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Accumulation of Elafin in Actinic Elastosis of Sun-Damaged Skin: Elafin Binds to Elastin and Prevents Elastolytic Degradation

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Elafin has a primary structure with two functional domains; a transglutaminase substrate domain at the N-terminus and a protease inhibitor domain at the C-terminus. Elafin expression has so far been reported only for epithelial tissues. Accumulation of elafin was immunohistochemically detected in the actinic elastosis of sun-damaged skin. Exposure of normal skin to UVA induced elafin expression that colocalized with elastic fibers. Incubation of synthetic transglutaminase substrate domain of elafin and elastin molecules in the presence of tissue transglutaminase *in vitro* resulted in the formation of a higher molecular complex on SDS-PAGE. Elafin expression was not detected in normal cultured skin fibroblasts, but was induced by UVA irradiation at both messengerRNA and protein levels. When radiolabeled insoluble elastin was incubated with recombinant full-length elafin and tissue transglutaminase, insoluble elastin became more resistant to neutrophil elastase digestion. These results indicate that (1) dermal fibroblasts potentially express elafin on UV irradiation, (2) UV-mediated elafin interacts with elastin, and (3) the elafin–elastin complex protects elastic fibers from elastolytic degradation, leading to the accumulation of elastic fibers in the actinic elastosis of sun-damaged skin. The transglutaminase substrate moiety of elafin plays an important role in anchoring elafin at its proper sites of action during UV-induced aging processes.

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INTRODUCTION

There are two different processes in skin aging; chronological, and photo-induced. Photoaging is related to severe UV-induced damage of the dermal extracellular matrix (Fisher *et al.*, 2002), which is characterized clinically by wrinkle formation and loss of recoil capacity, and histologically by accumulation of disintegrated abnormal elastic fibers in the upper dermis, which is defined as actinic elastosis. The mechanism of the accumulation of altered elastic fibers in the photo-damaged skin is unclear at present, but is important in the search for anti-photoaging drugs (Mera *et al.*, 1987; Sellheyer, 2003). Accumulation of abnormal elastic fibers is considered because of overproduction and/or decreased degradation of elastic fibers. Elastin and fibrillin expression in sun-damaged skin is reported to be increased several-fold (Bernstein *et al.*, 1994); however, no information on elastin degradation in sun-damaged skin is available. Several investigators have shown that exposure of skin to UV light leads to sustained elevation of matrix metalloproteinases (MMPs) (MMP-1, -2, -7, -8, and -12) (Fisher *et al.*, 1997; Saarialho-Kere *et al.*, 1999; Ohnishi *et al.*, 2000) and elastase secreted by infiltrating neutrophils or dermal fibroblasts (Tsukahara *et al.*, 2001; Teunissen *et al.*, 2002; Cavarra *et al.*, 2002), implying that degradation of elastic fibers by elastolytic enzymes is also involved in the formation of actinic elastosis. The activities of MMPs and elastase are regulated by the specific protease inhibitors, tissue inhibitor of metalloproteinases (TIMPs) and skin-derived antileukoproteinase (elafin), respectively. Previous studies have demonstrated that the effects of UV irradiation on TIMP expression are varied; UV irradiation causes no significant change in TIMP-1 and -2 expression in dermal fibroblasts (Brenneisen *et al.*, 1996; Kawaguchi *et al.*, 1996), or decreases TIMP-2 expression (Oh *et al.*, 2004), or increases TIMP-1 expression (Fisher *et al.*, 1997). Elafin expression in the epidermis of normal skin is reported to be increased by UVB irradiation (Pfundt *et al.*, 2001), but the effects of UV irradiation on elafin expression in dermal fibroblasts has never been studied.

The trappin gene family has a unique protein structure defined by (1) an N-terminal domain consisting of a variable number of repeats with the consensus sequence GQDXVK that can act as a substrate for transglutaminase crosslinking, and (2) a C-terminal four-disulfide core or whey acidic protein domain that harbors a functional motif involved in the

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Abbreviations: mRNA, messenger RNA; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase

