Degree of Ultraviolet-Induced Tortuosity of Elastic Fibers in Rat Skin Is Age Dependent

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To elucidate differential effects of ultraviolet (UV) exposure on three-dimensional networks of elastic fibers during maturation of rat skin, Sprague-Dawley rat hind limbs were irradiated with suberythemal doses of UV light (UVB, 130 mJ/cm^2, or UVA, 27 J/cm^2) in three different time courses of exposure: 3–9 weeks old, 9–15 weeks old, and 3–15 weeks old. Three-dimensional arrangement with special reference to linearity of elastic fibers was quantified by image analysis using a scanning electron microscope after a combination of intravascular resin injection and selective digestion technique using formic acid. Among the three irradiation groups, the group irradiated with UVB or UVA between 3 and 15 weeks old (UVB, three times per week; UVA, five times per week) elicited the most marked decrease in the linearity of elastic fibers. Despite the same irradiation period, there was a significant difference in the decreased linearity between the two irradiation groups of 3–9 and 9–15 weeks old, with the former irradiation group exhibiting greater loss of linearity than the latter irradiation group. The magnitude of the decreased linearity was greater in the UVB-exposed groups than in the UVA-exposed group. These findings indicate that the three-dimensional linearity of elastic fibers is more susceptible to disruption by UV exposures during the growth period than that after the growth period. ***Key words: skin/ultraviolet radiation/ three-dimensional network. J Invest Dermatol 105:254–258, 1995***

Chronic ultraviolet (UV) irradiation leads to alterations in the dermal connective tissues. Histologically, chronically sun-exposed skin displays a tremendous dermal accumulation of elastin-staining material [1–3] and these changes are believed to lead to photoaging of the skin. A striking visible feature of this photodamage is skin wrinkling. A number of investigators have reported success in the development of histologic animal models for solar-aged skin and indicated histologically detectable alterations in dermis with UV exposure [4–6]. In conjunction with the histologic changes, the three-dimensional architecture of connective tissue components, especially dermal elastin fibers, has been one of the key targets for understanding the mechanism leading to the formation of wrinkling by UV exposure, because it is likely that ultrastructural deformation of the elastic fibers may be responsible for the decline in skin elasticity that provides a rheologic basis for the development of sagging and wrinkling of the skin. In aged skin that develops wrinkling, ultrastructural changes in human dermal elastic fibers have been reported using an autoclave method that was developed for scanning electron microscopy [7–9]. Recently, Imayama and Braverman [10] evaluated the three-dimensional structure in tissues after intravascular resin injection and selective digestion using rat hind limb skin and reported loss of linearity of elastic fibers with age [11]. Previously, we demonstrated that the loss of three-dimensional linearity of elastic fibers is predominantly associated with the reduction of skin elasticity and wrinkling during the photoaging process [12]. These studies would provide a model for explaining or predicting how and to what extent photoaging skin develops wrinkling. In recent years, there have been arguments regarding the seriousness of damage caused by UV exposure to children's skin as compared with that of adults. However, there have been few studies focusing on comparison among different ages. Therefore, in this study, using the intravascular resin injection and selective digestion technique, we investigated differential effects of UV exposure during maturation of rat skin, by performing continuous UV irradiation during or after the growth period and comparing its effects on the three-dimensional structure of elastic fibers in the dermis.

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MATERIALS AND METHODS

Animals Male Sprague-Dawley (SD) rats, 3 weeks old, were purchased from Charles River Laboratories, Japan. They were fed a standard diet and water *ad libitum*, and housed in rooms where the lighting without UVB emission was automatically regulated on a 12-h light and dark cycle.

