Selective Inhibition of Skin Fibroblast Elastase Elicits a Concentration-Dependent Prevention of Ultraviolet B-Induced Wrinkle Formation

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We previously reported that wrinkle formation in the skin following long-term ultraviolet B irradiation is accompanied by decreases in skin elasticity and the curling of elastic fibers in the dermis. We further showed that wrinkles could be repaired by treatment with retinoic acid and that this was concomitant with the recovery of skin elasticity ascribed to the repair of damaged elastic fibers. Those studies suggested that decreasing the tortuosity of dermal elastic fibers is an important factor involved in inhibiting or repairing wrinkle formation. Therefore, it is of particular interest to determine whether the inhibition of elastase activity *in vivo* would prevent the damage of dermal elastic fibers and might abolish wrinkle formation associated with the loss of skin elasticity. Because the major elastase in the skin under noninflammatory conditions is skin fibroblast elastase, we used a specific inhibitor of that enzyme to assess its biologic role in wrinkle formation. The hind limb skins of Sprague–Dawley rats were irradiated with ultraviolet B at a suberythemal dose three times a week for 6 wk. During that period, 0.1–10.0 mM *N*-phenetylphosphonyl-leucyl-tryptophane, an inhibitor of skin fibroblast elastase, was applied topically five times a week. *N*-phenetylphosphonyl-leucyl-tryptophane application at concentrations of 0.1–1.0 mM abolished wrinkle formation in a concentration-dependent manner, with a peak for inhibition at 1.0 mM. This inhibition was accompanied by a continued low tortuosity of dermal elastic fibers and a maintenance of skin elasticity. Measurement of elastase activity after 6 wk of ultraviolet B irradiation demonstrated that whereas phosphoramidon-sensitive elastase activity was significantly enhanced in the ultraviolet B-exposed skin, there was no significant increase in that activity in the ultraviolet B-exposed, *N*-phenetylphosphonyl-leucyl-tryptophane-treated skin. These findings suggest that skin fibroblast elastase plays an essential part in the degeneration and/or tortuosity of elastic fibers induced by cumulative ultraviolet B irradiation. ***Key words: elastase inhibitor/elastic fibers/elasticity/photoaging/three-dimensional structure. J Invest Dermatol 117:671–677, 2001***

We recently reported that a decrease in the linearity of elastic fibers induced by ultraviolet (UV) B irradiation is an important regulatory factor reducing skin elasticity during wrinkle formation (Imokawa *et al*, 1995). The important role of the elastic fiber network in sustaining skin elasticity, the reduction of which is an essential factor in the formation of wrinkles, was corroborated by a subsequent paper that showed treatment with all-*trans* retinoic acid (Tsukahara *et al*, 1999) or with a CO_2 laser (Tsukahara *et al*, 2001) elicited the repair of wrinkles, which was accompanied by a recovery in the linearity of dermal elastic fibers responsible for maintaining skin elasticity.

Elastic fibers have been documented to be degraded by several types of proteases, such as serine protease (Werb *et al*, 1982; Lammers *et al*, 1986; Godeau and Hornebeck, 1988; Kafienah *et al*, 1998) derived from inflammatory infiltrating cells, MMP-12 (Shipley *et al*, 1996; Mecham *et al*, 1997) derived from macrophages, and skin fibroblast elastase produced by fibroblasts (Szendri *et al*, 1984; Schwartz *et al*, 1986). Other studies have shown an elevated elastase activity in skin with actinic elastosis elicited by long-term UVB irradiation (Schwartz *et al*, 1988). Among the types of elastase that are found in the skin, neutrophil elastase and skin fibroblast elastase have generally been reported (Godeau and Hornebeck, 1988), and the activity of skin fibroblast elastase increased after repeated fibroblast subculture (Homsy *et al*, 1988). Such evidence suggests a close involvement of skin fibroblast elastase in photoaging and chronologic aging. Elevated levels of IL-1β expression in aging human fibroblasts (Kumar *et al*, 1992), stimulation of human fibroblast elastase activity by IL-1β (Croute *et al*, 1991), and inhibition of elastin synthesis by IL-1β (Mauviel *et al*, 1993) have been reported. In mid-dermal elastolysis, which is characterized by the appearance of fine wrinkles in nonsun-exposed areas, fibroblast elastase activity in affected areas was upregulated in the absence of UVB irradiation, and elastic tissue in the dermal middle layer completely disappeared (Fimiani *et al*, 1995). This evidence allows us to speculate that the secretion and activation of

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Abbreviations: UVB, ultraviolet radiation in wavelength ranging 290–320 nm; NPLT, *N*-phenetylphosphonyl-leucyl-tryptophane.

skin fibroblast elastase by dermal fibroblasts responding to UVB irradiation and/or to cytokines released by UVB-exposed keratinocytes are responsible for the degeneration of the three-dimensional structure of elastic fibers during the formation of wrinkles. In considering the relationship between the degeneration of elastic fibers and wrinkle formation due to decreases in skin elasticity, it would be interesting to determine if inhibiting elastases during long-term UVB irradiation could prevent wrinkle formation in the skin by preventing the degeneration of the elastic fiber network. As there is no infiltration of inflammatory cells in the skin during wrinkle formation induced by UVB irradiation (Moloney and Learn, 1992), and thus no indication of neutrophil elastase involvement, we focused our attention on skin fibroblast elastase as the enzyme associated with wrinkle formation and we synthesized an inhibitor of that elastase to characterize its potential inhibitory effect on wrinkle formation.

