Inhibitory Effect of an Extract of *Sanguisorba officinalis* L. on Ultraviolet-B-Induced Photodamage of Rat Skin

Kazue TSUKAHARA, Shigeru MORIWAKI, Tsutomu FUJIMURA, and Yoshinori TAKEMA*

Biological Science Laboratories, Kao Corporation, 2606 Akabane, Ichikai, Haga, Tochigi 321–3497, Japan.
Received May 9, 2001; accepted June 27, 2001

We previously reported that chronic Ultraviolet-B (UVB) irradiation causes wrinkle formation, decreases skin elasticity, and damages/curls dermal elastic fibers. Those UVB-induced wrinkles can be improved by treatment with retinoic acid or with a CO_2 laser which results in a recovery of skin elasticity and a repair of elastic fiber linearity. We showed further that topical application of *N*-phenetyl-leucyl-tryptophane, an agent that specifically inhibits fibroblast-derived elastase, immediately after UVB irradiation inhibited UVB-induced wrinkle formation, maintained skin elasticity, and inhibited changes in the three-dimensional structure of dermal elastic fibers in a dose-dependent manner. In this study, the effects of an extract of *Sanguisorba officinalis* L., which also inhibits fibroblast-derived elastase, was evaluated for possible inhibition of UVB induced wrinkle formation, maintenance of skin elasticity, and prevention of damage to the 3-dimensional structure of dermal elastic fibers. Hind limb skins of 3-week-old Sprague–Dawley rats were irradiated with UVB at a suberythemal dose 3 times a week for 6 weeks. Simultaneously, an extract of *Sanguisorba officinalis* L. (at 0.2% (v/v) or 1% (v/v)) was topically applied 5 times per week immediately following each UVB irradiation and 1 d later. The extract of *Sanguisorba officinalis* L. inhibited wrinkle formation, maintained skin elasticity, and inhibited the decrease of dermal elastic fiber linearity in the rat hind limb skin in a dose-dependent manner. We have confirmed that the inhibition of elastase activity in fibroblasts immediately after UVB irradiation using an extract of *Sanguisorba officinalis* L. prevents chronic photodamage following UVB irradiation.

Key words photoaging; wrinkle; elastase; elastic fiber; *Sanguisorba officinalis* L; skin elasticity

We recently reported that short-term chronic Ultraviolet-B (UVB) irradiation of rat hind limb skin at a suberythemal dose reduces skin elasticity and decreases the linearity of dermal elastic fibers which induces wrinkle formation.[1,2] In order to clarify the 3-dimensional structure of elastic fibers after improvement of wrinkles, skin with UVB-induced wrinkles was treated topically with all-*trans* retinoic acid[3] or with a CO_2 laser,[4] which are widely used for treating wrinkles, and improvement in wrinkles and skin elasticity and recovery of dermal elastic fibers was observed. Subsequently, to evaluate the cause of elastic fiber curling, we noted that elastase is activated by UVB irradiation. Elastic fibers have been reported to be digested by serine proteinase in infiltrating cells that migrate after UVB irradiation,[5—8] by MMP-12 derived from macrophages,[9,10] and by elastase produced by fibroblasts.[11,12] Chatterjee *et al.* reported an increase in elastase activity after chronic UVB irradiation at a suberythemal dose in the skin of hairless mice.[13] Therefore, we evaluated elastase activity in rat hind limb skin after chronic UVB irradiation at a suberythemal dose and observed no increase in neutrophil elastase activity but a significant increase in phosphoramidon-sensitive metalloprotease activity.[14,15] Those findings suggested that the UVB-induced wrinkle formation and the increase in skin elasticity could be inhibited, and that curling of dermal elastic fibers could be avoided, by inhibiting the increase in metalloprotease activity. We synthesized a chemical compound (*N*-phenetyl-Leucyl-Tryptophane; NPLT) that specifically inhibits fibroblast-derived elastase which is in the metalloprotease family,[12,16] and we applied this agent topically to rat hind limb skin immediately after UVB irradiation and 1 d later. No increase in elastase activity was observed in the NPLT treated UVB irradiated skin, curling of elastic fibers did not occur, and the treated skin maintained elasticity, all of which resulted in significant decreases in wrinkle formation.[14,15]