Radiation Source The 3-week–old SD rats were divided into two groups from the same initial stock; group 1 was the UVB-irradiation experiment and group 2 was the UVA experiment. Group 1 UVB rats or group 2 UVA rats were placed in cages individually and irradiated by a bank of five Toshiba SE lamps (UVB) without any filtering or Toshiba BL black lamps (UVA) with glass filter (thickness is 0.5 mm). The latter lights have no detectable emission below 340 nm. The distance from the lamps to the animals' hind limbs was 42 cm (irradiance was approximately 0.72 mW/cm^2) for UVB or 6 cm (irradiance approximately 3.79 mW/cm^2) for UVA. A dose of 130 mJ/cm^2 (rat 1 minimal erythemal dose (MED) = 170 mJ/cm^2) or 27 J/cm^2 (rat 1 MED = 216 J/cm^2) was given three or five

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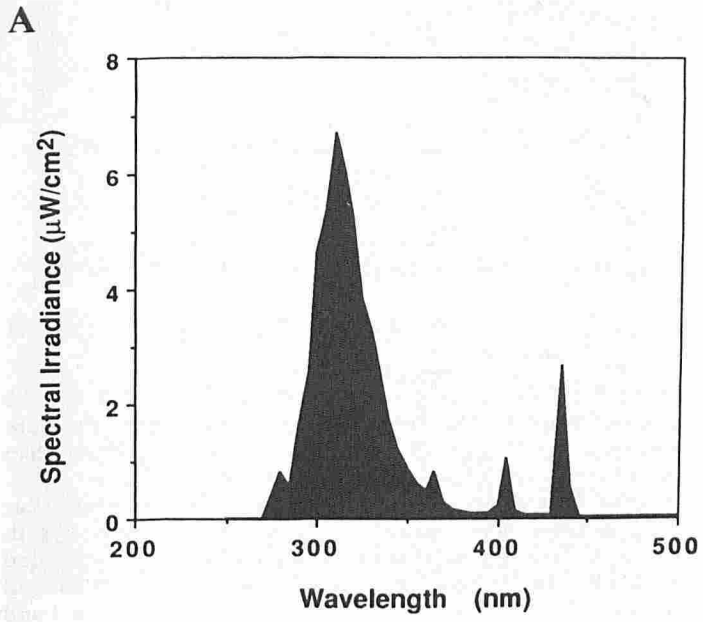

Figure 1. The spectrum of UV lights. *A*: The spectrum of Toshiba SE lamp used as UVB light source. *B*: The spectrum of Toshiba BL black lamp used for UVA light.

times weekly from 3 weeks of age for UVB or UVA. The energy output of the lamps was measured with a Topcon Co. Ltd. UV radiometer 305/365DII. The spectral irradiance of these lamps was measured with an Optical Science Co. Ltd. MSR7000 Radiospectrometer. The spectral output is shown in **Fig 1**.

Irradiation Schedule **Figure 2** shows changes in body weight of the rats with growth. SD rats grew to a constant body weight at about 9 weeks after birth, allowing reproduction. Therefore, in this study, a rat aged less than 9 weeks was considered to be in the growth period and after 9 weeks to be in the postgrowth period. The irradiation schedule was as follows: rats were divided into four groups (n = five rats, 10 limbs for each) for each of the UVB and UVA experiments; three groups were irradiated with UV during the growth period (between 3 and 9 weeks of age), after the growth period (between 9 and 15 weeks of age), or during and after the growth period (between 3 and 15 weeks of age). Animals in the other group were used as non-irradiated controls.

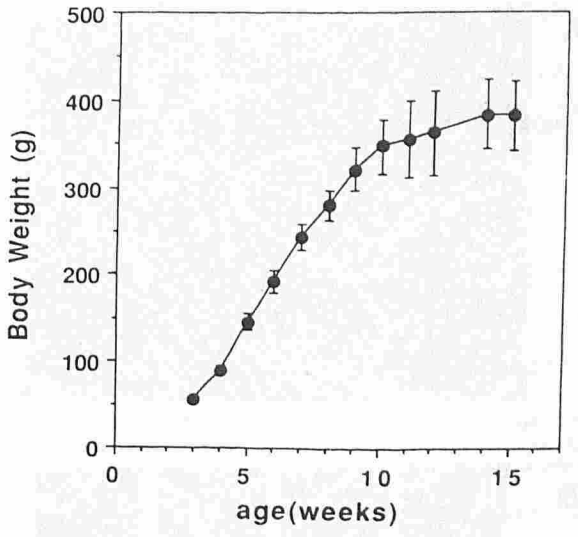

Figure 2. Sequential changes in body weight of the rats with growth.

