Degenerative Alterations of Dermal Collagen Fiber Bundles in Photodamaged Human Skin and UV-Irradiated Hairless Mouse Skin: Possible Effect on Decreasing Skin Mechanical Properties and Appearance of Wrinkles

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Dermal collagen fiber bundles (DCFB) are the major constructional element in the dermis. Although degenerative alterations of DCFB have been reported in chronologically aged skin, changes in photodamaged skin have not been fully investigated. We report ultrastructural alterations of DCFB, and their relation to skin elasticity using photodamaged human skin and UV-irradiated hairless mouse skin. The degree to which DCFB were intact and closely packed was evaluated and scored blindly. Exposed skin (outer forearm) exhibited marked ultrastructural degeneration. In UV-irradiated hairless mouse skin, the intact ultrastructural appearance of DCFB was gradually lost with increasing UV dosage; however, marked alterations in DCFB ultrastructure were absent in either human inner upper arm (unexposed) skin or nonirradiated age-matched control mouse skin. Skin mechanical properties were measured using a Cutometer SEM 474 suction extensometer, recording Ue* immediate deformation, Uv* viscous deformation, Uf* final deformation, and Ur* immediate contraction, all normalized for skin thickness. Uf*, Ue*, Uv*, and Ur/Uf were significantly decreased in exposed compared with unexposed skin. Significant positive correlations between degenerative alterations of DCFB and the decrease in Uf*, Ue*, and Uv* were seen. Changes of ''% area of wrinkles'' in UV-irradiated mouse skin was significantly correlated with degenerative changes of DCFB. Based on these results, we confirm observations made by others that chronic photodamage may have more severe effects on degeneration of DCFB than that of chronologic aging alone. Furthermore, degeneration of DCFB as detected ultrastructurally may, by its effect on skin elasticity, result in an increase in the appearance of wrinkles. ***Key words: connective tissue/photoaging/scanning electron microscopy/skin elasticity. Journal of Investigative Dermatology 117:1458–1463, 2001***

Various alterations in the dermal connective tissue occur in areas of skin exposed to solar irradiation. Typically the upper dermis exhibits solar elastotic degenerative change, with the histologic appearance of amorphous elastic staining tissue replacing the normal dermis. Elastic fibers become shorter and thicker. The fibrous meshwork of the dermis becomes more disorganized, with loss of fiber integrity, and cross-linking of collagen increases (Bissett *et al*, 1987; Kligman and Lavker, 1988; Gilchrest, 1989; Chatterjee *et al*, 1990). It is thought that these alterations are of major importance in determining the changes in mechanical properties that occur in photodamaged skin and are the putative reason for the development of changes in the skin surface texture, and in the development of fine lines and wrinkles (Kligman *et al*, 1985; Tsuji *et al*, 1986; Bissett *et al*, 1987; Bryce *et al*, 1988; Kligman *et al*, 1988; Gilchrest, 1989; Chatterjee *et al*, 1990; Chen *et al*, 1992; Moloney *et al*, 1992; Fisher *et al*, 1996). Warren *et al* showed more wrinkles, more elastosis and less collagen in the facial skin of a high sun exposure group *versus* a low exposure group (Warren *et al*, 1991). A large number of investigators have studied alterations of dermal connective tissue using both animal models and human skin. The effects of UV irradiation on the dermal collagen metabolism have been extensively reported. Although these studies have led to various and sometimes differing results, e.g., changes in ratio of type I/III collagen (Kligman *et al*, 1989; Schwartz *et al*, 1989; Chaqour *et al*, 1995; Talwar *et al*, 1995) or total collagen content (Chatterjee *et al*, 1990; Kligman *et al*, 1989; Chen *et al*, 1992; Schwartz *et al*, 1993; Chaqour *et al*, 1995; Neocleous *et al*, 1997), none have satisfactorily explained a causal relationship between alterations of dermal connective tissue and the changes of the skin mechanical properties, and the alterations of skin surface texture.

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Abbreviations: DCFB, dermal collagen fiber bundle; SEM, scanning electron microscopy; cutometer parameters, refers to parameters normalized to skin thickness: Ue*, immediate deformation; Uv*, viscous or delayed deformation; Uf*, final deformation; Ur*, immediate contraction*.