Screening of 480 types of plant extracts showed high inhibitory activities against fibroblast-derived elastase by extracts of *Sanguisorba officinalis* L. and the *Zingiber officinale* (L.) ROSE (about 72% and 45% inhibition, respectively, at a concentration of 1% (v/v)).[17] The extract of *Sanguisorba officinalis* L. has also been reported to have inhibitory activity *in vitro* against neutrophil elastase[18] and hyaluronidase[19] and to have an anti-oxidation action.[20] However, 3-dimensional changes elicited in dermal elastic fibers after application of this extract to UVB-irradiated rat skin have never been characterized. If the extract of *Sanguisorba officinalis* L. inhibits fibroblast-derived elastase activity, even if it is not specific, this extract may be useful for maintaining the linearity of dermal elastic fibers during and after UVB irradiation. Therefore, we have now evaluated the inhibitory effects of this extract on the curling of dermal elastic fibers, on the resulting maintenance of skin elasticity and on the inhibition of wrinkle formation induced by chronic UVB irradiation of rat skin.

MATERIALS AND METHODS

Animals Male Sprague–Dawley (SD) rats, 3 weeks 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.

Radiation Source Rats were placed in cages individually and were irradiated by a bank of 5 Toshiba SE lamps (UVB) without any filtering, for a total of 6 weeks. The distance from the lamp to the animal's hind limbs was 42 cm (irradiance was approximately 0.72 mW/cm^2), and a dose of

* To whom correspondence should be addressed. e-mail: 157481@kastanet.kao.co.jp

130 mJ/cm^2 (rat 1 sub-erythemal dose=170 mJ/cm^2) was given three times weekly. The energy output of the lamps was measured with a Topcon Co., Ltd. (Tokyo, Japan) UVB radiometer 305/365DII, and their spectral irradiance was measured with an Optical Science Co., Ltd. (Tokyo, Japan) MSR7000 Radiospectrometer. The spectral output of these lamps is as previously reported.[2] Briefly, the relative spectral contributions of the 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.

Plant Extract An extract of *Sanguisorba officinalis* L. was used which has an inhibitory action on fibroblast elastase.[17] The extract used in this study was purchased from Koei Perfumery Co., Ltd. (Tokyo, Japan). This extract was obtained by occasional stirring of a mixture of the root and rhizome (200 g) of *Sanguisorba officinalis* L. (produced in Dalian, China) and 50% 1,3-butylene glycol (1200 g) for 10 d at room temperature. The extract of *Sanguisorba officinalis* L. specifically inhibited normal fibroblast derived elastase (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), measured using *N*-succinyl-tri-alanyl-*p*-nitroanilide (Peptide Institute INC., Osaka, Japan) as a substrate, according to the method of Nakagawa *et al.*[21] A 1% (v/v) solution of the *Sanguisorba officinalis* L. extract inhibited fibroblast-derived elastase activity by 72%.[17]

Sample Treatment Rats were divided into 4 groups of 5 rats each: three of the groups (the treated groups) were exposed to UVB light, followed by topical applications of the extract of *Sanguisorba officinalis* L. (in 80% EtOH) at a dose of 0.2% (v/v) or 1.0% (v/v) or with the vehicle (80% EtOH) alone. The final group was another control group which was not exposed to UVB light or treated topically with any material. Samples were applied in 10 μl to the unilateral hind limb skin at the same time each day 5 times a week for 6 weeks. On days when animals were UVB irradiated, samples were applied immediately after the irradiation. A summary of the experimental procedure is shown in Fig. 1.