MATERIALS AND METHODS

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

UV radiation source Rats were placed in cages individually and were irradiated by a bank of five Toshiba SE lamps (UVB) without any filtering, three times a week for a total of 6 wk. The distance from the lamp to the animal's hind limbs was 42 cm (irradiance was approximately 0.72 mW per cm^2), and a dose of 130 mJ per cm^2 (rat 1 suberythemal dose = 170 mJ per cm^2) was given three times weekly. The energy output of the lamps was measured with a Topcon (Tokyo, Japan) UVB radiometer 305/365DII, and their spectral irradiance was measured with an Optical Science (Tokyo, Japan) MSR7000 Radiospectrometer. The spectral output is shown as previously reported (Imokawa *et al*, 1995). Briefly, the relative spectral contributions of Toshiba SE lamps used in this study were 63, 36, and 0.5% in the UVB (290–320 nm), UVA (320–400 nm), and UVC (< 290 nm) regions, respectively.

Measurement of elastase activity Elastase activity in the hind limb skin was measured 6 wk after irradiation of UVB. Enzyme activity was measured in the hind limb skin by the method of Nakagawa *et al* (1987). Briefly, the entire skins of both rat hind limbs were detached, washed twice with phosphate-buffered saline and placed with the dermis downward on ice in Petri dishes containing phosphate-buffered saline. The subcutaneous tissue was removed, and blood was washed out. The skin tissue was punched at four sites for each hind limb skin using a biopsy trepan (caliber, 4 mm, Kai Industries, Gifu, Japan). The eight skin specimens punched out from the hind limb skins of each rat were placed in a tube (1.5 ml), mixed with 1 ml lysis buffer (0.1% TritonX-100/0.2 M Tris–HCl [pH 8.0]), and sonicated using a Hitachi HG 30 Homogenizer and an Ultrasonic processor, and then centrifuged (Himac CR 15D Hitachi, Hitachi, Tokyo, Japan) (350 × *g*) at 4°C for 20 min. The supernatant was used as the source of enzyme to be measured. Each enzyme solution (100 μl) was preincubated at (37°C) for 15 min in the presence or absence of 1 μM phosphoramidon as a metalloprotease inhibitor (Aoyagi and Umezawa, 1975). *N*-succinyl-tri-alanyl-p-nitroanilide (Peptide Institute, Osaka, Japan) (62.5 mM) was used as a substrate, and 2 μl of the substrate solubilized in DMSO was incubated with 100 μl enzyme solution at 37°C for 2 h. The amount of released nitroaniline was measured by determining the absorbance at 410 nm using a spectrophotometer (Model 450 Microplate Reader, Bio-Rad Laboratories, CA). Elastase activity was expressed as unit per h per mg, 1 unit representing the activity for the release of 5×10^{-3} pmol nitroaniline per hour.

Elastase inhibitor Phosphoramidon, a metalloprotease inhibitor, penetrates poorly through the skin because of the hydrophilic rhamnose residue in its chemical structure. Among the various derivatives of phosphoramidon synthesized in our study, *N*-phenetylphosphonyl-leucyl-tryptophane (NPLT) (in which the rhamnose residue is replaced by a lipophilic phenetyl residue) (**Fig 1**) was found to inhibit normal fibroblast-derived elastase specifically (Dainippon Pharmaceutical, Osaka Japan), measured using *N*-succinyl-tri-alanyl-p-nitroanilide (Peptide Institute Inc.) as a substrate, according to the method of Nakagawa *et al* (1987). Thus, NPLT (10 μM) was able to inhibit fibroblast-derived elastase activity by 90.8% with an IC_{50} of 50 nM, a level similar to the

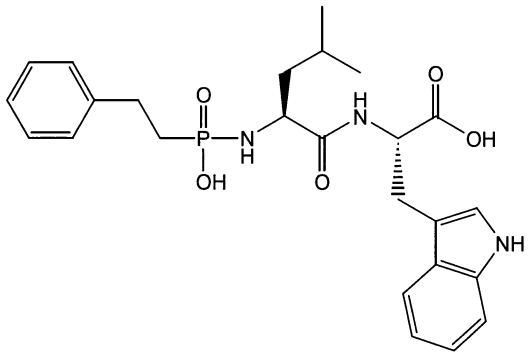

Figure 1. The structure of NPLT, a synthetic compound that specifically inhibits fibroblast-derived elastase. NPLT was synthesized by substituting the rhamnose area of phosphoramidon with a phenetyl group, as described in the *Materials and Methods*.