In the skin of patients with Ehlers Danlos syndrome, it has been shown that degeneration of dermal collagen fiber ultrastructure alters skin mechanical properties such as hyperextensibility and fragility (Vogel *et al*, 1979; Byers, 1989). Structural alterations of dermal collagen fibers in photodamaged skin have been reported, such as indistinct outlines and smaller diameter of collagen fibers (Lavker, 1979; Schwartz *et al*, 1989; Zheng and Kligman, 1993); however, few ultrastructural studies have reported the ultrastractural appearance of collagen fibres and DCFB in the skin aging processes. Lavker *et al* (1987) have reported that DCFB appear to unravel in chronologically aged inner upper arm skin. Similar alterations of DCFB, namely swollen and unravelled bundles, were reported by Fornieri *et al* (1989) in chronologically aged rat skin. On the other hand, only slight or negligible ultrastructural differences of DCFB were noted between females and males or in skin specimens taken from different sun-protected sites, i.e., the abdomen, the inner forearm, or the thigh (Quaglino *et al*, 1996). Based on the results obtained from these studies, it is suggested that DCFB degenerated with chronologic aging; however, the ultrastractural alterations of DCFB in sun-exposed, photodamaged sites have not been fully investigated. Therefore, in this study, we have attempted to investigate the ultrastructural alterations of DCFB in sun-exposed photodamaged human skin and their relation to skin elasticity. In addition, the UV-irradiated hairless mouse model was used to demonstrate ultrastructural changes in DCFB caused by photodamage.

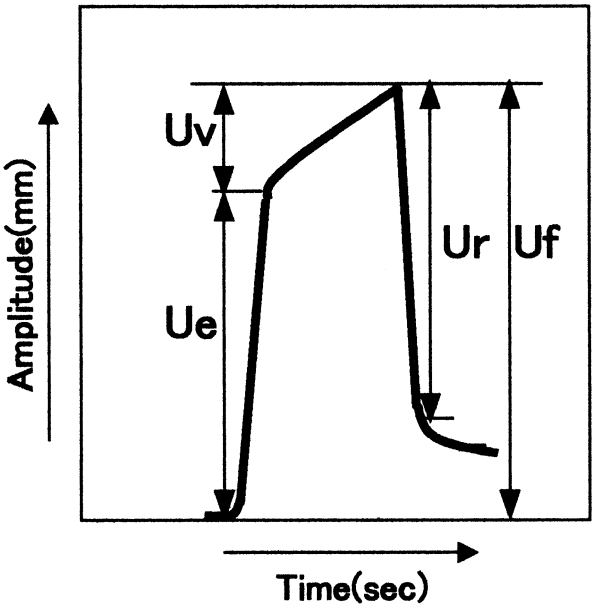

Figure 1. Parameters of skin mechanical properties. Ue is immediate deformation; Uv is delayed deformation; Uf is final deformation; Ur is immediate contraction.

MATERIALS AND METHODS

Subjects Twenty Caucasians (aged 31–81) who were clinically judged to have significant photodamage to their exposed skin were recruited with no evidence or history of other skin disorders likely to interfere with this study. Two body sites, the outer forearm (photodamaged site; moderate or severe photodamage was assessed by a dermatologist) and the central inner upper arm (control site; no obvious photodamage), were used for this study. After all noninvasive measurements had been completed, 4 mm punch biopsies were obtained from the two body sites. Each biopsy was halved. One half was prepared for scanning electron microscopy (SEM). The other half of each biopsy was fixed in 10% buffered formalin, processed in paraffin wax, sectioned at 5 μm and stained by Gomori's aldehyde fuschin method or the standard haematoxylin and eosin method.

Animal model of photodamage Five-week-old female albino hairless mice (Hr-/Kud) were obtained from Kyudo (Kumamoto, Japan). The animals were acclimatized for 3 wk prior to study. Forty-five female albino hairless mice were divided into two groups, one irradiated group (20 mice) and one nonirradiated group (25 mice). Irradiated animals were exposed to ultraviolet radiation (UVB dose of 60 mJ per cm^2 measured by the UVB probe of a Topcon radiometer) from a bank of four Toshiba SE lamps without any filtering, and placed 40 cm above the back of each animal (irradiance of UVB was approximately 1.25 mW per cm^2). As previously reported, the peak spectral output of this lamps is approximately 310 nm (Imokawa *et al*, 1995), with no energy detectable below 260 nm and approximately 0.6% between 260 and 280 nm (UVC), 72.7% between 280 and 320 nm (UVB), and 26.7% between 320 and 400 nm (UVA). The energy output of the lamps was measured with a Topcon UV radiometer 305/365D. Under these conditions, the minimal erythema dose (MED) of these animals was 70 mJ per cm^2 UVB. Animals were irradiated five times a week for 10 wk. Under these conditions, mice did not show any erythema, edema, or scaling, so the irradiations were below their MED thresholds. The total dose for a 10 wk period of predominantly UVB exposure amounted to 3.0 J per cm^2. Animals were fed with a standard diet and were allowed water ad libidum, and were housed in rooms where the lighting was free from both UVA and UVB emissions. A 12 h automated light and dark cycle was used.

