Possible Involvement of Gelatinases in Basement Membrane Damage and Wrinkle Formation in Chronically Ultraviolet B-exposed Hairless Mouse

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A number of studies indicate that matrix metalloproteinase might be involved in photoaging, but little is known about their direct contribution to ultraviolet-induced histologic and morphologic changes in the skin *in vivo*. This study reports the relationship between changes of matrix metalloproteinase activities and ultraviolet B-induced skin changes in hairless mouse. The role of matrix metalloproteinase in the skin changes was studied by topical application of a specific matrix metalloproteinase inhibitor. The backs of mice were exposed to ultraviolet B three times a week for 10 wk. Histologic studies showed that the basement membrane structure was damaged, with epidermal hyperplasia, in the first 2 wk of ultraviolet B irradiation, followed by the appearance of wrinkles, which gradually extended in the latter half of the ultraviolet B irradiation period. We observed enhancement of type IV collagen degradation activity, but not collagenase or matrix metalloproteinase-3 activity, in extracts of ultraviolet B-irradiated, wrinkle-bearing skin. Gelatin zymographic analysis revealed that gelatinases, matrix metalloproteinase-9 and matrix metalloproteinase-2, were significantly increased in the extract. *In situ* zymographic study clarified that the activity was specifically localized in whole epidermis of ultraviolet B-irradiated, wrinkled skin in comparison with normal skin. The activity was induced around the basal layer of the epidermis by a single ultraviolet exposure of at least one minimal erythema dose. Furthermore, topical application of a specific matrix metalloproteinase inhibitor, CGS27023A, inhibited ultraviolet B-induced gelatinase activity in the epidermis, and its repeated application prevented ultraviolet B-induced damage to the basement membrane, as well as epidermal hyperplasia and dermal collagen degradation. Ultraviolet B-induced wrinkles were also prevented by administration of the inhibitor. These results, taken together, suggest that ultraviolet B-induced enhancement of gelatinase activity in the skin contributes to wrinkle formation through the destruction of basement membrane structure and dermal collagen in chronically ultraviolet B-exposed hairless mouse, and thus topical application of matrix metalloproteinase inhibitors may be an effective way to prevent ultraviolet B-induced wrinkle formation.
Key words: basement membrane/matrix metalloproteinases/mice/photoaging of skin/ultraviolet rays. J Invest Dermatol 120:000–000, 2003

Symptoms of cutaneous aging, such as wrinkles, develop earlier in sun-exposed skin than in unexposed skin. This phenomenon is known as photoaging, and is characterized by histologic changes, including damage to collagen fibers, excessive deposition of abnormal elastic fibers, and increase of glycosaminoglycans (Sams and Smith, 1961; Smith *et al*, 1962; Kligman, 1969; Uitto *et al*, 1989). As it is ethically unacceptable to continue experimental ultraviolet (UV) irradiation of human skin for long enough to induce the wrinkle formation, a photoaging model that shows skin changes characteristic of photoaged skin, such as wrinkle formation, was developed by applying repeated low-dose UVB irradiation to the skin of hairless mice (Kligman, 1989), and since then, many studies have been carried out using the photoaging model (Kligman *et al*, 1985, 1989; Bissett *et al*, 1987, 1990a, b, c; 1991; Schwartz, 1988; Chatterjee *et al*, 1990; Kiss *et al*, 1991; Kochevar *et al*, 1993; Zheng and Kligman, 1993). As marked degradation and degenerative changes of the extracellular matrix in the dermis were observed during the process of wrinkle formation, matrix-degrading enzymes were assumed to be involved in wrinkle formation (Kligman *et al*, 1989).

Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases involved in the remodeling of the extracellular matrix,

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Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; MED, minimal erythema dose.

and play important parts in morphogenesis, angiogenesis, arthritis, skin ulcer, tumor invasion, and metastasis (Birkedal-Hansen, 1995). Five families of MMP have been recognized: collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMP. These enzymes are composed of several domains, including propeptide, catalytic, and hemopexin (except for matrilysin) domains, and are involved in the degradation of collagens, proteoglycans, and various glycoproteins (Birkedal-Hansen, 1995). Among them, gelatinase A (MMP-2 or 72 kDa type IV collagenase) and gelatinase B (MMP-9 or 92 kDa type IV collagenase) digest type IV and VII collagens, and stromelysins (MMP-3, MMP-10) degrade laminins of the basement membrane (Reynolds, 1996). MMP are secreted as inactive zymogens (pro-MMP), and their activation (to active MMP) is a prerequisite for function. Stimulation or repression of pro-MMP synthesis is mostly regulated at the transcriptional level by growth factors or cytokines (Reynolds, 1996). MMP are considered to be involved in photoaging as MMP-1, 2, 3, and 9 were increased by UV irradiation in experiments using human fibroblasts (Scharffetter *et al*, 1991; Herrmann *et al*, 1993; Wlaschek *et al*, 1995; Kawaguchi *et al*, 1996; Brenneisen *et al*, 1998) and human skin (Scharffetter *et al*, 1991; Koivukangas *et al*, 1994; Fisher *et al*, 1996, 1997). In particular, Fisher *et al* (1996) demonstrated an increase of MMP in human skin following exposure even to an extremely low level of UVB, and suggested that MMP are UV-induced aging factors. Although MMP were detected in skin of UV-irradiated mice (Chatterjee *et al*, 1990; Schwartz *et al*, 1998; Saariaho-Kere *et al*, 1999), it has still been unclear whether MMP are directly involved in UV-induced wrinkle formation in mice.