SEM Observation All procedures were carried out to preseve the three-dimensional arrangements of the elastic fibers by virture of the microvascular scaffolding. The technique employed in this study was identical to that described previously [10,12]. Briefly, the sample animals were anesthetized with sodium pentobarbital (30 mg/kg, injected intraperitoneally), and perfusion was performed through a cannula inserted into the abdominal aorta. The hind limbs were flushed with Ringer's solution briefly to remove the blood and then injected with Mercox resin (Dainihon Inc., Tokyo, Japan). After polymerization of the resin, limbs were dissected and immersed in a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer for a minimum of 4 d at 4°C. After fixation, pieces of skin measuring 3 to 4 mm in depth and including the underlying muscles were removed using a scalpel. These tissue blocks were incubated in 88% formic acid solution at 45°C for 5 to 6 d to remove tissue components mainly composed of collagen fibrils. After incubation, specimens were carefully washed in 0.002 N HCl for 4 to 5 h to remove residual collagen fibrils and then immersed in 1% tannic acid solution for 1 h. After rinsing in water, the samples were post-fixed in 2% osmium tetroxide for 1 h, dehydrated through a graded ethanol series, and dried by the critical point drying technique. Specimens were then mounted, sputtercoated with gold, and examined using a HITACHI Model S-4000 field emission scanning electron microscope at 5 kV. After the scanning electron microscope observation, most of the specimens were embedded in Spurr's resin and studied by transmission electron microscopy to confirm the surface morphology that was seen under the scanning electron microscope.

Elastic Fiber Linearity Samples were prepared after a combination of intravascular resin injection and selective digestion technique using formic acid and analyzed on scanning electron micrographs. Elastic fiber linearity was quantified at magnifications of 1000 using a personal image analyzer system (PIAS LA-555 Pias Co. Ltd. Japan), as shown in **Fig 3**. Two electron micrographs at 1000 × magnification were taken from representative areas of five scanning electron microscopy blocks from each hind limb, providing ten electron micrographs for each sample **(Fig 3*A*)**. The electron microphotographs were enlarged and evenly divided into 16 sections **(Fig 3*B*)**. From these divisions, representative fragmented lines of elastic fibers were traced onto a clear plastic sheet (OHP film, Kao Corporation) **(Fig 3*C*)**. The tracings were then analyzed under the above image-analysis system. Before calculation of the linearity of each fragmented line, the width of each tracing was set to constant value (eight pixel width) to eliminate artifacts at tracing. **Figure 4** shows the calculation methods. The length and width of the minimum rectangle enclosing one fragmented line of elastic fiber, which were automatically calculated by computing, were designated as C and B respectively, and the area of fragmented elastic fiber as A. The linearity of fragmented elastic fibers was expressed as A/(B × C). For example, in a straight fragmented elastic fiber, the linearity is 1.

Statistical Analysis Elastic fiber linearity is expressed as mean ± SD. Differences between means were checked for significance using the Student t test.