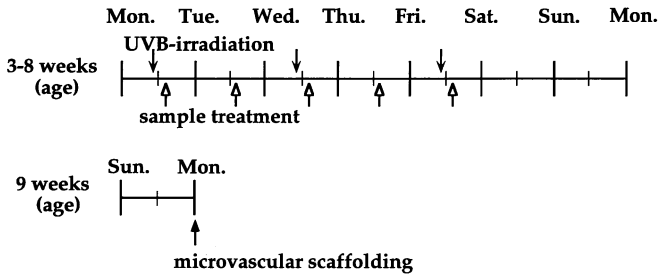

Figure 2. Procedures for UVB irradiation and sample treatment.

IC_{50} of phosphoramidon. In contrast, NPLT (at 10 μM) inhibited neutrophil elastase activity by only 1.4%, collagenase I activity by 9.3%, and collagenase IV by 3.6%.[1] As a spectrophotometric feature, there is a negligible absorption of NPLT in the UVB wavelength range (290–320 nm) whereas there is a marked absorption of octyl methoxycinnamate (Parsol-MCX, p-MCX; Hoffman Laroche, Givaudan, NJ), a typical UVB absorbent (Harrison *et al*, 1991) at that wavelength range.

Sample treatment Rats were divided into eight groups (five rats each): five of the groups (treated groups) were exposed to UVB light, followed by topical applications of NPLT (in 80% EtOH) at doses of 0.1, 0.5, 1.0, 5.0, or 10.0 mM. Two other groups (UVB control groups) were exposed to UVB light as above, followed by treatment with 80% EtOH alone or with 10 mM p-MCX in 80% EtOH. The final group was used as a control group that was not exposed to UVB light or treated topically with any materials. Samples were applied in 10 μl to the unilateral hind limb skin at the same time each day five times a week for 6 wk, 1 h or 24 h after UVB irradiation. A summary of the experimental protocol is shown in **Fig 2**.

Wrinkle scoring and image analysis Wrinkles in the rat hind limb skin were then assessed according to the scoring system of Bissett *et al* (1987) (grade 0, no coarse wrinkles; grade 1, a few shallow coarse wrinkles; grade 2, some coarse wrinkles; grade 3, several deep coarse wrinkles). A photograph of each rat hind limb was taken, using a Minolta α707si with a macro100 lens system. Replicas were obtained using Exafine hydrophilic vinyl silicon impression material (GC, Tokyo, Japan) and were processed so that their backs became flat, and image analysis of wrinkles was performed on a 5 × 10 mm area by our previously reported method (Takema *et al*, 1996a) using a PIAS LA-555 personal image analysis system (PIAS, Osaka, Japan). The percentage of area of wrinkles in the image analysis area was then calculated as previously described (Imokawa and Takema, 1993).

Skin elasticity The skin elasticity was measured with a Cutometer Skin Elasticity Meter 575 (Courage$^+$ Khazaka, Cologne, Germany) just before the animals were killed, as detailed in previous papers (Imayama *et al*, 1994; Takema *et al*, 1994). This instrument measures the elastic

properties of skin, based on the principle of suction elongation, using an optical measuring unit described by Elsner *et al* (1990). Briefly, the time/strain mode was used with application of a 500 mbar load for 3 s followed by 3 s of relaxation. The skin deformation was then plotted as a function of time. The parameters used were immediate distension (Ue), measured at 0.1 s, delayed distension (Uv), immediate retraction (Ur), and final distension (Uf), as described by Agache *et al* (1980).

SEM observation All procedures were carried out to preserve the three-dimensional arrangements of elastic fibers by virtue of the microvascular scaffolding. The technique employed in this study was identical to that described previously (Imayama *et al*, 1994; Imokawa *et al*, 1995). Briefly, animals were anesthetized and a cannula was inserted into their abdominal aortas. The hind limbs were flushed with Ringer's solution and were then injected with Mercox resin (Dainihon, Tokyo, Japan). After the resin had polymerized, the limbs were fixed in 4% paraformaldehyde and incubated in 88% formic acid. After incubation, specimens were washed and postfixed in 2% osmium tetroxide, dehydrated in graded ethanols, and dried by the freeze-dry method. Specimens were then mounted, sputter-coated with gold, and examined using a Hitachi Model S-4000 field emission scanning electron microscope (SEM) at 5 kV.

Elastic fiber linearity Elastic fiber linearity was quantified at a magnification of $\times 1000$ using a personal image analyzer system, as detailed previously (Imayama *et al*, 1994; Imokawa *et al*, 1995). Briefly, the length and width of the minimum rectangle enclosing one fragmented line of an elastic fiber, automatically calculated by the computer, were designated as C and B, respectively, and the area of the fragmented elastic fiber as A. The linearity of each fragmented elastic fiber is expressed as $A/(B \times C)$, and for example, in a straight fragmented elastic fiber, the linearity is 1.