In this study, we examined the role of MMP in the process of UVB-induced wrinkle formation. We found that UVB irradiation induced the production of gelatinases in the epidermis of hairless mouse skin, that basement membrane at the dermal–epidermal junction was damaged during the wrinkle formation process, and that topical treatment with a synthetic MMP inhibitor blocked wrinkle formation and basement membrane damage.

MATERIALS AND METHODS

Animals Male albino hairless HOS:HR-1 mice were purchased from Hoshino Laboratory Animals Co., Ltd. (Saitama, Japan). These animals were approximately 6 wk old at the start of the experiment. They were fed water and a commercial diet (CRF-1, Oriental Yeast Co., Ltd, Tokyo, Japan) ad libitum.

MMP inhibitor *N*-Hydroxy-2(R)-[[(4-methoxyphenyl) sulfonyl](3-picolyl) amino]-3-methylbutanamide hydrochloride, CGS27023A (**Fig 1**), was synthesized at Shiseido Co. Ltd. (Yokohama, Japan) according to the method described in the literature (MacPherson *et al*, 1997). Its Ki values against MMP were: MMP-1, 33 nM; MMP-2, 20 nM; MMP-3, 43 nM; and MMP-9, 8 nM. The purity of the compound was 99.2% as determined by high-performance liquid chromatography analysis (detection: UV absorbance measurement at 254 nm). As the spectrum of CGS27023A showed a peak at 245 nm, and no or little absorbance was detected above 290 nm (UVB: 290–315 nm), topically applied CGS27023A did not absorb UVB.

UVB irradiation As a source of UVB, 10 Toshiba FL-20 SE fluorescent lamps (Toshiba Electric, Tokyo, Japan) were used in the same way as in other studies (Haratake *et al*, 1997; Naganuma *et al*, 2001). These lamps emit UV light mainly in the range of wavelengths from 280 to 340 nm, with the maximum wavelength of 305 nm as shown in **Fig 2** (Haratake *et al*, 1997). The intensity of irradiation was set at 0.3 mW per cm^2 by using UV-Radiometer (CVR-305/365D II, Topcon, Tokyo, Japan). The time of irradiation was changed to control the UVB energy applied to the dorsal region of each mouse. The value of the minimal erythema dose (MED) for the hairless mouse was about 54 mJ per cm^2.

Wrinkles were formed by long-term repeated UVB irradiation according to the method described by Schwartz *et al* (1998), which was partially modified so that wrinkle formation could be induced within a shorter period of time while minimizing the occurrence of severe inflammatory changes, such as edema, and skin cancer. Briefly, the initial dose was set at 36 mJ per cm^2, which was subsequently increased to 54, 72, 108, 144, 162, 180, and 198 mJ per cm^2 at 1 wk intervals and finally to 216 mJ per cm^2 at weeks 9 and 10. The frequency of irradiation was set at three times per week (30 doses/10 wk). The total exposure dose was about 4.6 J per cm^2. In this protocol, wrinkles began to be observed macroscopically in the dorsal region from about 5 wk after initiation of irradiation, and deep wrinkles were formed at 10 wk.

Evaluation of wrinkle formation At 7 and 10 wk after initiation of repeated UVB irradiation, each hairless mouse was anesthetized, and the UVB-exposed dorsal area (site of wrinkle formation) was photographed. Three investigators individually determined the degree of wrinkle formation from the photograph of each animal according to the grading scale described in **Table I**, whereas the name of the animal group was kept blind; this is a modification of the method described by Bissett *et al* (1987). The statistical analysis of intergroup differences was conducted by using the Mann–Whitney U test.

Topical treatment with MMP inhibitor CGS27023A was dissolved in a solvent (ethanol/propylene glycol = 7 : 3) at a concentration of 1% (w/v), and 100 μl of this solution was applied to the dorsal region three times weekly just after UVB exposure, to avoid UV absorption by CGS27023A. Moreover, the skin surface was wiped with ethanol before each session of UV irradiation to remove residual drug from the skin surface. In this protocol, even substances having a higher degree of UV absorption than

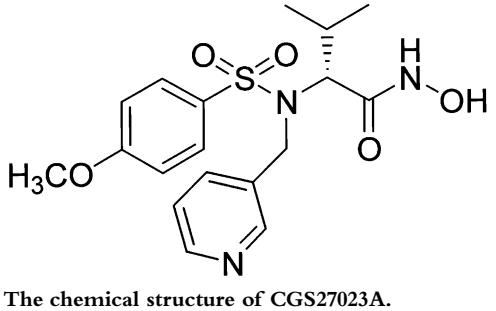

Figure 1. The chemical structure of CGS27023A.