Wrinkle Scoring and Image Analysis The following measurements were performed under Nembutal anesthesia. Wrinkles in the rat hind limb skin were assessed according to the scoring system of Bissett *et al.*[22] (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 camera (Minolta Co., Ltd., Tokyo, Japan) with a macro100 lens system. Skin impression replicas were made of the hind limb skin using Exafine hydrophilic vinyl polysiloxane impression material (GC Co., Ltd., Tokyo, Japan). We set the impression replicas on the sample stand so that the measurement surface was horizontal, and we produced wrinkle shadows by illumination with light of a fixed intensity at an angle of 30 degrees, using a fiber optic light source (Nikon Corp., Tokyo, Japan). The shadow images at the center of the hind limb skin (5 mm×10 mm area) were photographed with a still video camera (MS-C1100, Minolta) and a digital image recorder (MS-R1100, Minolta) with a macro 50 lens system, and were input into an image analyzer (PIAS LA-555 personal image analysis system, PIAS Corp, Osaka, Japan). Binary images were obtained by extracting shaded areas of each image at a constant gray level. We measured the shadow area for all shadows in one image, using the image analyzer, and calculated the ratio of wrinkle area (%), which is defined as the ratio of the sum of the shadow area to the measured area, as previously described.[23,24]

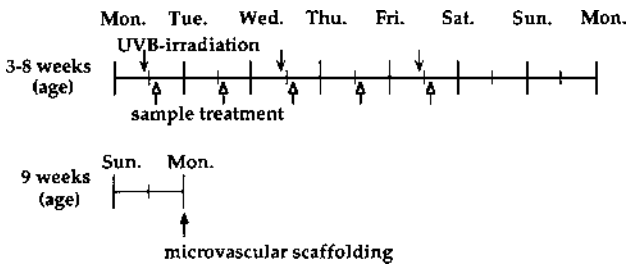

Fig. 1. Protocol Timing for UVB Irradiation and Skin Treatment

Skin Elasticity The skin elasticity was measured with a Cutometer SEM 575 (Courage$^+$ Khazaka, Cologne, Germany) under Nembutal anesthesia just before the animals were sacrificed, as detailed in our previous papers.[1,25] 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.*[26] 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.*[27]

Scanning Electron Microscope (SEM) Observations This process was carried out in the following sequence: photography, wrinkle scoring, measurement of skin elasticity and replica generation. 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.[1,2] Briefly, each animal was anesthetized and perfused through a cannula inserted into its abdominal aorta. The hind limbs were flushed with saline to remove the blood and were then injected with 10 ml of Mercox resin (Dainihon Inc., Tokyo, Japan). After the resin had polymerized, the limbs were dissected and immersed in a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer for 3—4 weeks at 4 °C. After fixation, small pieces of excised skin (3 mm×3 mm) including the underlying muscles were incubated in an 88% formic acid solution at 45 °C for 7 d to selectively remove tissue components mainly composed of collagen fibrils. After incubation, specimens were carefully washed in 0.002 N HCl daily for 5 d to remove residual collagen fibrils and were then immersed in 1% tannic acid solution for 1 h. After rinsing in water, the residual components including elastic fibers were post-fixed in 2% osmium tetroxide for 1 h, dehydrated in a graded ethanol series, and freeze-dried. Specimens were then mounted, sputter-coated with gold, and examined using a HITACHI Model S-4000 field emission SEM at 5 kV.

Elastic Fiber Linearity Elastic fiber linearity was quantified at a magnification of 1000 using a personal image analyzer system, as detailed previously.[1,2] 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 was designated as A. The

linearity of each fragmented elastic fiber is expressed as A/(B×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 were embedded in paraffin. Specimen sections were cut and then stained with hematoxylin and eosin (H & E) and Luna.

Statistics Results are expressed as means±standard deviation (S.D.) and standard error of the mean (S.E.M.). Differences between means were checked for statistical significance using the Student *t*-test and Mann–Whitney's *U* test.