Skin elasticity in human skin In human skin only elasticity was measured using a Cutometer SEM474 suction extensometer (Courage & Khazaka, Cologne, Germany) as previously reported (Agache *et al*, 1980). Briefly, suction was applied to a 2 mm diameter area of the volunteer's skin surface. The suction probe acted as a guard ring to preclude skin involvement outside of the area measured. The probe was fitted with a spring that ensured that it was applied to the skin at a constant pressure. Skin elongation was measured by an optical method with an accuracy of 0.01 mm using an infrared diode. The time/strain mode was used with an elementary load cycle consisting of a deformation by a 400 mbar strain, maintained for 2 s and then released and followed by a 2 s relaxation period. This elementary load cycle was repeated five times successively, but data were only taken on the fifth cycle to improve reproducibility. Four parameters of skin mechanical properties were calculated from the recorded fifth cycle (**Fig 1**). These four parameters showed skin mechanical properties related to skin elasticity, as described previously by Agache *et al* (1980).

Scanning electron microscopic observations We have investigated the ultrastructure of DCFB on both human and mouse skin specimens using SEM because, as Lavker discussed in his histologic study of aged skin (Lavker *et al*, 1987), the geometry of the connective tissue is best displayed by SEM. The three-dimensional organization of DCFB structures was examined as previously reported (Ohtani, 1987; Ohta *et al*, 1990). Briefly, after removing the subcutaneous fat layer, the specimens were fixed in 2% glutaraldehyde for 3–7 d. The samples were then immersed in a 2-N NaOH solution for 4–7 d to remove noncollagenous material. The epidermis was removed, and only dermal collagenous architecture was remaining after this treatment. After rinsing in distilled water for a minimum of 4 d, the specimens were stained with 1% tannic acid, and were then postfixed in 1% osmic acid. After dehydration in a graded series of ethanol, the specimens were frozen in liquid nitrogen and fractured. The samples were dried in a HCP-2 critical point dryer (Hitachi, Japan) using liquid carbon dioxide. The specimens were then coated with platinum and the surface features and the DCFB were observed under a JSM U3 field emission SEM with an accelerating voltage of 15 kV. In the human biopsy skin study, only half of a 4 mm punch biopsy from each volunteer was available for the freeze fracture process. We were therefore only able to successfully process seven pairs of specimens from the 20 subjects.

Grading of dermal collagen fiber bundles Electron micrographs of the seven pairs of human skin specimens were taken at a magnification of ×1000 from three random areas (avoiding surrounding areas such as hair folicles, sweat ducts, erector pilli muscle) at each of four depths (approximately 100–500 μm depth from the uppermost dermis) of each fractured section. Electron micrographs of mouse skin specimens were taken at a magnification of ×2500 from three random areas, also avoiding surrounding areas of cutaneous appendages in the upper dermis (approximately 30–100 μm depth from the uppermost dermis). Using these micrographs, the ultrastructure of DCFB was graded using a four point scale in a blind manner using the following grades descriptions:

Grade 0: Complete absence of intact collagen fiber bundles.

Grade 1: A few intact bundles seen.

Grade 2: Intact bundles seen in more than half area of a micrograph; a few degenerated bundles seen.

Grade 3: Intact bundles seen over the whole area of the micrograph (**Fig 2**).