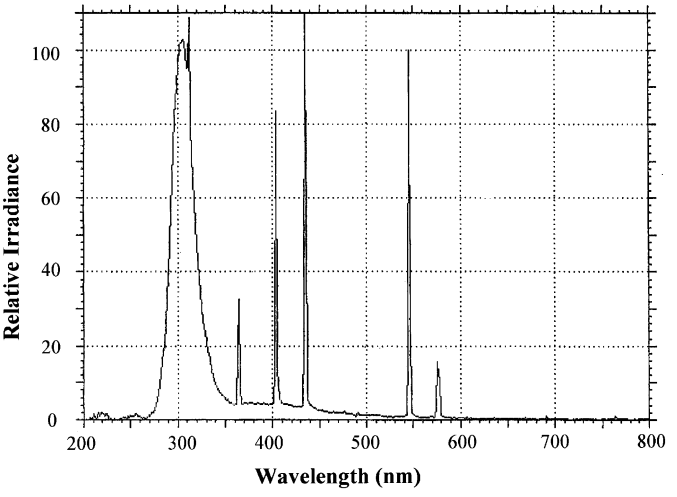

Figure 2. Emission spectrum of Toshiba FL-20 lamps.

Table I. Grading of mouse skin wrinkles

Grade	Evaluation criteria
0	No wrinkles
2	A few shallow wrinkles across the back skin are observed occasionally
4	Shallow wrinkles across the back skin are observed on the whole surface
6	Some deep, long wrinkles across the back skin are observed
8	Deep, long wrinkles across the back skin are observed on the whole surface

CGS27023A did not influence the UVB-induced skin changes, so the effects of UVB absorption of CGS27023A were considered to be negligible in these experiments.

Detection of MMP in the skin extract Hairless mice were sacrificed under ether anesthesia and skin was collected from the dorsal region. The skin extract solution was prepared as described below. The subcutaneous tissue was removed using a shaving scalpel, and the skin was washed several times with 0.05 M Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl. In order to improve the extraction efficiency of MMP from the tissue, the tissue was heated in phosphate-buffered saline at 60°C for 10 s and minced, then 50 mg wet weight of the tissue was placed in 1 ml of 0.05 M Tris–HCl buffer, pH 7.4, containing 0.2 M NaCl, 5 mM $CaCl_2$, and 0.1% Triton X-100, and homogenized on ice at 20,000 r.p.m. twice for 20 s each using a Polytron PT 3100 (Kinematica, Littau, Switzerland). The homogenate was centrifuged at $8000 \times g$ at 4°C for 20 min, and the supernatant was used as the skin extract solution for experiments.

The activities of type I collagenase, type IV collagenase, and MMP-3 in the skin extract solution were measured using a commercially available kit according to the manufacturer's protocol (Yagai, Yamagata, Japan).

Gelatin zymography was conducted as follows. The skin extract solution containing 6.6 μg protein, as determined by the bicinchoninic acid (BCA) method (Pierce, Rochfold, IL), was loaded on to 10% sodium dodecyl sulfate–polyacrylamide gel copolymerized with 0.2% gelatin (Bio-Rad, Tokyo, Japan) and subjected to electrophoresis at 100 V for 1.5 h. In order to remove sodium dodecyl sulfate, the gel for electrophoresis was washed twice with 2.5% Triton solution for 15 min each, rinsed with incubation buffer (0.05 M Tris–HCl buffer pH 8.0, 5 mM $CaCl_2$, 5 μM $ZnCl_2$), and incubated at 37°C overnight. The gel was stained with 0.5% Coomassie Brilliant Blue R-250, which was dissolved in water containing 25% 2-propanol and 10% acetic acid, at room temperature for 30 min and destained with water containing 10% methanol and 10% acetic acid. Gelatinases in the skin extract solution were detected as unstained gelatin degraded zones. These zones were quantified using an image analyzer (Fluor-S MultiImager, Bio-Rad).

***In situ* gelatin zymography** The tissue samples collected from the dorsal region of hairless mice were frozen with Tissue-Tek OCT embedding compound (Sakura Finetechnical Co., Tokyo, Japan). *In situ* gelatin zymography was carried out using cross-linked gelatin membrane with a thickness of 7 μm on polyester film (FIZ-GN, Fuji Photo Film Co., Ltd, Tokyo, Japan), which is suitable for detecting gelatinase activity in tissues (Nakamura *et al*, 1999; Nemori and Tachikawa, 1999; Ohashi *et al*, 2000). Each frozen tissue sample was sliced to 5 μm sections using a cryostat (CM3050S, Leica, Nussloch, Germany) and placed on FIZ-GN. After 18 h of incubation in a moist chamber at 37°C, the tissue was stained with 0.8% Ponceau 3R solution (Wako Pure Chemical Industries Ltd, Osaka, Japan) at room temperature for 6 min. The gelatinases in the tissue section were detected as negative staining areas with Ponceau 3R solution. To confirm the specificity of *in situ* gelatin zymography, 10 μl of tissue inhibitor of metalloproteinase (TIMP)-2 (recombinant human TIMP-2; Fuji Chemical Industries, Takaoka, Japan) solution at a concentration of 20 μg per ml or the same amount of water (control) was added to the sections at the beginning of the incubation.