RESULTS

Topical Application of an Extract of *Sanguisorba officinalis* L. Inhibits UVB-Induced Wrinkle Formation The extract of *Sanguisorba officinalis* L. was applied topically to the rat hind limb skin at different concentrations 60 min or 24 h after UVB irradiation for 6 consecutive weeks. Visual assessment of close-up photos showed significant inhibition of UVB-induced wrinkle formation by the extract at a concentration of 1.0% compared with the irradiated and vehicle treated controls (Figs. 2, 3). Assessment by replica image analysis showed a significant inhibition of wrinkle formation at an extract concentration of 0.2% and virtually no wrinkles (*i.e.* similar to the unirradiated/untreated group) at a concentration of 1.0% (Figs. 4, 5).

H & E staining revealed no neutrophil infiltration of any of the skin specimens in any irradiated group. As we previously reported,[3,4] no actinic elastosis was observed in the UVB irradiated group.

Topical Application of an Extract of *Sanguisorba officinalis* L. Inhibits the UVB-Induced Decrease in Skin Elasticity The results of measurements of skin elasticity (physical parameters: Ue, Uf, Ur, and Uv) using a Cutometer following the various treatments are shown in Table 1. A significant inhibition in the decrease of skin elasticity was observed in terms of 3 parameters (Ue, Uf and Ur) in rat skin treated topically with an extract of *Sanguisorba officinalis* L. (at concentrations of 0.2% or 1.0%) during the 6-week UVB irradiation period compared with the control group treated topically with EtOH only and with the unirradiated control group.

Topical Application of an Extract of *Sanguisorba officinalis* L. Inhibits the UVB-Induced Disruption of the 3-Dimensional Structure of Elastic Fibers SEM observation revealed marked curling of elastic fibers in the control group treated topically with EtOH compared with the unirradiated/untreated group (Fig. 6). However, topical application of the extract of *Sanguisorba officinalis* L. inhibited curling at a concentration of 0.2% and maintained the high linearity of elastic fibers similar to the unirradiated/untreated control group at a concentration of 1.0%.

Quantitative assessment of the linearity of elastic fibers by replica image analysis showed complete inhibition of the UVB-induced curling of elastic fibers by the extract of *Sanguisorba officinalis* L. at a concentration of 1.0% (Fig. 7).

DISCUSSION

In this study, topical application of an extract of *Sanguisorba officinalis* L., which inhibits fibroblast-derived elastase,[17] over the course of chronic UVB irradiation of rat hind limb skin resulted in a dose-dependent inhibition of wrinkle formation. This was accompanied by prevention of the decrease in skin elasticity and the degeneration in the 3-dimensional structure of elastic fibers to levels observed in the non-