Skin surface features To evaluate the degree of wrinkling in mouse skin, we applied the image analysis technique for assessment of human wrinkles reported by Grove *et al* (1989). Briefly, surface replicas of the skin of the animals were taken with silicon rubber. The replica was illuminated normal to the wrinkles at a 20° angle using an optical fiber connected to a Xenon lamp. Percentage area of shadows in a 1 × 1 cm area was calculated using an image analyzer (LA-555, PIAS, Japan). In this study, we designated this percentage area of shadows as a semiquantitative measurement of degree of wrinkling ''percentage area of wrinkles''. Finally, the replicas were coated with platinum, and the skin surface features were observed under a JSM U3 field emission SEM (JEOL, Hachioji, Japan) with an accelerating voltage of 15 kV.

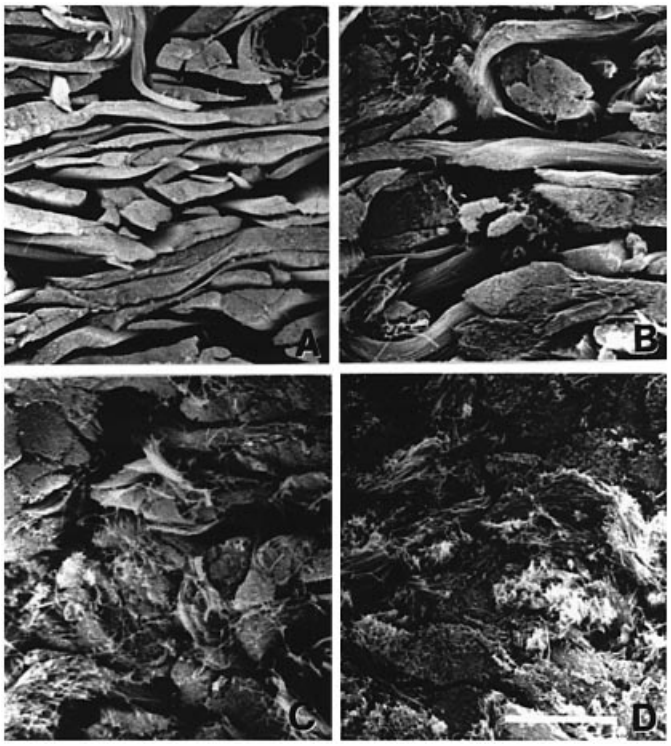

Figure 2. Reference scale for the evaluation of DCFB. (*A*) grade 3 (normal mouse skin), (*B*) grade 2, (*C*) grade 1, (*D*) grade 0. *Scale bar*: 15 nm.

Statistical evaluation Statistical significance of the differences in measurements of mechanical properties between the inner upper arm site and the outer forearm site and the percentage area of wrinkles between UV-irradiated mice and age-matched control mice were determined by Student's t test. Statistical significance of the differences in mean grading score of the DCFB between two body sites and between predominantly UVB-irradiated mice and age-matched control mice were determined by the Wilcoxon test. Correlation coefficients between the mean grading score of the DCFB and the skin mechanical properties were determined by Spearman's correlation test.

RESULTS

Dermal collagen fiber bundles and skin mechanical properties in photodamaged human skin Microscopic examination and measurement of elastic stained materials by image analysis demonstrated that there was significantly more elastic materials in the outer forearm skin than in the skin of the inner upper arm (**Table I**). It was concluded therefore that the outer forearm could be designated as a photodamaged site.

SEM of freeze fractured specimens from the human inner upper arm skin, revealed intact and closely packed DCFB with distinct outlines (**Fig 3*A–C***). In contrast, the DCFB from the photodamaged outer forearm skin demonstrated frayed bundles with indistinct outlines (**Fig 3*D–F***).

Using the cutometer suction device generates several numerical indices. Briefly, parameter Ue demonstrates immediate deformation, Uv demonstrates delayed deformation, and Uf demonstrates final deformation. The parameter Ur represents immediate contraction, Ur/Uf demonstrates the ratio between immediate retraction and total distension, and Uv/Ue demonstrates the ratio between the viscoelastic properties of the skin and immediate distension. **Figure 1** demonstrates the absolute parameter definitions. All of the absolute parameters are influenced by skin thickness. Only ratios of parameters (Ur/Uf and Uv/Ue) are independent of thickness. Therefore, we present absolute parameters normalized by skin thickness (Uf*, Uv*, Ue*, Ur*); however the non-normalized values yield the same results as the normalized values.

The measurements of skin mechanical properties (Uf*, Ue*, Uv*, Ur/Uf) in the outer forearm skin were significantly decreased compared with the inner upper arm skin (**Table I**). There were significant positive correlations between the loss of intact DCFB and the decrease in Uf*, Ue*, and Uv* (**Table II**).