Light microscopy The dorsal skin was collected from hairless mice, fixed with acetone at 4°C, and embedded by the AmeX method (Sato *et al*, 1992). Briefly, the acetone-fixed skin was embedded in paraffin after substitution with methyl benzoate and xylene. The block was sliced to 3 μm sections. The sections were stained as usual with hematoxylin and eosin and observed morphologically. Immunostaining was performed by the SAB (streptavidin–biotin) method using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). After blocking with 10% rabbit serum, the 3 μm sections were incubated at 4°C overnight using mouse monoclonal anti-laminin 5 antibody (BM165; Rousselle and Aumailley, 1994) and anti-type VII collagen antibody (NP185; Sakai *et al*, 1986) as the primary antibodies. The tissue was washed with phosphate-buffered saline, and allowed to react with biotin-labeled anti-mouse immunoglobulin as the secondary antibody at room temperature for 1 h. After 30 min of incubation with peroxidase-labeled streptavidin as the tertiary antibody at room temperature, the tissue was allowed to develop a color with diaminobenzidine, and the nucleus was stained with hematoxylin for observation under a light microscope.

Electron microscopy After each hairless mouse was weighed, a Nembutal injection (5 mg per ml) was administered intraperitoneally at a dose of 0.1 ml per 100 g. Under anesthesia, an incision was made in the thoracic region, and an aliform needle (21 gauge) was inserted into the left ventricle. Then an incision was made in the right arterial auricle under perfusion with 0.1 M phosphate-buffered saline, and the animal was bled to death. Using a Perista pump, the flow rate was adjusted to about 2 ml per min. After perfusion with about one-half volume (with respect to body weight) of 0.1 M phosphate-buffered saline buffer (pH 7.4), picric acid-colored 4% paraformaldehyde/0.1 M phosphate-buffered saline equivalent to about 1.5 or 2 times the body weight was perfused to fix the skin, and the dorsal skin was collected and fixed with Karnovsky fixative in 0.1 M cacodylate buffer, for 2 h and 1% osmium tetroxide for 1 h at 4°C. The tissues were dehydrated through a graded ethanol series and embedded in Epon 812. Ultrathin sections were cut using an ultramicrotome (Reichert Ultracut S, Reichert, Vienna, Austria), stained with saturated uranyl acetate and lead citrate, and observed under a transmission electron microscope (Hitachi H-7100, Hitachi Co. Ltd, Tokyo, Japan).

Quantitative analysis of collagen The quantitative analysis of collagen was carried out according to the Sirius red-staining method described by Lopez-De and Rojkind (1985). Briefly, sections 16 μm in thickness were stained with a mixture of Sirius red F3BA and fast green FCF (Collagen Research Center, Tokyo, Japan) at room temperature for 30 min and photographed. The tissue was washed with distilled water, and then discolored five times with 200 μl each of discoloring solution, and the solution was collected. The absorbance of the discoloring solution was measured at 530 and 605 nm, and the amount of collagen in the tissue was calculated. The photograph was scanned into NIH Image, and the area of the dermis was calculated to obtain the amount of collagen per unit area. Six portions were stained and the mean ± SD was calculated for each of the drug and vehicle groups.

RESULTS

Skin changes induced by UVB irradiation Epidermal thickness significantly increased from week 2 and the increase progressed with time after UVB irradiation, as determined by microscopic observation of hematoxylin and eosin-stained skin sections (**Fig 3*a***). The epidermal thickness began to change prior to the development of wrinkle formation, as there was no change in the visible appearance of the skin at 2 wk after starting UVB irradiation, whereas wrinkles became apparent from about week 5 (**Fig 3*b***). The degree of wrinkle formation increased with time after initiation of UVB irradiation (**Fig 3*b***). Ultrastructural analysis revealed that a decrease in the number of hemidesmosomes and partial separation of epidermis from the basement membrane were already apparent at week 2 after initiation of UVB irradiation (**Fig 4*a***). The degree of separation and the disruption of the epidermal basement membrane became more severe at week 10 (**Fig 4*d***). The epidermal basement membrane damage also began to appear earlier than the wrinkle formation.

MMP activities in the extract of wrinkle-bearing skin Activities of MMP-1, MMP-3, and type IV collagenase in skin extracts were compared between unexposed normal skin and the wrinkle-bearing skin, as MMP are capable of degrading

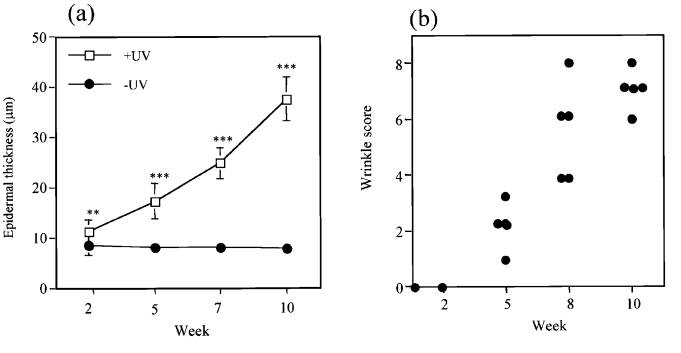

Figure 3. Changes of epidermal thickness and wrinkle formation during UVB irradiation. (*a*) Change of epidermal thickness on UVB-nonirradiated normal skin (–UV) or UVB-irradiated skin (+UV). $^{**}p < 0.01$; $^{***}p < 0.001$. (*b*) Changes of wrinkle formation on the back of hairless mouse during repeated UVB irradiation.