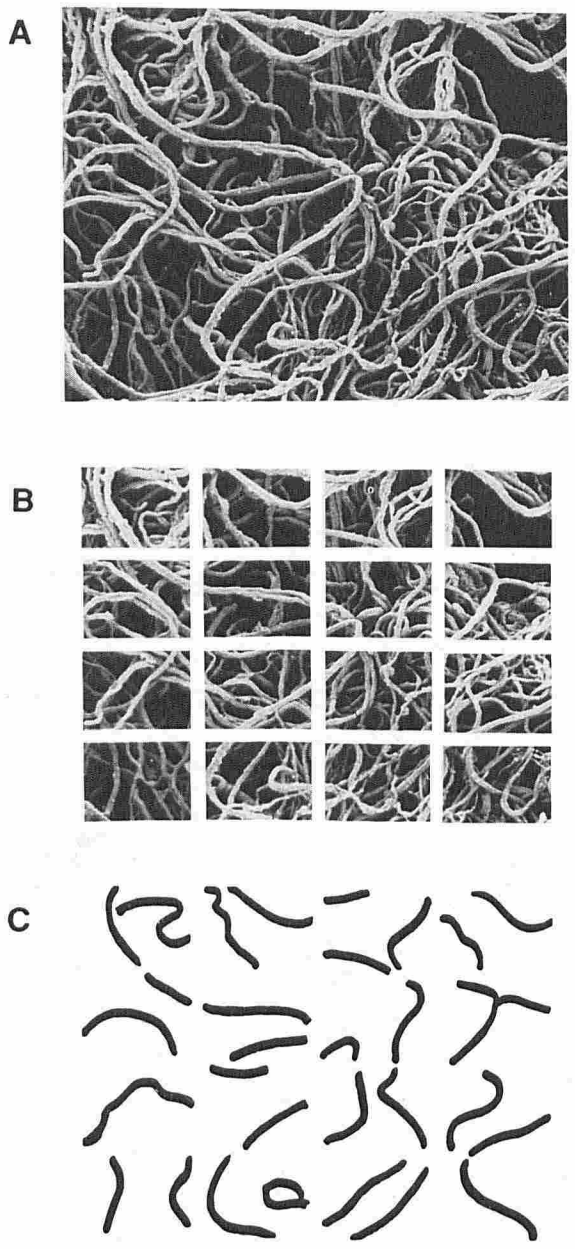

Figure 3. Procedure for quantifying elastic fiber linearity under image analyzer. *A*: A representative micrograph. *B*: Photos divided into 16 sections. *C*: Representative elastic fibers traced onto a clear plastic sheet.

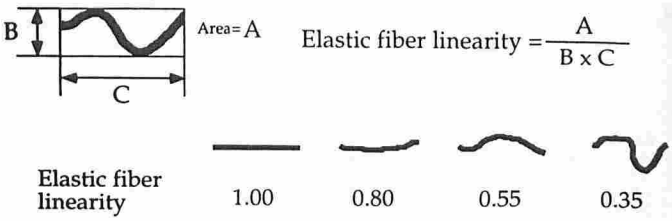

Figure 4. Method for calculation of elastic fiber linearity.

RESULTS

Three-dimensional architecture of the connective tissue was well preserved by virtue of the microvascular scaffolding and directly observed under the scanning electron microscope. **Figures 5 and 6** show representative scanning electron micrographs after ultraviolet irradiation followed by intravascular injection and selective digestion at the age of 15 weeks. In non-irradiated controls at the age of 15 weeks, elastic fibers in the dermis of hind limbs were arranged in a multilayered, ordered meshwork of relatively straight fibers **(Fig 5*A*)**. After UVB irradiation between 3 and 9 weeks, elastic fibers became tortuous **(Fig 5*B*)**. After UVB irradiation between 3 and 15 weeks, tortuousness of elastic fibers was more marked **(Fig 5*C*)**. After UVB irradiation between 9 and 15 weeks, tortuousness of elastic fibers was detectable, but less marked than that in the above two groups **(Fig 5*D*)**. In UVA experiments, as in UVB experiments, the linearity of elastic fibers showed the most marked deterioration after UVA irradiation between 3 and 15 weeks old **(Fig 6*B*)**, whereas tortuousness of elastic fiber was more prominent after UVA irradiation between 3 and 9 weeks old than that between 9 and 15 weeks old **(Fig 6*C*,*D*)**.

Figure 7 shows changes in the linearity of elastic fibers after UVB irradiation. The linearity of elastic fibers was decreased in each condition compared to the control group; the group irradiated with UVB between 3 and 15 weeks old showed the most marked decrease, followed in order by the group irradiated between 3 and 9 weeks old and that irradiated between 9 and 15 weeks old. Thus, even with the same irradiation period, the decrease in the linearity of elastic fibers was significantly more marked in the group irradiated between 3 and 9 than between 9 and 15 weeks old. These results suggest that UVB irradiation during the growth period significantly decreases the linearity of elastic fibers as compared with UVB irradiation after the growth period.