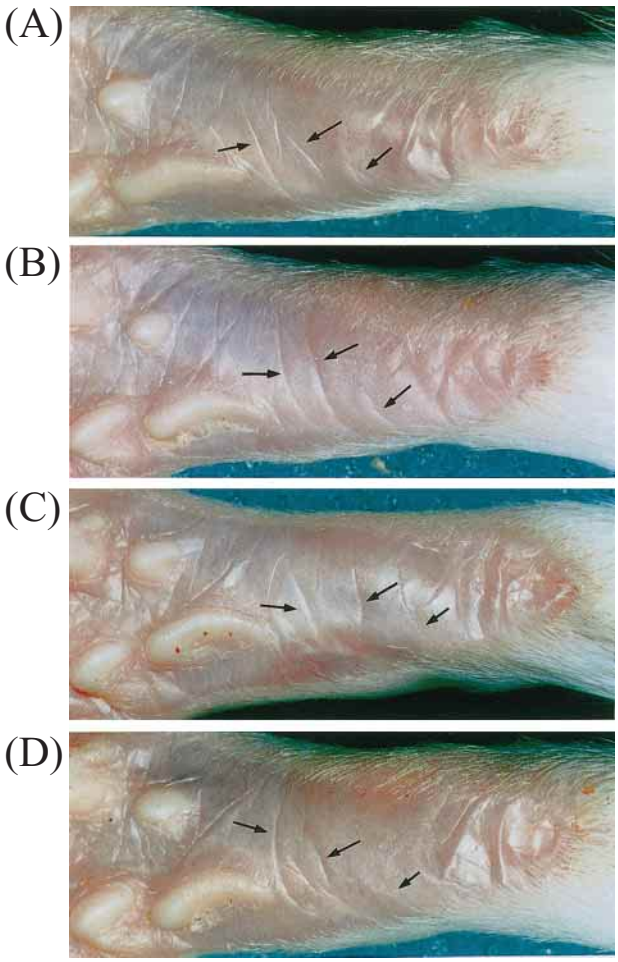

Fig. 2. Close-Up Photos after Sample Application (5 Times a Week for 6 Weeks) during the Period of UVB Irradiation (3 Times a Week for 6 Weeks) of Rat Hind Limb Skin

(A) Unirradiated and untreated skin; (B) irradiated and ethanol treated skin; (C) irradiated and 0.2% *Sanguisorba officinalis* L. extract treated skin; (D): irradiated and 1.0% *Sanguisorba officinalis* L. extract treated skin.

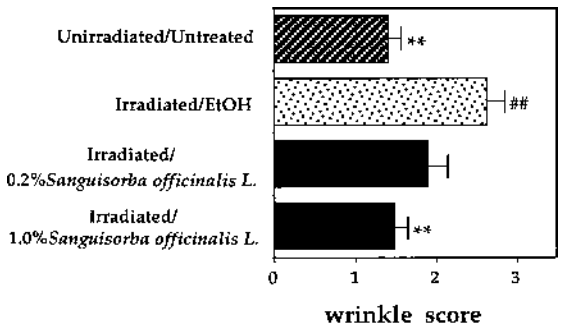

Fig. 3. Visual Scoring of Wrinkles

Wrinkle scoring was performed by the method of Bissett *et al.*[22] after sample application (5 times a week for 6 weeks) during the period of UVB irradiation (3 times a week for 6 weeks) of rat hind limb skin. Each bar represents the mean±S.E.M. (n=10). **: $p<0.01$ (*vs.* irradiated/EtOH). ##: $p<0.01$ (*vs.* unirradiated/untreated).

UVB-exposed controls.

Studies of elastic fibers using animal models have typically used tissue sections of hairless mice,[22,28,29] but some methods of observing the 3-dimensional structure of dermal elastic fibers in the skin have been reported.[30—34] Tsuji *et al.* examined skin samples consisting of elastic fibers alone using SEM,[30—32] and observed that the shape and arrangement of elastic fibers in human skin becomes more complicated with aging. Imayama and Braverman perfused and fixed rat hind limbs, then digested the skin with formic acid, and observed the elastic fibers and blood vessels in the skin by SEM in a state similar to that found *in vivo.*[33] They found losses in the linearity of dermal elastic fibers in the rat skin with aging,[34] and they speculated that curling of elastic fibers reduces skin elasticity, which results in wrinkle formation. Therefore, in earlier studies, we irradiated the hind limb skin of 3-week-old rats with UVB at a suberythemal dose, 3 times a week for 6 weeks (using Imayama's method) and we observed losses in the linearity of dermal elastic fibers and decreases in skin elasticity, which were similar to the changes observed with aging.[1,2] We suggested that elastase, which degrades elastin, may be involved in the curling of elastic fibers after consecutive suberythemal UVB irradiation. Elastin has been reported to be degraded mainly by serine protease and by metalloprotease.[5—12] When elastase activity was measured in the rat skin after chronic UVB irradiation, phosphoramidon-sensitive metalloprotease activity was increased.[14,15] Therefore, an inhibitor of fibroblast elastase (a member of the metalloprotease family[12,16]) was synthesized and was applied topically after each UVB irradiation. Such treatment inhibited increases in phosphoramidon-sensitive metalloprotease activity, and in a dose-dependent manner inhibited wrinkle formation, inhibited the decrease in skin elasticity, and inhibited damage of the 3-dimensional structure of elastic fibers.[14,15] Those results suggest the importance of inhibiting fibroblast elastase in preventing the curling of elastic fibers after chronic UVB irradiation.