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binding of proteinases (Schalkwijk *et al.*, 1999). Elafin/skin-derived antileukoproteinase or trappin-2 is a member of the trappin gene family, and has a transglutaminase substrate domain at its N-terminal region that is also called ''cementoin'' (Nara *et al.*, 1994) and a proteinase inhibitor domain at its C-terminal region. The protease inhibitor domain carries an inhibitory function against at least three serine proteinases, human leukocyte elastase, porcine pancreatic elastase, and human leukocyte proteinase 3 (Wiedow *et al.*, 1991; Ying *et al.*, 1994; Pfundt *et al.*, 1996). The transglutaminase substrate domain possesses a GQDXVK sequence and is considered to crosslink with epidermal proteins, keratin 1, and loricrin by transglutaminase (Steinert and Marekov, 1995). Elafin is exclusively expressed by epithelial cells, including the skin epidermis, and mucous epithelium in the trachea, esophagus, and vagina. In the skin epidermis, elafin is extensively found in the subcorneal differentiated keratinocytes of psoriatic epidermis, and absent from normal keratinocytes (Schalkwijk *et al.*, 1993, 1999; Nonomura *et al.*, 1994). Previous studies have been focused on epithelial cells, and no information on their expression in mesenchymal cells such as dermal fibroblasts is available at present.

We, here, found elafin accumulation in the degenerated elastic fibers of photo-damaged skin. Because dermal fibroblasts are responsible for the production/degradation of dermal extracellular matrix proteins, the result led us to investigate whether (1) UV irradiation induces elafin expression in cultured dermal fibroblasts, (2) elafin induced by UV irradiation in fibroblasts interacts with elastin via the transglutaminase substrate domain *in vitro*, and (3) crosslinked elafin may protect elastic fibers from elastolytic degradation. This paper highlights the importance of elafin in elastin accumulation in sun-damaged skin.

RESULTS

Elafin immunoreactivity with elastic fibers of photo-damaged skin

Immunohistochemical studies using mAb against elafin revealed that there was no immunoreactivity in the skin obtained from a sun-exposed area (face) of a 9-year-old child (case 20 in Table 1) (Figure 1a), whereas in the skin obtained from sun-exposed areas (face) of 57- and 79-year-old patients (cases 11 and 8, respectively, in Table 1), a strong elafin immunoreactivity was observed in the degenerated elastic fibers of actinic elastosis (Figure 1b and c). In addition, the subcorneal region of the epidermis from the aged patients reacted with anti-elafin antibody, whereas the epidermis from the 9-year-old child did not (Figure 1a–d). This is consistent with the previous report (Pfundt *et al.*, 2001), and supports the specificity of the antibody used. Thirty-two sun-exposed and sun-protected skin samples derived from subjects of various ages (7–92 years) were immunohistochemically examined (Table 1). No elafin immunoreactivity was detected in sun-protected skins (cases 21–32; 7–90 years), or in eight sun-exposed skin samples from subjects of younger age with low-grade actinic elastosis (G0 and 1) (cases 13–20; 9–49 years). A strong, moderate, or partial positive reaction was observed in sun-exposed skin with high-grade actinic elastosis (G3 and 4) (cases 1–10; 77–92 years) (Table 1). Polyclonal antibody against the transglutaminase substrate domain showed essentially similar results, but the extent of immunoreactivity was lower than that with the mAb (not shown). mAbs against TIMP-1 and -2 showed no significant immunoreactivity with actinic elastosis (not shown).

Table 1. Immunoreactivity of sun-exposed and sun-protected skins with monoclonal anti-elafin antibody

Case no[1]	Age/sex	Site	Degree of actinic elastosis[2]	Reactivity with anti-elafin antibody[3]
1	92/F	Face	G4	±
2	85/F	Face	G4	+
3	85/F	Face	G3	+
4	84/F	Face	G4	+
5	80/F	Face	G4	±
6	80/M	Face	G3	+
7	79/M	Face	G3	±
8	79/M	Face	G3	++
9	77/F	Face	G3	±
10	77/F	Face	G4	±
11	57/F	Face	G2	+
12	52/F	Face	G2	−
13	49/F	Face	G1	−
14	32/M	Face	G1	−
15	28/F	Face	G0	−
16	24/M	Face	G0	−
17	20/F	Face	G0	−
18	19/F	Face	G0	−
19	13/F	Face	G0	−
20	9/F	Face	G0	−
21	90/F	Abdomen	G0	−
22	86/F	Abdomen	G0	−
23	80/M	Abdomen	G0	−
24	70/M	Abdomen	G0	−
25	69/F	Back	G0	−
26	64/M	Abdomen	G0	−
27	61/M	Abdomen	G0	−
28	33/F	Buttock	G0	−
29	26/F	Buttock	G0	−
30	13/M	Buttock	G0	−
31	8/M	Abdomen	G0	−
32	7/F	Buttock	G0	−

Abbreviations: F, female; M, male.
[1]Cases 1–20 indicate sun-exposed skins and cases 21–32 indicate sun-protected skins.
[2]Degree of actinic elastosis was scored according to the criteria by Kligman (1969).
[3]++, strongly positive; +, moderately positive; ±, partially positive; −, negative.