Figure 8 shows change in the linearity of elastic fibers after UVA irradiation, indicating decreases similar to those observed after UVB irradiation. UVA irradiation during the growth period significantly reduced the linearity of elastic fibers compared with that after the growth period. In general, a more marked decrease in the linearity of elastic fibers was observed by UVB than UVA irradiation under the present experimental conditions.

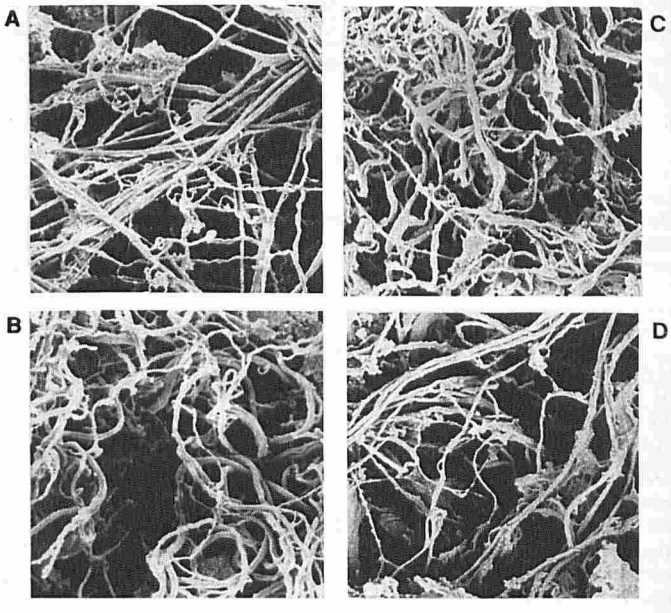

Figure 5. Scanning electron micrographs after UVB irradiation followed by intravascular injection and selective digestion at the age of 15 weeks. *A*: Non-irradiated control. *B*: UVB irradiation between 3 and 15 weeks old. *C*: UVB irradiation between 3 and 9 weeks old. *D*: UVB irradiation between 9 and 15 weeks old.

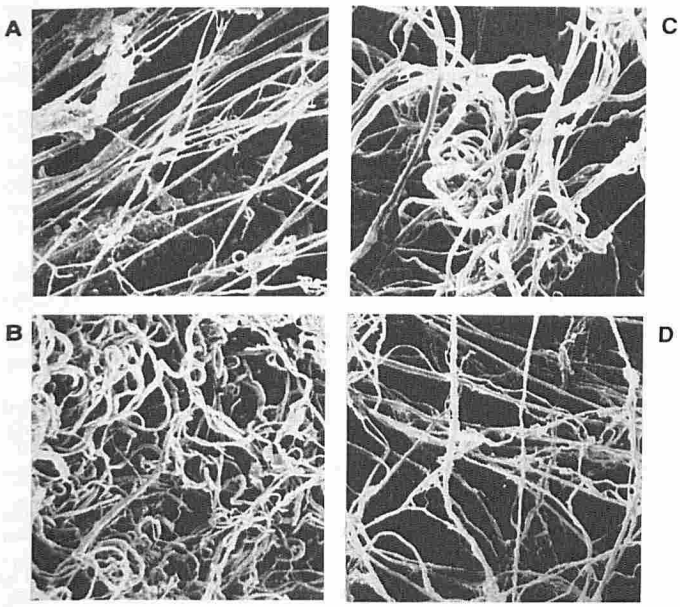

Figure 6. Scanning electron micrographs after UVA irradiation followed by intravascular injection and selective digestion at the age of 15 weeks. *A*: Non-irradiated control. *B*: UVA irradiation between 3 and 15 weeks old. *C*: UVA irradiation between 3 and 9 weeks old. *D*: UVA irradiation between 9 and 15 weeks old.

