-week chronic UVB exposure was significantly larger than that of the control mice (non = UVB exposed). ($N = 14$) The grade of skin wrinkles in mice was assessed according to the method described by Bissett et al. [2]. Briefly, the severity of wrinkling was graded from 1 to 4: grade 1, no wrinkles; grade 2, a few shallow coarse wrinkles; grade 3, some coarse wrinkles; grade 4, several deep coarse wrinkles. Quarter-intermediate grades (1.25, 1.5, 1.75, 2.25, 2.5, 2.75, 3.25, 3.5, 3.75) were also used in this evaluation. Unlike human skin, hairless mice have no wrinkles without UVB irradiation, so their grades of wrinkles in control mice are all grade 1

of keratin expression are quite unique and distinct. The expression of K6 and K16 lasts for several weeks after completion of the chronic UVB exposure, without any obvious reduction in normal keratin species, K10 and K5 (Figs. 2, 3). Furthermore, the long-term increases in K6 and K16 expression are not accompanied by aberrant differentiation and hyperproliferation of the epidermis, which was demonstrated by HE staining and Ki-67 immunostaining, respectively (Figs. 6, 7, 8). K6 and K16 can also be induced by several other factors including topical phorbol ester or skin injury. Such induction, however, does not last long after the inducing stimulus is terminated. These events also accompany the hyperproliferation and aberrant differentiation of the epidermis and often correlate with the reduction of the constructive keratins K1 and K10 [16].

On the other hand, a single acute UVB exposure causes relatively brief changes in keratin expression. After the single UVB exposure, the induction of expression of K6 and K16 almost ceased at 2 weeks after the UVB exposure (Fig. 4). This short duration of expression is in accordance with other studies which reported that a single UVB exposure induces K6 expression lasting for about 2–3 weeks [4, 6]. Some reports have mentioned that K6 expression is concomitantly diminished with a reduction of K1 and K10.

From a mechanistic point of view, our data suggest that the apparent increase of K6 and K16 expression would have no direct relationship to keratinocyte proliferation and epidermal thickening. This is because the induced expression of K6 and K16 lasts beyond the period of keratinocyte hyperproliferation (as revealed by Ki-67 staining) and epidermal thickening. This is supported by a report that the expression of K6 and K16 is not necessarily related to proliferation [1].

Immediately after the chronic UVB exposure, the expression of K6 and K16 seems proliferation-related since they accompany considerable increases of epidermal thickness and mitotic activities, suggesting hyperproliferation and aberrant differentiation (Figs. 3, 5b, f, 6b, 7, 8b). However, only the expression of K6 and K16 persists after the completion of the 9-week chronic UVB exposure. Aberrant differentiation and hyperproliferation soon diminish and epidermal differentiation and proliferation returns to baseline levels (Figs. 2, 3, 5c, g, 6c, 7, 8c).

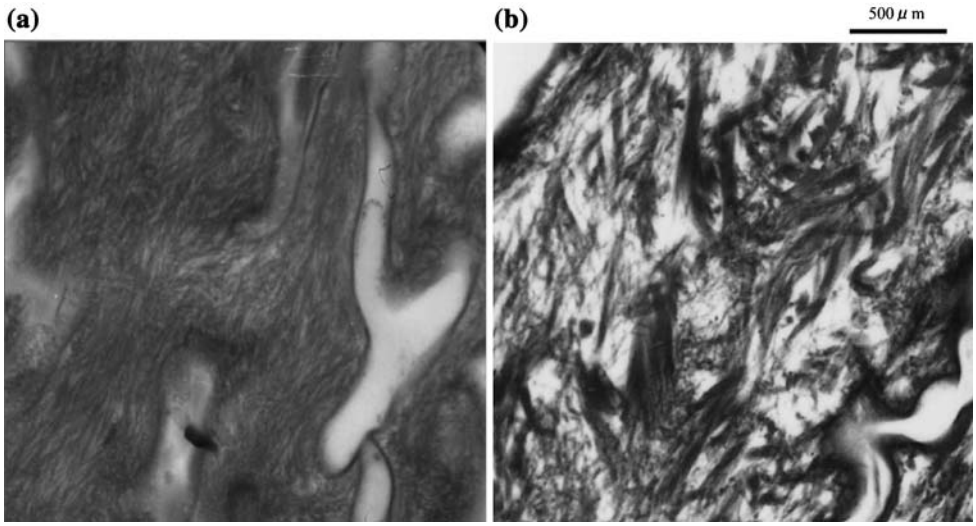

Fig. 10 Typical TEM images of the stratum corneum. **a** Non-UVB exposed control. **b** At 6 weeks after the 9 weeks of chronic UVB exposure**.** Microfibrils with an ultrastructure similar to the keratin pattern consisting of KIFs, were evident in corneocytes of control skin (non-UVB exposed) (**a**). The fibrous ultrastructure had completely disappeared in corneocytes of skin at 6 weeks after the 9-week chronic UVB expoxure, where the orientation of KIFs became distorted and random (**b**)