Dermal collagen fiber bundles and skin surface features in UV-irradiated mouse skin These observations were similar to those from human skin. The intact structure of DCFB was observed in nonirradiated mouse skin (**Fig 4*A***), and an intact structure of distinct bundles of DCFB was observed in the whole dermis. Following predominantly UVB irradiation, these intact DCFB were gradually lost, especially in the upper dermis (**Fig 4*B–D***). In contrast, we did not observe any remarkable ultrastructural changes in the DCFB in age-matched control mice (data not

Table I. Measurements of skin thickness, mechanical properties, and mean grading scale of DCFB in human skin

	Skin thickness (mm)	% area of elastic materials	Mean grading scale of DCFB	Measurements of mechanical properties				
				Uf*	Ue*	Uv*	Ur/Uf	Uv/Ue
Inner upper arm								
mean	1.069	8.632	1.778	0.415	0.280	0.135	0.669	0.488
SD	0.312	3.121	0.326	0.124	0.086	0.042	0.114	0.075
n	20	20	7	20	20	20	20	20
Outer forearm								
mean	1.499	18.724	1.084	0.174	0.111	0.053	0.482	0.508
SD	0.418	6.852	0.366	0.059	0.040	0.018	0.118	0.097
n	20	20	7	20	20	20	20	20
p value (paired t test #Wilcoxon test)	$p < 0.001$	$p < 0.001$	$p < 0.01$#	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS

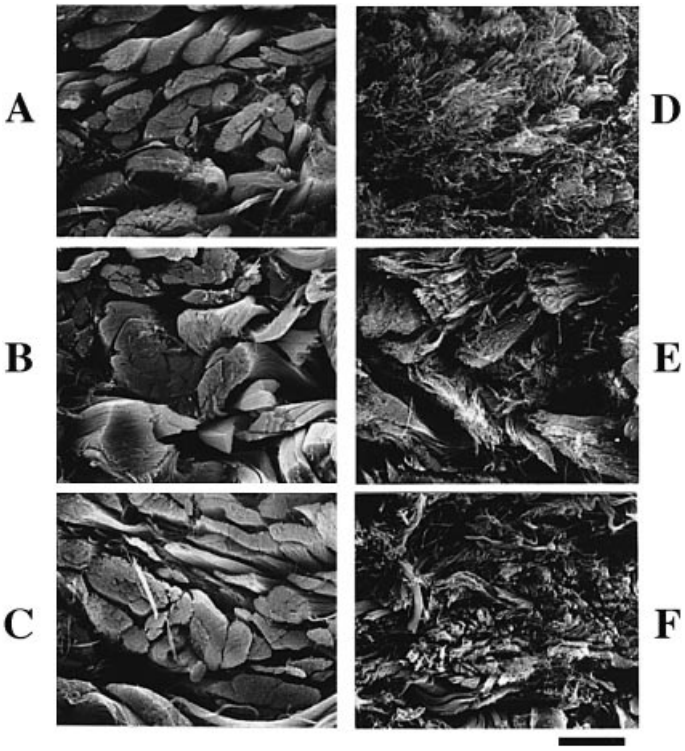

Figure 3. SEM of DCFB in human skin. (*A*, *D*) 47-y-old male, (*B*, *E*) 55-y-old female, (*C*, *F*) 75-y-old female. (*A–C*) The inner upper arm site, (*D–F*) the outer forearm site. *Scale bar*: 100 nm.

Table II. Correlation coefficients of DCFB grade *versus* difference in cutometer-derived mechanical parameter between inner and outer arm

	Parameters of mechanical properties				
	Uf*	Ue*	Uv*	Ur/Uf	Uv/Ue
Spearman's correlation coefficient (n = 14)	0.668	0.732	0.780	0.276	0.276
p value	$p < 0.01$	$p < 0.01$	$p < 0.01$	NS	NS

shown). A very organized skin surface texture was observed in nonirradiated mouse skin (**Fig 4*E***). With predominantly UVB irradiation, clear wrinkling appeared after 5 wk and some qualitative changes in the appearance of skin surface roughness became apparent (**Fig 4*G***, ***H***).