We also topically applied 10 mM parsol MCX[35] as a UVB absorber, *i.e.* at the same concentration as the elastase inhibitor NPLT, after each UVB irradiation but that had no effect on wrinkle formation, decreased skin elasticity, or curling of dermal elastic fibers.[14,15] Thus we concluded that the prevention of photoaging effects by the topical application of NPLT are not due to UVB absorption but rather are due to

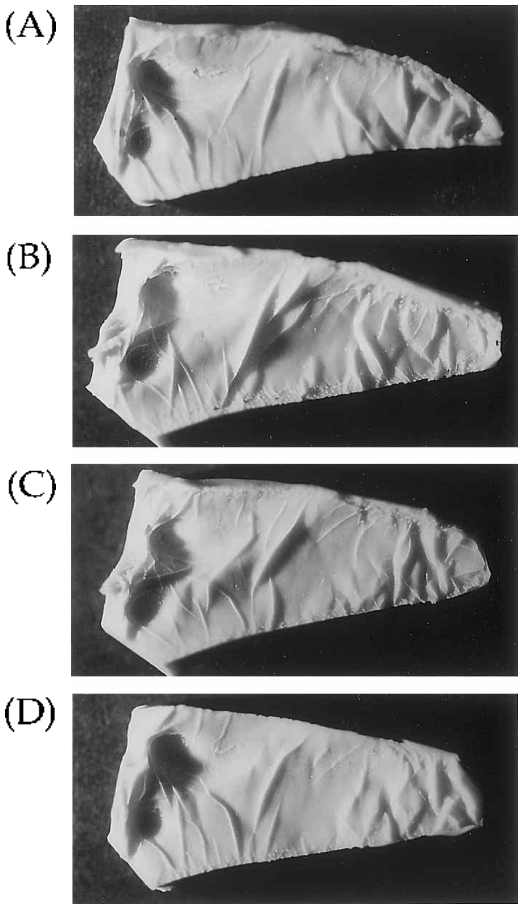

Fig. 4. Replica Photos Obtained after Sample Application (5 Times a Week for 6 Weeks) during the Period of UVB Irradiation of Rat Hind Limb Skin (3 Times a Week for 6 Weeks)

(A) Unirradiated and untreated skin; (B) irradiated and ethanol treated skin; (C) irradiated and 0.2% *Sanguisorba officinalis* L. extract treated skin; (D) irradiated and 1.0% *Sanguisorba officinalis* L. extract treated skin.

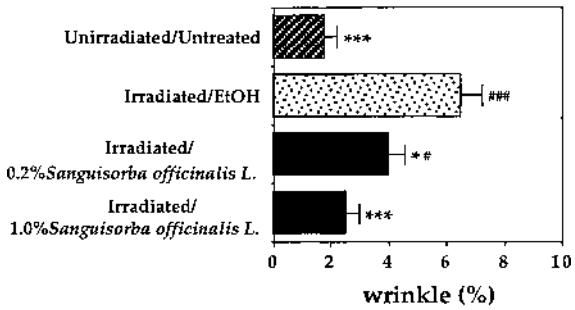

Fig. 5. Results of Replica Analysis Image after Sample Application (5 Times a Week for 6 Weeks) during the Period of UVB Irradiation (3 Times a Week for 6 Weeks) of Rat Hind Limb Skin

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±S.E.M. (n=10). ***,*: p<0.005, 0.05 (*vs.* irradiated/EtOH). ###,#: p<0.005, 0.05 (*vs.* unirradiated/untreated).