Histology For light microscopy, skin specimens obtained from rat hind limbs were fixed with formalin and embedded in paraffin. Sections of the specimens were stained with hematoxylin and eosin and Luna.

Statistics Results are expressed as mean ± SD. Differences between means were checked for significance using Student's t test.

RESULTS

Inhibition of elastase activity prevents wrinkle formation following UVB irradiation When NPLT was applied topically for 6 wk at various concentrations on rat hind limb skin, 1 h or 24 h after each UVB exposure, the formation of wrinkles (evaluated by visual score based on corresponding photographs) was significantly suppressed (**Figs 3 and 4**). Inhibition occurred in a concentration-dependent manner at concentrations greater than 0.1 mM compared with controls. This suppressive effect reached a plateau at concentrations greater than 1.0 mM. In contrast, a similar treatment with a UVB sunscreen (p-MCX) at a concentration of 10 mM did not elicit any suppressive effect on wrinkle formation, indicating no involvement of sunscreen effects in that process. When assessed by image analysis of replicas, the NPLT treatment significantly decreased the formation of wrinkles at concentrations greater than 0.5 mM, reaching a plateau at concentrations greater than 1 mM (**Figs 5 and 6**), whereas again, the UVB sunscreen p-MCX had no suppressive effect.

There was no visible infiltration of any inflammatory cells, including neutrophils (data not shown) as assessed by histochemistry with hematoxylin and eosin or Luna staining. Actinic elastosis was not seen in any of the UVB-irradiated skins, which was consistent with a previous report (Tsukahara *et al*, 1999).

The elastase inhibitor NPLT prevents a reduction in skin elasticity following UVB irradiation The results of measurements of skin elasticity using a Cutometer are shown in **Table I**. Whereas 6 wk of UVB irradiation elicited marked decreases in skin elasticity (expressed as the parameters, Ue, Uf, Ur, and Uv) 6 wk of treatment with NPLT during those exposures abolished those decreases in skin elasticity at concentrations greater than 0.1 mM (for Ue, Uf, and Ur) or 1.0 mM (for Uv). In contrast, a similar treatment with the UVB sunscreen, p-MCX, at a concentration of 10 mM did not prevent the decrease in skin elasticity.

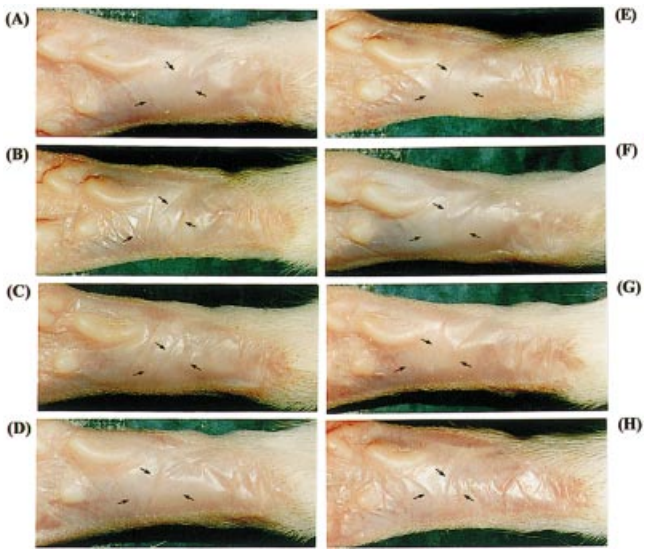

Figure 3. Close-up photos after sample application (five times weekly for 6 wk) during the period of UVB irradiation (three times weekly for 6 wk) on rat hind limb skin. (*A*) Unirradiated and untreated group. (*B*) Irradiated and ethanol treated group. (*C*) Irradiated and 0.1 mM NPLT-treated group. (*D*) Irradiated and 0.5 mM NPLT-treated group. (*E*) Irradiated and 1.0 mM NPLT-treated group. (*F*) Irradiated and 5.0 mM NPLT-treated group. (*G*) Irradiated and 10.0 mM NPLT-treated group. (*H*) Irradiated and 10 mM p-MCX treated group. *Arrows* represent areas where wrinkles appear.

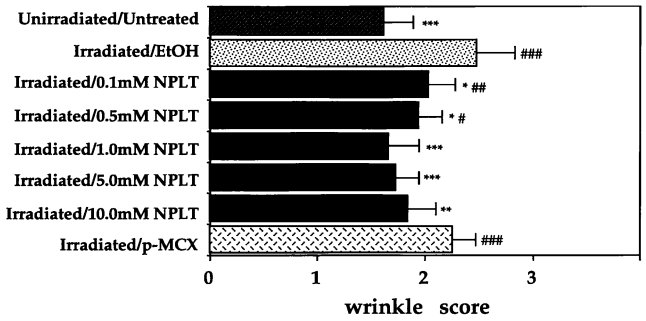

Figure 4. Visual scoring (0–3) of wrinkles. The scoring was performed by the method of Bissett *et al* (1987) after sample application (five times weekly for 6 wk) during the period of UVB irradiation (three times weekly for 6 wk) on rat hind limb skin. Each bar represents the mean ± SD (n = 10). ***p <0.005, **p <0.01, *p <0.05 (*vs* irradiated/EtOH). ###p <0.005, ##p <0.01, #p <0.05 (*vs* unirradiated/untreated).