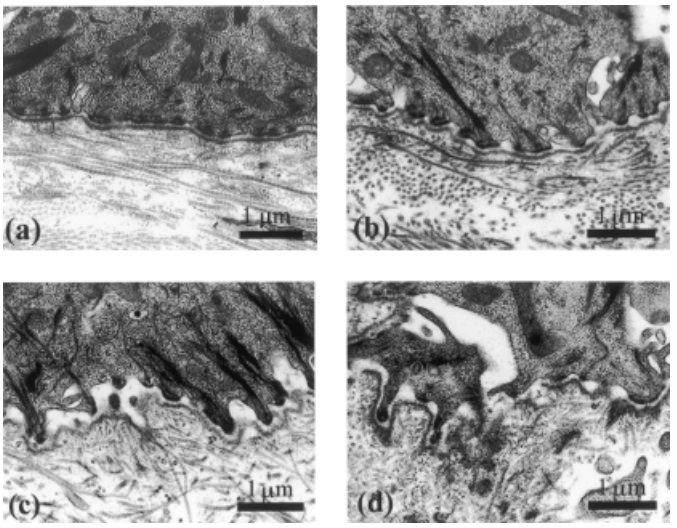

Figure 4. Changes of basement membrane ultrastructure in UVB-irradiated mouse. Mouse skin was irradiated with UVB (total 5 J per cm^2) for 10 wk. (*a*) Non-irradiated control skin. (*b*) Basement membrane began to separate from basal cells after 2 wk of UVB irradiation. (*c*) The basement membrane damage became more severe after 5 wk of UVB irradiation. (*d*) Many disruptions of basement membrane were observed after 10 wk of UVB irradiation. *Scale bars*: 1 μm.

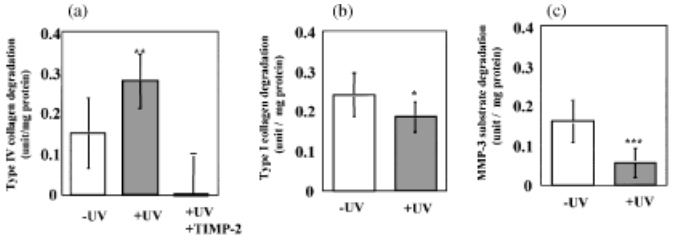

Figure 5. Type IV collagen-degrading activities, type I collagen-degrading activities and MMP-3 activities in the skin extracts. Skin extracts were prepared from UVB-nonirradiated normal skin (−UV) or chronically UVB-exposed skin (+UV). (*a*) Type IV collagen degradation activity in the extracts was increased by UVB exposure and TIMP-2 (200 ng) inhibited the type IV collagen degradation (+UV+TIMP2). Type I collagen degradation activity (*b*) and MMP-3 substrate degradation activity (*c*) in the extracts were reduced by chronic UVB irradiation. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

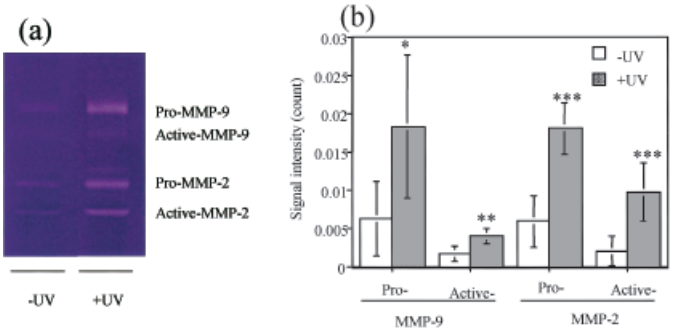

Figure 6. Gelatinase activities in UVB-exposed (wrinkle bearing) or nonexposed control skins. (*a*) Typical gelatin zymographic pattern. Gelatinase activities were increased in chronically UVB-exposed skin extract. (*b*) Each gelatinase band was densitometrically quantified by computer image analysis. Each bar represents the mean ± SD (n = 7). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