DISCUSSION

There have been many three-dimensional studies of the fiber components in the dermis. However, most have analyzed the arrangement of collagen and elastic fibers [13–15]. For three-dimensional analysis of elastic fibers in the dermis, the autoclave method [8,9] and formic acid digestion after perfusion fixation are generally used. Tuji *et al* [8] observed age-related changes in elastic fibers in the human skin and reported that the shape and arrangement of elastic fibers become complex with age. They also observed the skin in solar elastosis by a similar method and found three patterns of change in elastic fibers; i.e., fibers that appear to be normal, winding thick round fibers, and a large number of degenerated fibers [16]. Among age-associated changes, Zheng *et al* [13] reported increased density of elastic fibers, increased irregularity in their arrangement, and a decrease in fibers running

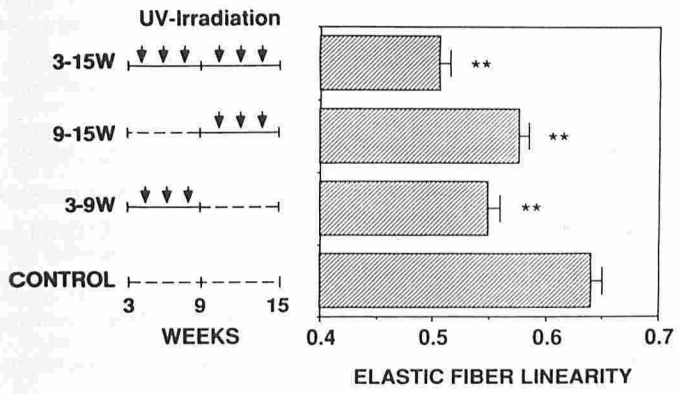

Figure 7. Changes in the linearity of elastic fibers after UVB irradiation with different time courses as measured by image analysis. $^{**}p < 0.01$ as compared to control. Each *bar* is the mean ± SD. Total fragmented line: n = 318–488. Animal, n = 5; limb, n = 10. There are significant differences at $p < 0.05$ between 9–15 weeks and 3–9 weeks, and at $p < 0.01$ between 3–9 weeks and 3–15 weeks.

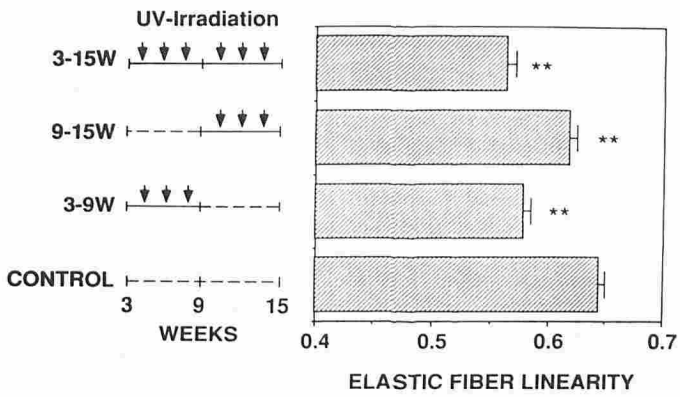

Figure 8. Changes in the linearity of elastic fibers after UV-A irradiation with different time courses as measured by image analysis. $^{**}p < 0.01$ as compared to control. Each *bar* is the mean ± SD. Total fragmented line: n = 598–755. Animal, n = 5; limb, n = 10. There are significant differences at $p < 0.01$ between 9–15 weeks and 3–9 weeks, and at $p < 0.05$ between 3–9 weeks and 3–15 weeks.

vertically. Imayama *et al* [11] considered that deformation in elastic fibers occurs at the time of tissue cutting and developed a new method to cut tissue after fixation by perfusion of blood vessels with resin. In an animal experiment using this method, the arrangement of elastic fiber was shown to be initially linear but to curve with age [11]. Imayama *et al* suggested that these changes cause a decrease in skin elasticity, resulting in wrinkles [12]. Based on these results, they suggested the importance of elastic fibers in the early stage of wrinkle formation and speculated the following mechanism. Elastic fibers are straight at first but curve with age. With growth, collagen fibers become predominant and push elastic fibers aside. Because collagen fibers obstruct regeneration of elastic fibers, the latter cannot form linearly.

Recently, we demonstrated that continuous UVB irradiation elicited a similar decrease in linearity of elastic fibers that was accompanied by a simultaneous decrease in skin elasticity [12]. These studies suggested that the deformation of elastic fibers may be responsible for the reduction in skin elasticity, because the tight fit of the skin is associated with the ability of elastic fibers to resume a short, straight configuration after being stretched [11]. Thus, tortuousness of elastic fibers may be a good indicator of the damage in dermal matrix proteins that leads to the loss of elasticity.