The elastase inhibitor NPLT prevents the disruption of the three-dimensional structure of elastic fibers induced by UVB irradiation Whereas 6 wk of UVB irradiation elicited marked disruption of the three-dimensional structure of elastic fibers, treatment with NPLT following those UVB exposures over the 6 wk obviously abolished that disruption of elastic fibers at concentrations greater than 0.1 mM (**Fig 7**). In contrast, a similar treatment with the UVB sunscreen (p-MCX) at a concentration of 10 mM did not elicit any preventive effect on the disruption of elastic fibers. Quantitative measurement by image analysis of the disruption of elastic fibers based upon elastic fiber linearity revealed that, whereas 6 wk of UVB irradiation induced a distinct decrease in elastic fibers with a high linearity, 6 wk of treatment with NPLT following the UVB exposure significantly abolished that decrease in a concentration-dependent manner at concentrations greater than

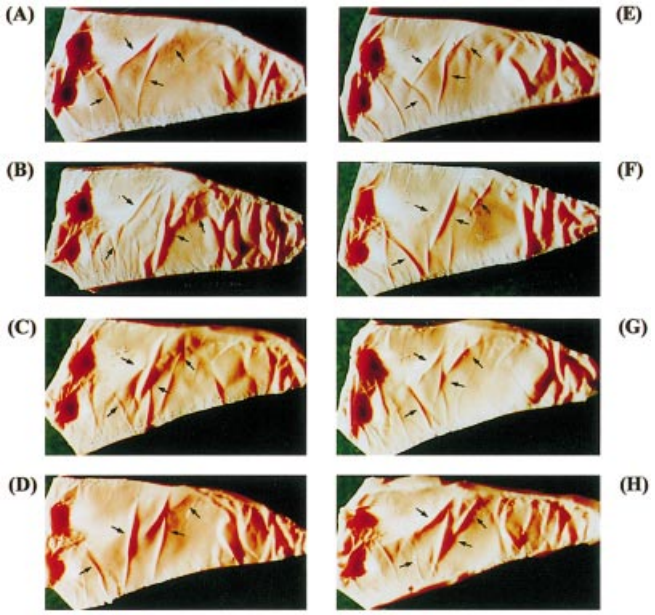

Figure 5. Replica photos obtained after sample application (5 times weekly for 6 wk) during the period of UVB irradiation on rat hind limb skin (3 times weekly for 6 wk). (*A*) Unirradiated and untreated group. (*B*) Irradiated and ethanol-treated group. (*C*) Irradiated and 0.1 mM NPLT-treated group. (D) Irradiated and 0.5 mM NPLT-treated group. (*E*) Irradiated and 1.0 mM NPLT-treated group. (*F*) Irradiated and 5.0 mM NPLT-treated group. (*G*) Irradiated and 10.0 mM NPLT-treated group. (*H*) Irradiated and 10 mM p-MCX-treated group. *Arrows* represent areas where wrinkles appear on each replica.

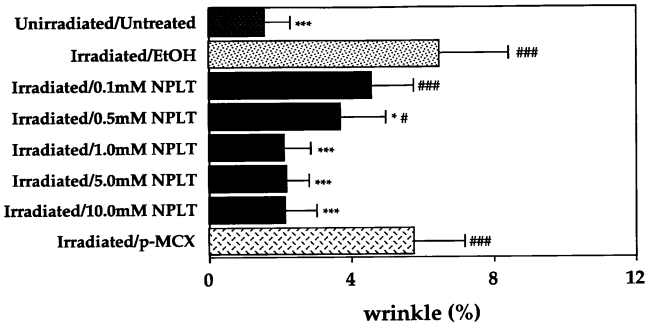

Figure 6. Results of analysis of replica images after sample application (five times weekly for 6 wk) during the period of UVB irradiation (three times weekly for 6 wk) on rat hind limb skin. An area of 5 × 10 mm was analyzed, and the percentage of the wrinkle area to the analysis area was calculated. Each bar represents the mean ± SD (n = 10). ***p < 0.005, *p < 0.05 (*vs* irradiated/EtOH). ###p < 0.005, #p < 0.05 (*vs* unirradiated/untreated).

0.1 mM (**Fig 8**). In contrast, a similar treatment with the UVB sunscreen (p-MCX) at a concentration of 10 mM did not elicit any preventive effect on the decrease in elastic fibers with high linearity.