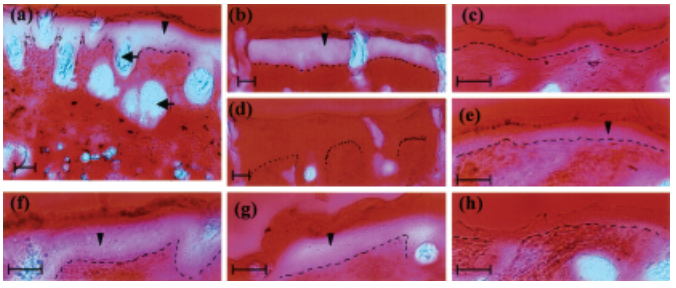

Figure 7. *In situ* zymographic localization of gelatinolytic activity in hairless mouse skin. (*a,b*) Ten week UVB-irradiated skin (wrinkles have formed). Note strong gelatinolytic activity (*arrowhead*) in the epidermis. (*c*) UVB-nonirradiated normal skin. (*d*) Administration of TIMP-2 at the beginning of the incubation for *in situ* zymography of 10 wk UVB-irradiated skin. Note blockage of the potent activity in the epidermis. (*e*) Skin at 48 h after 1 MED of UVB exposure. (*f*) Skin at 48 h after 3 MED of UVB exposure. (*g*) Skin at 48 h after five exposures to 1 MED of UVB every other day. (*h*) CGS27023A was topically applied to skin during irradiation five times with 1 MED of UVB each. Broken lines show the junction between the epidermis (*top*) and the dermis (*bottom*). *Arrowheads* show gelatinolytic activities in the epidermis. *Arrows* show gelatinolytic activities in sebaceous glands. *Scale bars:* 50 μm.

basement membrane components such as type IV collagen, type VII collagen, laminins, etc. The activities of MMP-1 and MMP-3 were rather lower than those in the nonirradiated control skin, whereas the type IV collagen degrading activity was higher in the wrinkle-bearing skin (**Fig 5**). As gelatinases, MMP-2 and MMP-9, are known to be type IV collagenases, TIMP-2 was added to the reaction mixture. The type IV collagenase activities were almost completely inhibited by TIMP-2 (**Fig 5*a***). The results of gelatin zymography confirmed the increase of active forms of MMP-2 and MMP-9 in the extract of wrinkle-bearing skin, in addition to the increase of pro-MMP-2 and pro-MMP-9 as compared with those in control skin (**Fig 6**).

Localization of gelatinase activity in the skin tissue The localization of gelatinolytic activity in the skin was investigated by *in situ* gelatin zymography. As shown in **Fig 7**, gelatinolytic activities were detected as a negative staining (white signal) area, representing the region in which gelatin had been digested, and were present throughout the epidermal layer of wrinkled skin and around some dermal cells and sebaceous glands in dermis of wrinkled skin (**Fig 7*a*,*b***), but not in the epidermis of UVB-unexposed control skin (**Fig 7*c***). The gelatinolytic activities in the whole epidermal layer were almost completely blocked by TIMP-2 added exogenously to the skin section (**Fig 7*d***). Gelatinolytic activities were induced in an energy-dependent manner by a single UVB exposure of 54 mJ per cm^2 (corresponding to 1 MED in mice) or 162 mJ per cm^2 (3 MED) in normal mice, and were present around the epidermal basal and spinal layers (**Fig 7*e*** and ***f***, respectively). Repeated irradiation (five times every other day) increased the area of negative staining from the basal layer toward the cornified layer (**Fig 7*g***).

Effect of a MMP inhibitor on the UVB-induced skin changes In order to clarify the role of gelatinases in the appearance of the UVB-induced skin changes, the effect of a potent synthetic inhibitor of MMP-2 and MMP-9, CGS27023A (MacPherson *et al*, 1997), was assessed. We first examined whether topically applied CGS27023A could inhibit UVB-induced

gelatinase activity in the epidermis. As shown in **Fig 7(*h*)** when CGS27023A was topically administered after each UVB irradiation of the skin (1 MED of UVB five times every other day), little gelatinase activity was observed in the epidermis by *in situ* gelatin zymography, although strong activities were detected in control UVB-exposed skin (**Fig 7*g***). Staining with toluidine blue showed that UVB-induced epidermal thickening (90.7 ± 12.1 μm, $n = 3$) was inhibited by CGS27023A treatment (54.0 ± 6.7 μm, $p < 0.001$, $n = 3$) (**Fig 8**). Immunostaining of laminin 5 and type VII collagen as constituents of the basement membrane showed linear staining of the epidermal basement membrane, with no difference in staining between the CGS27023A-treated and control groups (data not shown). Electron microscopic observation of the basement membrane structure at the dermal–epidermal junction, however, revealed that its separation from epidermal cells and rupture were inhibited and the continuity of the lamina densa was well maintained in the CGS27023A-treated group as compared with the control group (**Fig 9**). Furthermore, the density of collagen fibers underneath the basement membrane was observed to be higher in CGS27023A-treated skin (**Fig 9*b***) than in the control (**Fig 9*a***). When the collagen content of the dermis was determined by staining collagen in the tissue with Sirius red, the amount of collagen per 0.1 cm^3 in the vehicle-treated UVB-exposed mice was significantly reduced by 21.4% as compared with that in control mice without UVB exposure (**Fig 10**). Topical application of CGS27023A inhibited the reduction of collagen content by UVB exposure, and there was no significant difference in collagen content between the CGS27023A-treated group and the nonirradiated group (**Fig 10**). The UVB-induced wrinkle formation was significantly inhibited by the topical application of CGS27023A as compared with that in the vehicle-treated group at both 8 and 10 wk after initiation of UVB irradiation (**Fig 11**).