In this study, we observed a decrease in the linearity of elastic fibers after continuous exposure to UVB and UVA, and a more marked decrease in this linearity after UV irradiation during the growth period than after the growth period. These structural changes were irreversible, and no recovery was observed even after discontinuation of UV irradiation. These findings, together with those of our previous studies, suggest that exposure to UV during the growth period predisposes skin to the decline in skin elasticity as a long-term effect.

There are several possible mechanisms responsible for loss of linearity in elastic fibers due to UV. One hypothesis involves degradation of elastin by UV irradiation and subsequent interference of linear formation of elastic fibers at the time of reconstruction by extracellular matrix components, especially collagen fibers. The other is that fibroblasts originally have the ability to form elastic fibers linearly, but their function is impaired by UV irradiation. The first hypothesis *may be* supported by the following findings. Elastic fibers are known to be degraded by elastase produced by inflammatory cells [17,18] and fibroblasts [19,20]. Considerable destruction of elastic fibers occurs with inflammation in emphysema [21]. Elastase activity in the UVB-exposed skin of hairless mice is documented to increase significantly after 6 weeks of UV exposures [22]. Increased elastase activity in actinic elastosis has also been reported [23]. It is well known that the exposure of

keratinocytes in culture [24] or the skin *in vivo* [25,26] to UV irradiation induces a marked release of several cytokines including interleukin-1 (IL-1) and tumor necrosis factor-α. The UV-inducible IL-1 has also been elucidated to stimulate strongly the synthesis of elastase by normal human fibroblast [27]. Although direct degradation of elastic fibers by UV was also reported in an *in vitro* study [28], it is likely that UV irradiation enhances the production of elastase in dermal fibroblasts through paracrine or autocrine linkage of IL-1 derived from keratinocytes or fibroblasts, resulting in the degradation of elastic fibers. On the other hand, recent studies have shown an increase in elastin synthesis after UV radiation§ and in elastin mRNA *in situ* and culture in chronic photodamaged skin¶ and also up-regulation of the elastin mRNA level by IL-1β [29] and its down-regulation by tumor necrosis factor-α [30]. Elastin synthesis has also been reported to be enhanced by elastin degradation products [31]. Thus, it is conceivable that the synthesis and degradation of elastin are regulated by some cytokines and degradation products. Elastic fibers may be degraded by UV irradiation and then reconstructed and, at the time of reconstruction, their linearity may be impaired. Alternatively, fibroblasts may form elastic fibers linearly. Based on this hypothesis, fibroblasts should sense a certain direction, suggesting the presence of mechanical receptors. Fibroblasts adhere to elastic fibers *via* integrin or elastonectin [32] and sense external force *via* these molecules [33]; these molecules might be damaged by UV irradiation.

The present study revealed that there is a more marked decrease in the linearity of elastic fibers after UV irradiation during than after the growth period. The mechanism of age-dependent vulnerability of the three-dimensional network of elastic fibers to disruption by UV irradiation is presently unknown. This may be associated with more active degradation and synthesis of elastic fibers during the growth period. Other studies have shown higher susceptibility to degradation in elastic fibers [34], more active elastin synthesis [35], and higher elastase activity in cells during than after the growth period [36]. Thus, more active degradation and synthesis after UV irradiation may be responsible for more marked impairment in their linearity during the growth period. In agreement with this, the minimal erythema dose to UV has been shown to be lower during than after the growth period [37].

Through attention to environmental changes such as destruction of the ozone layer by freon gas, skin damage due to UV has attracted attention regarding light-associated aging and cancer. A previous study suggested that the majority of human UV exposure occurs before the age of 18 years [38]. Therefore, investigation of the effects of UV exposure during the growth period on the dermis is very important. Our study showed that exposure to UV during the growth period has irreversible effects on the three-dimensional structure of dermal elastic fibers. This suggests the importance of protection from UV during the growth period.

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