Semi-quantitative measurements of the degree of wrinkling "% area of wrinkles" increased in contrast to the degenerative changes of the DCFB with predominantly UVB irradiation (**Fig 5**). The above ultrastructural changes were not observed in age-matched control mice over the same study period (8–18 wk old). Significant correlation was detected between "% area of wrinkles" and mean grading score of the DCFB (Spearman's correlation coefficient $r_s = -0.711$, $p < 0.01$, $n = 45$).

DISCUSSION

The mouse model here described used a light source that contains small amounts of UVC and some UVA wavelengths. The small amount of UVC radiation present will not penetrate deeply and its relative spectral effectiveness for erythema (RSEE) is low. Because this study is primarily concerned with dermal changes, the presence of small amounts of UVC in the irradiation source is not considered significant.

The mouse model shows that dermal changes similar to those seen in photodamaged human skin can be induced by UV irradiation. It does not give any insight as to which spectral components are responsible for the dermal changes seen in humans. The fact that the mouse dermal changes can be seen to progress with increasing doses of UV irradiation, and that they are similar to those seen in human photodamaged but not relatively photoprotected skin, supports the widely held conclusion that solar damage in human skin is largely caused by the UV spectral region.

Human DCFB showed more degeneration in the outer forearm skin than in the inner upper arm skin. In UV-irradiated hairless mouse, the intact ultrastructure of the DCFB was gradually lost with increasing UV irradiation, but noticeable ultrastructural alterations were not observed in the nonirradiated age-matched control mice in this study period (from 8 to 18 wk old). Degenerative alterations of the DCFB have been previously reported by other investigators using chronologically aged rat and human skin (Fornieri *et al*, 1989; Quaglino *et al*, 1996); however, in this study, we did not find any significant alterations of DCFB in the sun-protected inner upper arm skin and in the nonirradiated age-matched control mouse skin. These different results might be due to the age differences of subjects. In this study, we used comparatively younger skin than those previous authors did. Based on these results, we can confirm that chronic photodamage may

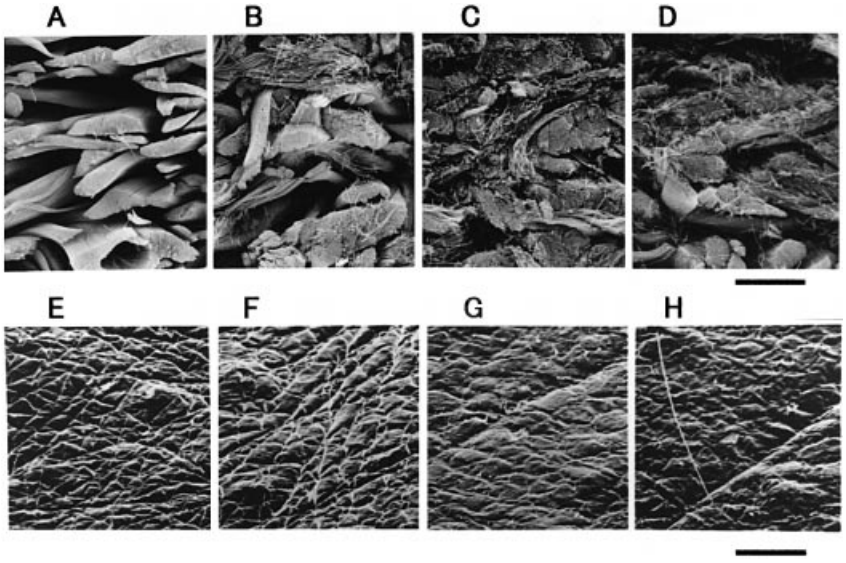

Figure 4. Changes of DCFB, skin, and dermal surface features with predominantly UVB irradiation in hairless mice. (*A–D*) SEM of the DCFB in the upper dermis; (*E–H*) SEM of silicon replicas from the skin surface; (*A*, *E*) nonirradiated control (8-wk-old); (*B*, *F*) irradiation for 2 wk (10-wk-old); (*C*, *G*) *irradiation for 5 wk (13-wk-old);* (*D*, *H*) irradiation for 10 wk (18-wk-old). *Scale bars*: (*A–D*) 10 nm; (*E–H*) 500 nm.