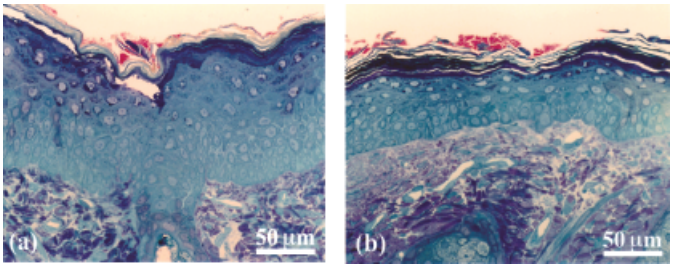

Figure 8. Topical application of CGS27023A suppressed epidermal hyperplasia induced by chronic UVB irradiation. One percent of CGS27023A was topically applied to UVB-irradiated skin for 10 wk. Vehicle-treated skin showed severe epidermal hyperplasia (*a*); CGS27023A reduced the epidermal hyperplasia (*b*). *Scale bars*: 50 μm.

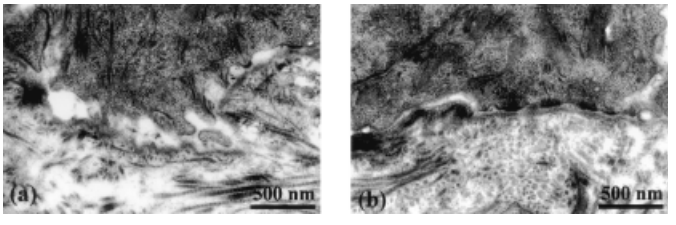

Figure 9. Effect of CGS 27023A topical application on basement membrane damage in UVB-irradiated mouse. In vehicle-treated skin, basement membrane showed many disruptions and wide separation from basal cells (*a*). In CGS27023A-treated skin, basement membrane showed better structure in terms of the continuity of lamina densa and structure of hemidesmosomes (*b*). *Scale bars*: 500 nm.

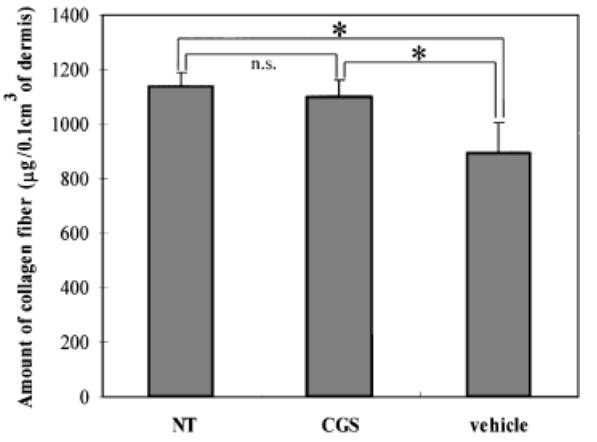

Figure 10. Effect of CGS27023A on collagen degradation in UVB-induced wrinkled skin. Collagen content per 0.1 cm^3 in the dermis was measured by the Sirius Red-staining method. Collagen content in the dermis was reduced by UVB irradiation, and topically applied CGS27023A inhibited the degradation. $^*p < 0.01$.

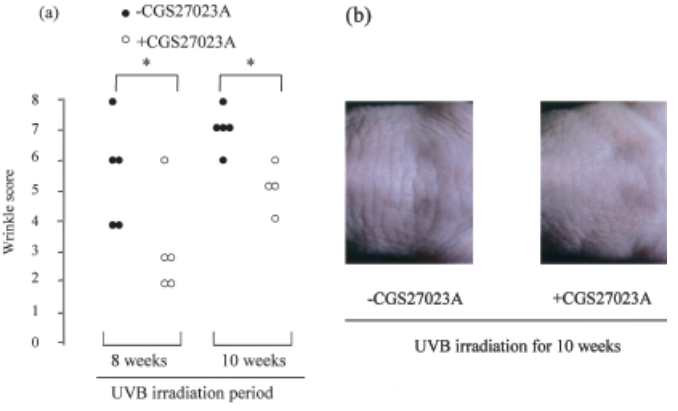

Figure 11. Effect of topically applied CGS27023A on wrinkle formation in UVB-irradiated mice. (*a*) Wrinkle score. CGS27023A inhibited the wrinkle formation. $^*p < 0.05$. (*b*) Typical photograph of wrinkles with or without CGS27023A treatment in hairless mice irradiated by UVB for 10 wk.

DISCUSSION

The increase of gelatinases (MMP-2 and MMP-9) in UVB-induced wrinkled skin was clarified by using three independent methods, i.e., type IV collagenase assay, gelatin zymography, and *in situ* gelatin zymography. The type IV collagenase activity in the wrinkle-bearing skin extract seemed to be due to activated forms of MMP-2 and MMP-9, as it was inhibited by TIMP-2. Indeed, the active forms of MMP-2 and MMP-9 were detected in the skin extract, in addition to pro-MMP-2 and pro-MMP-9, by gelatin zymography. The gelatinase activity was found in the whole epidermis and in the spotted dermis of wrinkle-bearing skin by *in situ* gelatin zymography, whereas epidermal gelatinase activity was not detected in unirradiated skin. On the other hand, type I collagen-degrading activity and MMP-3 were reduced in the UVB-induced wrinkled skin. Similarly, skin collagenase activity per gram wet weight skin and steady-state mRNA levels of collagenase and stromelysin (MMP-3) were reported to be reduced in chronically UVB-irradiated skin of hairless mice (Chatterjee *et al*, 1990; Schwartz *et al*, 1998, respectively). Therefore, gelatinases may be involved in the process of wrinkle formation.