Table 1. Effect of Topical Application of the *Sanguisorba officinalis* L. on Elasticity of UVB-Irradiated Rat Hind Limb Skin

Physical parameters	Unirradiated/Untreated	Irradiated/EtOH	*Sanguisorba officinalis* L. Irradiated/0.2%	Irradiated/1% (mean±S.D.)
Ue	0.068±0.015***	0.026±0.003###	0.038±0.015***,###	0.042±0.015***,#
Uf	0.085±0.020***	0.035±0.010###	0.055±0.012**,###	0.062±0.010***,#
Ur	0.052±0.018***	0.022±0.015###	0.031±0.008*,#	0.040±0.007***
Uv	0.019±0.008	0.014±0.010	0.018±0.012	0.019±0.020

Skin elasticity is expressed as values of Ue, Uv, Ur and Uf, measured with a Cutometer just before the animals were sacrificed. This measurement was carried out at the age of 9 weeks. ***,**,* p<0.005, 0.01, 0.05 (*vs.* irradiated/EtOH treatment), ###,# p<0.005, 0.05 (*vs.* unirradiated/untreated). n=10. Ue, immediate distension measurement at 0.1 s; Uv, delayed distension; Ur, immediate retraction; Uf, final distension.

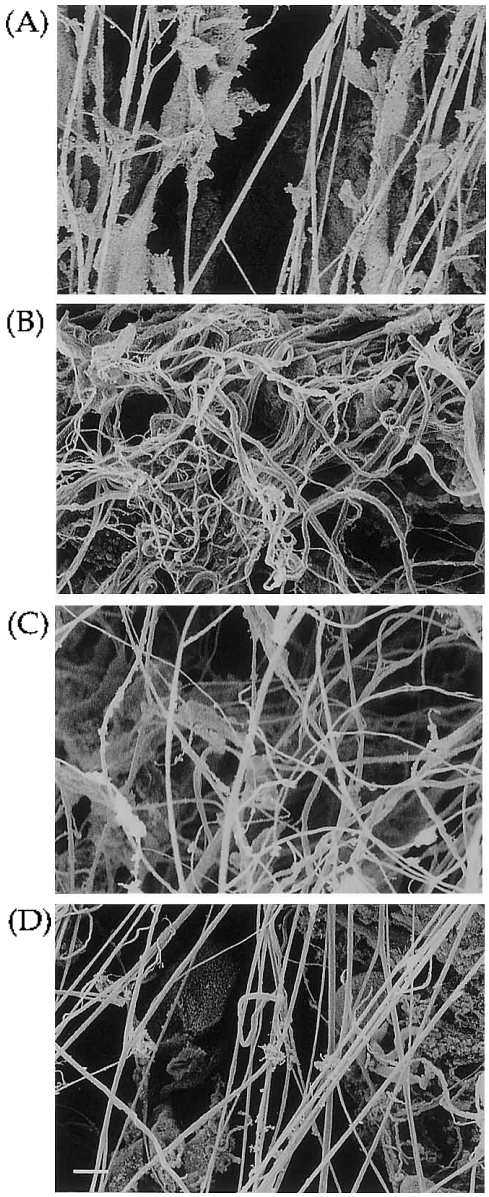

Fig. 6. Scanning Electron Micrographs after Sample Treatment Followed by Intravascular Injection and Selective Digestion at 9 Weeks

(A) Unirradiated and untreated skin; (B) irradiated and ethanol treated skin; (C) irradiated and 0.2% *Sanguisorba officinalis* L. extract treated skin; (D) irradiated and 1.0% *Sanguisorba officinalis* L. extract treated skin. Scale bar=5 μm.

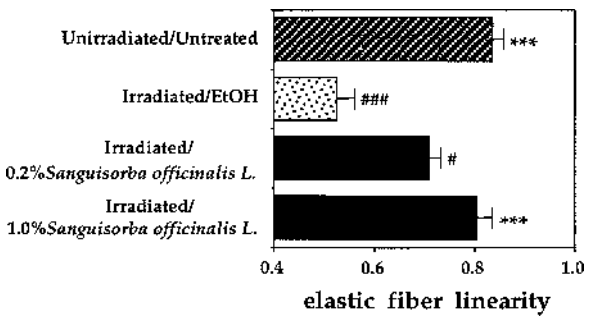

Fig. 7. Changes in the Linearity of Dermal Elastic Fibers after Sample Application, Measured by Image Analysis