Our results on chronic UVB exposure may be explained by a combination of two distinct responses to UVB radiation, one a shorter term event, while the other is a longer term event. The shorter term event, which lasts for about 2 weeks, includes aberrant differentiation, hyperproliferation, and induction of K6 and K16 expression. All those changes are likely to be similar to those induced by a single acute UVB exposure. The longer term event, which lasts for more than 6 weeks, could include increased expression of K6 and K16, but maintains almost normal differentiation and proliferation of the epidermis. This process seems unique to chronic UVB exposures. These two distinct responses might be initiated after the chronic UVB exposure and cause the characteristic alterations of skin such as wrinkles found in this study.

The sustained expression of K6 and K16 may be a sign of modification of the keratinocyte differentiation-regulating process

The nature of K6 and K16 induction in the epidermis has been controversial in relation to hyperproliferation, and K6 and K16 are sometimes called hyperproliferation-related keratins. This notion is based on the fact that, besides the outer root sheath of hair follicles, K6 and K16 are expressed in stratified epithelia undergoing chronic hyperproliferation or abnormal differentiation such as in psoriasis and in cancer [16, 17, 19]. However, the identification of those keratin species should not be oversimplified as hyperproliferation-related. The synthesis of K6 and K16 occurs without the concomitant up-regulation of proliferation-specific nuclear antigen, as revealed by Ki-67 staining [8]. Bernot et al. reported that the expression of K6 and K16 are not necessarily related to proliferation [1]. From our two stage hypothesis mentioned above, the induction of K6 and K16 could be explained differently. Their early induction may be caused in the shorter term by hyperproliferation due to UVB stimulation. However, the long-lasting expression of K6 and K16 may indicate modification of the differentiation-regulating process in keratinocytes as the longer term event. Normal murine epidermis renews itself approximately in 8.5 days; in other words, the turnover time of murine epidermis is about 8.5 days [20]. The 6 to 7-week period of sustained expression of K6 and K16 found in our study is far beyond the usual turnover time of the epidermis. Therefore, the influence of chronic UVB exposure is somehow incorporated or imprinted in living keratinocytes, thereafter modifying the keratin biosynthesis system toward the sustained expression of K6 and K16, even without further UVB exposures. The biochemical mechanism of this imprinting remains to be clarified.

The relationship between intermediate filaments and expression of K6 and K16

Interestingly, our results show that the long-lasting expression of K6 and K16 is accompanied by a drastic disruption of the ordered ultrastructure of KIFs in the stratum corneum (Fig. 10). This suggests that the altered keratin expression might trigger the disruption of KIF ultrastructure although no direct causative relationship is shown between the two events.

It is well established that KIFs play an important role in sustaining the structural resilience of the epidermis [15]. Some reports strongly suggest that aberrant expression of K6 and K16 disrupts KIF ultrastructure and subsequently affects the mechanical resilience of keratinocytes. Paladini et al. [19] reported that the forced expression of human K16 in keratinocytes of transgenic mouse skin elicits the retraction of keratin filaments from the cell periphery. Takahashi et al. [24] reported that over-expression of K16 in transgenic mice disrupts the keratinocyte cytoarchitecture and causes structural alterations in desmosomes at the cell surface. Taken together, these studies support the idea that the abnormal expression of various keratins could trigger the ultrastructural disruption.

We previously reported that a chronic 12 week UVB exposure regimen resulted in the reduction of epidermal elasticity and in the formation of relatively shallower wrinkles [22]. We demonstrate here that 6 weeks after the 9-week chronic UVB exposure, wrinkles were formed (Fig. 9). Wrinkle formation represents a typical sign of skin aging. There have been many studies on the formation of wrinkles including histological studies and studies of mechanisms underlying their formation [7, 12, 13, 18, 26, 27]. Those studies have indicated that chronic solar exposure causes the formation of wrinkles and that wrinkle formation is closely linked to the loss of elastic properties of the skin, which results from denatured elastic fibers in the dermis. In addition to these mechanisms, we suspect that the sustained expression of K6 and K16 would play an essential role in altering the physical properties of the epidermis through the disruption of KIFs. This process could also be of relevance to an early event of wrinkle formation caused by chronic UV exposure.

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