Gelatinase activities were detected in the basal and spinal layers of epidermis in single-dose UVB-exposed skin by *in situ* gelatin zymography. Gelatin zymography showed that most of the gelatinase activity was due to MMP-9, but MMP-2 was also increased slightly at 48 h after single exposure to 1 or 3 MED of UVB (data not shown). These results are consistent with the finding in human tissue that MMP-9 was markedly overexpressed in the epidermis of single-dose UV-exposed skin, whereas MMP-2 also showed a weak increase (Fisher *et al*, 1997). On the other hand, in chronically UVB-exposed wrinkled skin, MMP-2 and MMP-9 both showed similar increases as detected by gelatin zymography (**Fig 6**). Schwartz *et al* (1998) reported that steady-state mRNA levels of α1 and α2 integrins were increased by 2.5 times and by 12.3 times, respectively, in skin treated for 5 wk with UVB and that the immunostaining intensity of α1 integrin was increased in fibroblasts of skin treated for 10 wk with UVB (Schwartz *et al*, 1998). MMP-2 is known to be predominantly synthesized in dermal fibroblasts in skin (Amano *et al*, 2001). Therefore, functionally altered dermal fibroblasts in the structurally modified dermis during chronic UV irradiation may be involved in the increased expression of MMP-2 in chronically UVB-exposed skin.

The increase of gelatinase activities in response to a single exposure to 1 MED of UVB was greater in the basal layer than in the spinal layer of the epidermis, as judged from the gelatin zymography results, whereas the whole epidermis was strongly positive in 10 wk UVB-exposed wrinkle-bearing skin. Therefore, UVB irradiation may induce the synthesis of gelatinases in the basal keratinocytes in the epidermis, and then gelatinase-containing basal keratinocytes may start to differentiate and move upwards.

Gelatinases, MMP-2 and MMP-9, are known to degrade type IV collagen and type VII collagen specifically (Seltzer *et al*, 1989; Aimes and Quigley, 1995), which are components of the epidermal basement membrane. In skin-equivalent models, increased levels of gelatinases inhibited the formation of basement membranes at the dermal–epidermal junction (Amano *et al*, 2001). As expected, the epidermal basement membranes were damaged, and detachment of the epidermis from basal lamina and disruption of the continuity of lamina densa were observed at the dermal–epidermal junction (**Fig 4**). In human skin, similar basement membrane damage, such as reduplication and disruption of lamina densa, was reported to be present at the dermal–epidermal junction of sun-exposed skins (Lavker, 1979; Amano *et al*, 2000). Therefore, the increased gelatinase activity around the basal layer in UVB-irradiated epidermis may cause detachment and disruption of the basement membrane structure by cleavage of basement membrane components, including type IV collagen and type VII collagen, as an MMP inhibitor, CGS27023A, suppressed ultrastructural changes of the basement membrane upon topical administration (**Fig 9**).

It is well known that connective tissue alterations, such as degradation of type I collagen and abnormal deposition of elastin are observed in UV-exposed skin (Kligman *et al*, 1989; Uitto *et al*, 1989). Our electron microscopic study and Sirius Red staining study showed that dermal collagen content tended to be reduced in chronically UVB-exposed mouse skin (**Fig 10**). The decrease was prevented by topical administration of CGS27023A. A recent study has shown that gelatinases are also capable of degrading type I collagen (Kerkvliet *et al*, 1999). *In situ* gelatin zymography did not clarify the change or localization of activity in the dermis because of unclear staining on the dermis, but gelatin zymography showed a tendency for increase in the protein levels of gelatinases in the dermal extract (data not shown). Upregulation of gelatinases caused by UVB may also be involved in the metabolism of collagen fibers in the dermis. On the other hand, we could not detect any effect of the MMP inhibitor on the changes of elastic fibers by immunostaining elastic fibers (data not shown). It is still unclear whether MMP are involved in the alteration of elastic fibers in this experiment.

Previous studies found that UVB-induced wrinkle formation in this model was prevented by various substances, some of which were known to have indirect MMP-inhibitory effects, such as anti-inflammatory agents (Bissett *et al*, 1990a, b, c). The wrinkle-formation-preventing effects of MMP-specific inhibitors, such as hydroxamic acid derivatives, however, have not been studied. We also found a wrinkle-preventing effect of doxycycline, which has a completely different chemical structure from CGS27023A; it does inhibit MMP activities, although less potently than CGS27023A (data not shown). These results suggest that the MMP, especially gelatinases, increase in activity over wide areas of mouse skin during chronic UVB exposure, and contribute to wrinkle formation through destruction of the basement membrane structure, followed by degradation of extracellular matrix components, such as collagen fibers. Topical application of MMP inhibitors may be an effective way to prevent wrinkle formation caused by UVB.

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