UVB irradiation stimulates elastase activity and NPLT abolishes stimulation Exposure of rat hind limb skin to UVB light for 6 wk at a dose of 130 mJ per cm^2 significantly stimulated elastase activity in the exposed skin (**Fig 9**). Concomitant application of NPLT at a concentration of 10 mM completely abolished that stimulation. When treated additionally with phosphoramidon at a concentration of 1 μM, the elastase

Table I. Effect of topical application with NPLT on elasticity of UVB-irradiated rat hind limb skin[a]

Physical parameters	Unirradiated /Untreated	Irradiated /EtOH	Irradiated /0.1mM NPLT	Irradiated /0.5mM NPLT	Irradiated/ 1.0mM NPLT	Irradiated/ 5.0mM NPLT	Irradiated/ 10.0mM NPLT	Irradiated/ p-M
Ue	0.040 ± 0.01***	0.019 ± 0.01###	0.025 ± 0.01###*	0.029 ± 0.01***	0.041 ± 0.01***	0.038 ± 0.01***	0.035 ± 0.01***	0.024 ± 0.00##*
Uf	0.059 ± 0.01***	0.032 ± 0.01###	0.041 ± 0.01###**	0.047 ± 0.01###***	0.056 ± 0.01***	0.053 ± 0.01***	0.050 ± 0.01***	0.033 ± 0.00###
Ur	0.039 ± 0.01***	0.017 ± 0.01###	0.024 ± 0.00###*	0.030 ± 0.01###***	0.040 ± 0.01***	0.033 ± 0.01***	0.032 ± 0.01**	0.018 ± 0.01####
Uv	0.023 ± 0.00***	0.040 ± 0.00####	0.040 ± 0.00####	0.040 ± 0.00####	0.040 ± 0.00*	0.040 ± 0.00***	0.040 ± 0.01*	0.040 ± 0.00####

[a]The skin elasticity expressed as physical parameters, Ue, Uv, Ur and Uf was measured with a Cutometer. This measurement was carried out at age of 9 wk.
***, **, *: p<0.005, 0.01, 0.05 (vs Irradiated/EtOH treatment) ###, ##, #: p<0.005, 0.01, 0.05 (vs Unirradiated/Untreated). Ue, immediate distension measured at 0.1s; Uv, delayed distension; Ur, immediate retraction; Uf, final distension. n = 10, each value represents mean ± SD.

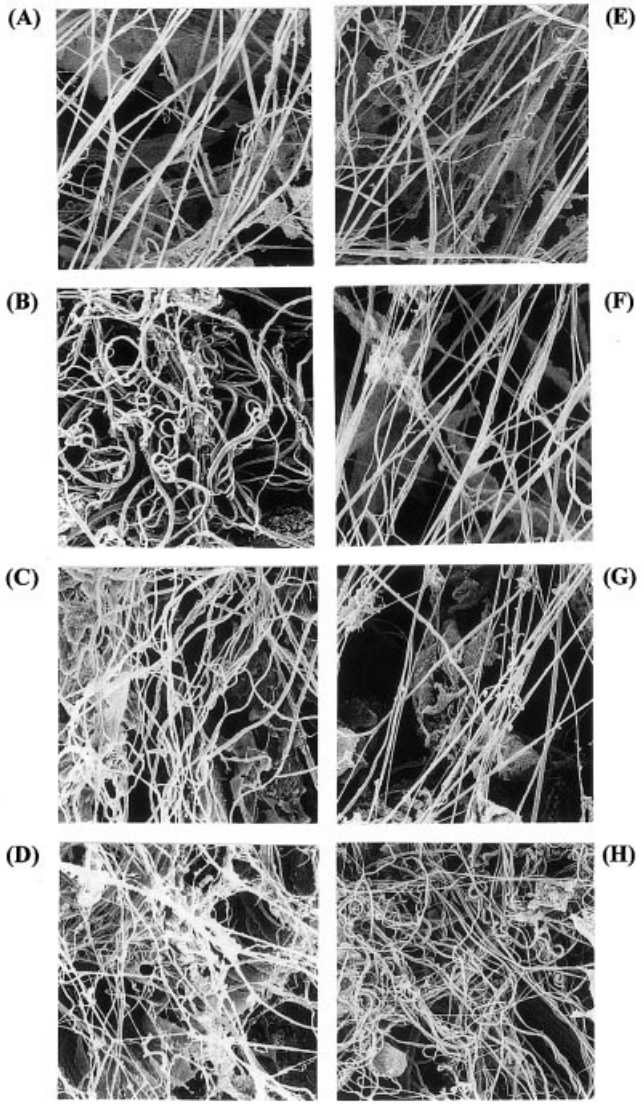

Figure 7 SEM micrographs after sample treatment followed by intravascular injection and selective digestion at 9 wk. (*A*) Unirradiated and untreated group. (*B*) Irradiated and ethanol-treated group. (*C*) Irradiated and 0.1 mM NPLT-treated group. (*D*) Irradiated and 0.5 mM NPLT-treated group. (*E*) Irradiated and 1.0 mM NPLT-treated group. (*F*) Irradiated and 5.0 mM NPLT-treated group. (*G*) Irradiated and 10.0 mM NPLT-treated group. (*H*) Irradiated and 10 mM p-MCX treated group.