Fibers were characterized on SEM micrographs. Each bar represents the mean±S.E.M. from 20 SEM microphotographs. Total fragmented line: n=320 (16 fragmented lines×20 microphotographs); Animal, n=5; limb, n=10. ***: $p<0.005$ (*vs.* irradiated/EtOH). ###,#: $p<0.005$, 0.05 (*vs.* unirradiated/untreated).

the inhibition of elastase activity itself. Although the UVB absorbing effects of topical application of an extract of *Sanguisorba officinalis* L. to the rat skin are unclear, it is unlikely that its potential UVB absorbing ability contributes to the inhibition of wrinkle formation, the decrease in skin elasticity, or the curling of dermal elastic fibers.

Extracts of *Sanguisorba officinalis* L. have been reported to slightly reduce neutrophil elastase activity *in vitro*.[18] Neutrophil elastase has been reported to degrade elastic fibers[5—8] more markedly than does fibroblast-derived elastase.[7] Therefore, it is possible that activation of neutrophil elastase by UVB irradiation damages the 3-dimensional structure of dermal elastic fibers. However, the UVB irradiation dose used in this study was 1 minimal erythemal dose (MED) or less, and no neutrophils were observed in any of the UVB irradiated groups. In addition, in our rat hind limb skin model, serine protease activity does not increase after UVB irradiation.[14,15] Therefore, under the present conditions, the influence of neutrophil elastase is probably negligible. In contrast, hyaluronic acid has been reported to decrease with age,[36,37] and inhibition of hyaluronidase activity by the extract of *Sanguisorba officinalis* L. has been shown *in vitro*.[19] However, in photoaging, an increase in hyaluronic acid has also been reported.[38,39] In photoaging evaluation systems such as the present experimental system, the *in vivo* effects of the hyaluronidase inhibition activity of this extract are unclear. Other researchers have shown slight *in vitro* antioxidation effects of the *Sanguisorba officinalis* L. extract.[20] Chronic topical application of ascorbic acid or α-tocopherol to hairless mouse skin before UVB irradiation inhibited wrinkle formation and reduced collagen damage in the skin tissue.[40] Therefore, under the present conditions, the inhibition of photoaging in the rat hind limb skin by the application of the *Sanguisorba officinalis* L. extract is considered to be based on its inhibition of fibroblast-derived elastase. However, it is possible that the ability of this extract to inhibit hyaluronidase activity and its anti-oxidant properties have additive or synergistic effects. Therefore, as cosmetic agents, the extract of *Sanguisorba officinalis* L. may be more useful than synthetic compounds that specifically inhibit fibroblast-derived elastase, such as NPLT.

The primary component of the *Sanguisorba officinalis* L. extract is tannin, and various types of tannin have been recovered from this extract.[41,42] Many studies have shown the effects of tannin, such as its anti-oxidant properties[43—45] and its capacity to inhibit hyaluronidase[46,47] and neutrophil elastase[48,49] activities. However, we have found that tannin extracted from green tea does not inhibit fibroblast-derived elastase (data not shown). Therefore, the presence of tannin in the extract of *Sanguisorba officinalis* L. can not explain the maintenance of the linearity of elastic fibers (which is based on the inhibition of fibroblast-derived elastase), the associated maintenance of skin elasticity or the inhibition of wrinkle formation. However, since tannin has an inhibitory action on various enzymes and since it functions as an antioxidant, as discussed above, its contribution to the *in vivo* inhibition of wrinkles and photoaging observed in this study can not be excluded.

In conclusion, we used a plant extract to confirm that agents with inhibitory activity against fibroblast elastase, when applied topically during chronic UVB irradiation, in-

hibit wrinkle formation in a dose-dependent manner, inhibit the decrease in skin elasticity, and also maintain the linearity of dermal elastic fibers.

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