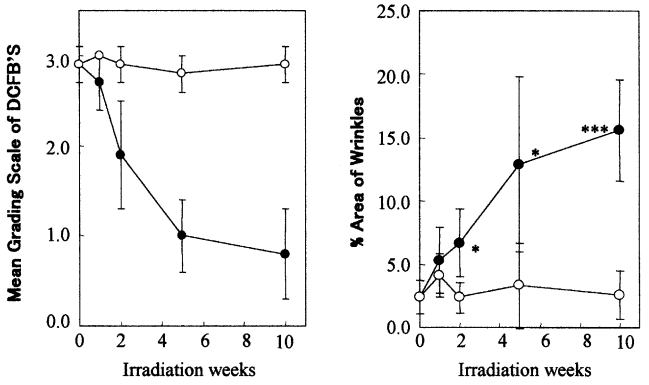

Figure 5. Development of wrinkles and degeneration of DCFB with predominantly UVB irradiation in hairless mice. *Left*: Changes in mean grading scale of the DCFB with predominantly UVB irradiation. *Right*: Changes in "% area of wrinkles" with predominantly UVB irradiation. *Open circles* indicate mean scales or measurements of the nonirradiated, age-matched control mice, and *closed circles* indicate those of the irradiated mice. *Error bars*, SD (n = 5): "% area of wrinkles" of the irradiated mice is significantly (*$p < 0.05$, ***$p < 0.001$) different from the age-matched control mice.

have more acute and severe effects on degeneration of the DCFB than intrinsic, chronologic aging.

Takema *et al* have reported that skin mechanical properties Ur/Uf and Uv*, Ue* were significantly decreased with age in human facial skin (Takema *et al*, 1994). They also showed a significant increase of Ue* and Uv* in the ventral forearm skin with age. They concluded that sun exposure appears to have a considerable effect on the mechanical properties of facial skin. Our measurement results of Uv* in the two body sites support their conclusions. The Uv* parameter was significantly decreased in the outer forearm skin compared with the inner upper arm skin. Loss of skin mechanical properties in both chronologic and actinic aged skin has been mainly investigated in relation to degenerative changes of elastic fibers (Hirose and Kligman, 1988; Uitto *et al*, 1989; Imayama *et al*, 1994; Takema *et al*, 1994). In this study, however, we detected a significant positive correlation between the loss of "intact" DCFB and the decrease in Uf*, Ue*, and Uv*. Imayama *et al* reported that during adulthood, there was a tortuosity of the distorted elastic fibers coupled with an incomplete rebuilding of the elastic fiber network that would normally be laid down in a way that interweaves with the collagen bundles (Imayama and Braverman, 1989). They hypothesized that the tortuous and fixed appearance of these elastic fibers implied that they have been stretched and that they have lost their original elasticity and their ability to become short and straight. Therefore, we think that the degeneration, unravelling of intact bundles, and loss of intact and distinct bundle macro structure of the DCFB, may have some effects on skin properties such as elasticity or viscoelasticity.

We would like to suggest two possible explanations of the relationship between the degeneration of DCFB and the decrease in skin elasticity: (1) DCFB are the main constructional element of the dermis. Therefore, degenerative changes of the DCFB may disturb the three-dimensional linearity of the elastic fibers, and the resulting tortuous elastic fibers are not able to regain their original elasticity. Another possible explanation for the relationship between the bundle structure and skin elasticity may be due to a more direct effect of the bundle macro structure. (2) The intact and distinct bundle macro structure of DCFB may in itself have some elasticity, and the loss of this structure in the photodamaged skin directly contributes to the decrease in skin elasticity. We think that both the direct and indirect effects of the degeneration of DCFB are responsible for the decrease of skin elasticity, particularly in photodamaged skin.

In line with the report by Kligman *et al* (1985), we could not find any remarkable ultrastructural changes of the DCFB in wrinkles that were distinguishable from the surrounding skin. On the other hand, we found that changes of the skin surface features "% area of wrinkles" produced by predominantly UVB irradiation was significantly correlated with degenerative changes of the DCFB. In their histologic and quantitative study of human facial skin, Warren *et al* have reported that a group with higher sun exposure had more wrinkles, more severe elastosis, and less collagen than a comparable but less sun-exposed group (Warren *et al*, 1991). Based on our and these results, we conclude that the ultrastructural changes of the DCFB may have some relation to skin surface feature alteration, and the degenerative effects on the development of wrinkles may be, in part, caused by either indirect or direct ultrastructural effects due to the decreased skin elasticity.

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