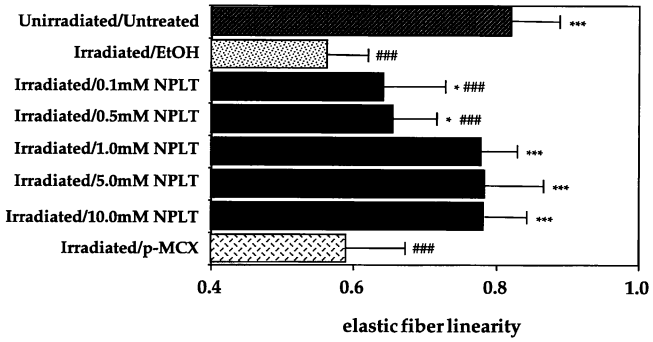

Figure 8. Changes in the linearity of dermal elastic fibers after sample application, measured by image analysis. Fibers were characterized on SEM micrographs. Each bar represents the mean SD from 20 SEM microphotographs. Total fragmented line: n = 320 (16 fragmented lines × 20 microphotographs); Animal, n = 5; limb, n = 10 ***p < 0.005, *p < 0.05 (*vs* irradiated/EtOH). ###p < 0.005 (*vs* unirradiated/untreated).

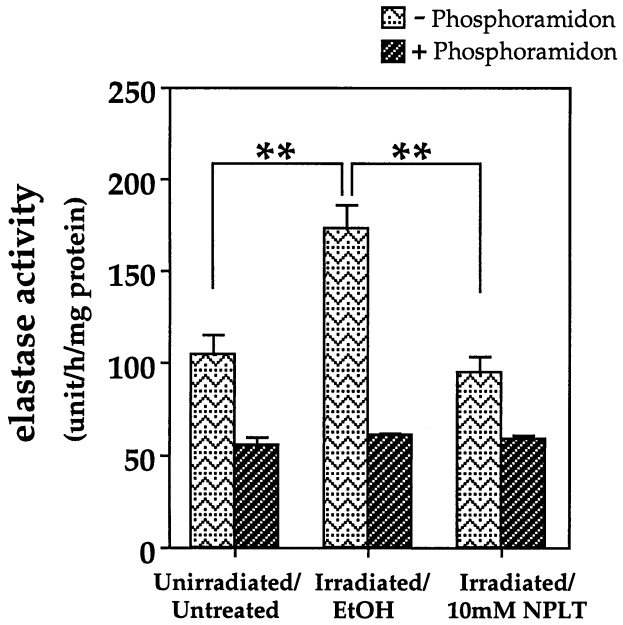

Figure 9. UVB irradiation stimulates elastase activity and NPLT abolishes that stimulation in UVB-exposed skin. Rat hind limb skins were exposed three times weekly for 6 wk, with or without subsequent applications of NPLT. Plus phosphoramidon represents the addition of phosphoramidon to each skin sample to eliminate existing metalloprotease activity. Each bar represents the mean ± SD (n = 3). **p < 0.01.

activities of unirradiated/untreated skin, irradiated/EtOH-treated skin and irradiated/NPLT-treated skin were diminished to similar levels, thus indicating that UVB stimulation of elastase activity is primarily responsible for phosphoramidon-sensitive metalloproteases.

DISCUSSION

In this study, the topical application of NPLT, which specifically inhibits skin fibroblast elastase,[1] resulted in a concentration-dependent inhibition of wrinkle formation in rat hind limb skin. This effect was accompanied by prevention of the decrease in skin elasticity and of degeneration in the three-dimensional structure of elastic fibers to levels observed in non-UVB-exposed controls. The induction of wrinkling by UVB irradiation and its inhibition by NPLT was associated with a significant upregulation of elastase activity in the UVB-exposed skin and the significant inhibition of that activity when the UVB-exposed skin was treated with the elastase inhibitor. The possibility that the effect of NPLT inhibiting wrinkle formation elicited by UVB irradiation might result from sunscreen effects (even though there is no absorption by NPLT in the UVB wavelength range[1]) was ruled out by data showing that a typical UVB sunscreen, p-MCX (Harrison *et al*, 1991), applied in the same manner had no similar inhibitory effect on wrinkle formation.

After intravascular resin injection and selective digestion with formic acid, Imayama and Braverman (1988, 1989) and Imayama *et al* (1994) used SEM to observe the three-dimensional structures of elastic fibers during aging. They found linear structures in young rats but curling structures with age, suggesting that the linearity of elastic fibers is necessary for maintaining skin elasticity. Using that same method, it has been previously reported that UVB irradiation

at a suberythemal dose induced degeneration of dermal elastic fibers (which consist mainly of curling elastic fibers), which resulted in decreased skin elasticity at an early phase and in turn led to wrinkle formation (Imokawa *et al*, 1995). In addition, the direct relevance of disrupted microstructures of elastic fibers to wrinkle formation was also corroborated by a recent study in which the application of all-*trans* retinoic acid (Tsukahara *et al*, 1999) or CO_2 laser treatment (Tsukahara *et al*, 2001) repaired wrinkles elicited by UVB irradiation. This correlated with the recovery in linearity of elastic fibers, which was in turn accompanied by a concomitant recovery of skin elasticity.

We previously proposed several possible mechanisms that might be involved in the curling of dermal elastic fibers after long-term UVB irradiation at a suberythemal dose (Imokawa *et al*, 1995). One possibility is that elastic fibers are degraded by elastases that are secreted by surrounding cells or that are induced directly in fibroblasts by UVB irradiation, which would result in the curling and/or reproduction of elastic fibers. Another possibility is that the reproduction of elastic fibers that occurs after the digestion of elastic fibers is prevented by existing collagen fibers, leading to a similar tortuosity of newly generated elastic fibers (Imayama *et al*, 1994). Yet another possibility is that the ability of fibroblasts themselves to pull elastic fibers straight is weakened by UVB irradiation, which would result in the curling of elastic fibers (this mechanism would reflect no involvement of elastase). In our present study, the linearity of elastic fibers was substantially sustained by the topical application of NPLT, a specific inhibitor of skin fibroblast elastase, at concentrations greater than 1 mM. This process occurred in a concentration-dependent manner and at 1 mM approached levels of non-UVB exposed controls. It should be noted that there was no substantial change in content of elastin fibers in Luna-stained section of UVB-exposed or the inhibitor-treated skin (data not shown). Furthermore, the formation or the prevention of wrinkling were also accompanied by the significant upregulation or suppression of elastase activity, respectively, in the treated skin. The sum of these data suggest that the loss of linearity, or the induction of tortuosity, of elastic fibers is associated with elastase activity, which is markedly enhanced in UVB-exposed skin. Thus, the first possibility listed above of the biologic mechanism involved in wrinkle formation is most likely.

The NPLT derivative used in this study inhibited fibroblast-derived elastase quite efficiently but did not inhibit neutrophil elastase.[1] Some studies have shown degradation of elastic fibers by neutrophil elastase (Werb *et al*, 1982; Lammers *et al*, 1986; Godeau and Hornebeck, 1988; Kafienah *et al*, 1998), and degradation of elastic fibers by neutrophil elastase was actually higher than by skin fibroblast elastase (Godeau and Hornebeck, 1988). Therefore, if neutrophil elastase is activated by UVB light, it could damage the three-dimensional structure of dermal elastic fibers; however, the UVB irradiation dose used in this study was less than 1 minimal erythemal dose and no infiltrating cells were observed in any of the UVB-exposed skin samples. Consistently, the assay of elastase activity in this study demonstrated that the majority of elastase stimulated by UVB irradiation was a phophoroamidon-sensitive metalloprotease. Taken together, it is suggested that the contribution of neutrophil elastase to that process may be negligible under these conditions. Experimental studies on animals have shown decreases in insoluble collagen and total collagen in hairless mice after continuous UVB irradiation (at 20 mW per cm^2) (Alpermann and Vogel, 1978). Conversely, no change of total collagen in hairless mice after UVB irradiation (less than 1 minimal erythemal dose) has been reported (Chatterjee *et al*, 1990; Moloney *et al*, 1992; Trautinger *et al*, 1994; Takema *et al*, 1996b; Neocleous *et al*, 1997; Starcher *et al*, 1999). There have been studies on the effects of UVB irradiation on collagen fibers, which showed a decrease in the amount of total collagen in human skin (posterior neck) exposed chronically to UVB compared with nonexposed areas (Schwartz *et al*, 1993), or no changes in collagen synthesis *in vivo* after short UVB treatment of human abdominal skin (Autio *et al*, 1994). Thus, as the sum of evidence has demonstrated that there are no substantial changes in the amounts of total collagen following UVB irradiation at less than 1 minimal erythemal dose in animals, it is conceivable that there is no relationship between quantitative changes in collagen fibers and wrinkle formation following UVB irradiation. Based upon these lines of evidence, we hypothesize that changes in collagen fibers during wrinkle formation may not be a direct cause of wrinkle formation but rather may be a phenomenon secondary to the structural damage of elastic fibers due to the activation of fibroblast elastase. This in turn suggests that the inhibition of skin fibroblast elastase leads to an inhibition of wrinkle formation as a result of the maintenance of skin elasticity by avoiding damage to the three-dimensional structure of dermal elastic fibers following UVB exposure.

In conclusion, these findings collectively suggest that skin fibroblast elastase plays an essential part in the degeneration and/or curling (tortuosity) of elastic fibers induced by cumulative UVB irradiation, which is intrinsically associated with wrinkle formation due to the reduction of skin elasticity.

[1]Imokawa G, Tsukahara K, Tsuji N, *et al*: Inhibition of fibroblast-derived elastases can prevent UVB-induced wrinkle formation. J Invest Dermatol 110:594, 1998 (Abstr.)

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