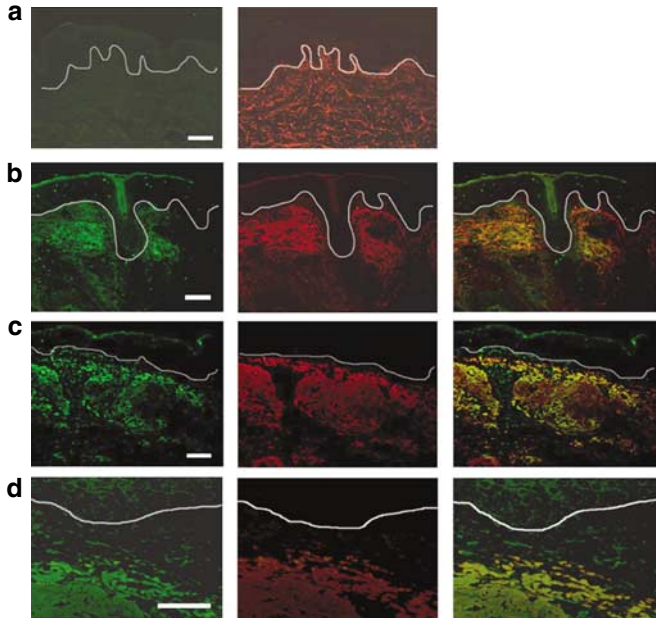

Figure 1. Elafin accumulation in elderly sun-exposed skin. Paraffin-embedded skin specimens were obtained from sun-exposed areas (face) of (**a**) a 9-year-old, (**b**) a 56-year-old, and (**c**) a 79-year-old patient. The skin sections were incubated with monoclonal anti-elafin antibody (1:50) (left) and polyclonal anti-elastin antibody (1:100) (middle), or both (right), then with secondary Ig antibodies. High magnification of the area of the dermal–epidermal junction in (**c**) is presented to show the presence of (**d**) elafin-immunoreactive epidermis. (**a**–**d**) Solid lines indicates the dermal–epidermal junction. (**a**–**d**) Bar = 100 μm.

Single exposure to UVA (10 J/cm^2) of normal skin taken from a sun-protected area of an 8-year-old patient induced elafin-immunoreactive fibers in the dermis (Figure 2a and b) compared to UVB irradiation (0.5 J/cm^2), which minimally induced them (Figure 2c). Double immunofluorescence studies using elafin and tropoelastin antibodies revealed that elafin immunoreactivity induced by UVA irradiation colocalized with elastic fibers (Figure 2d–f). Similar immunohistochemical results were obtained for the other skin samples from 18-, 21-, 23-, and 24-year-old patients (data not shown). *In vivo* irradiation with UVA to the back skin of a patient with suspected photosensitive dermatitis also induced elafin-immunoreactive elastic fibers (Figure 2g–i). The results suggest that elafin is induced by UVA irradiation and exhibits a distribution similar to that of elastic fibers in the dermis.

In vitro crosslink formation by elafin and tropoelastin

To prove that elafin binds to elastin via tissue TGase *in vitro*, two species of synthetic elafin peptides (elafin a, N-terminal crosslinking domain; elafin b, C-terminal protease inhibitor domain) and recombinant tropoelastin were incubated in the presence of tissue TGase, and the resulting elafin–elastin complex was examined *in vitro* by immunoblotting assays. When the blots were incubated with anti-elastin antibody, a higher molecular complex of elastin (75 and ~200 kDa) appeared only in the combination of elafin a (cementoin), tropoelastin, and tissue TGase (Figure 3a, lane 4). In other combination sets, the elastin molecule (68 kDa) and its multiple degradation products (below 68 kDa) were detected (Figure 3a, lanes 1–3 and 5). When the blots reacted with polyclonal anti-elafin antibody against the N-terminal domain, elafin peptide a (migration position shown by arrowhead), and TGase produced protein complexes with molecular weights of 40 and 46 kDa, which are considered self-aggregation forms of elafin a (Figure 3b, lane2). The combination of elafin a and tropoelastin in the presence of tissue TGase produced several high molecular weight (55, 68, 75, 120, and ~200 kDa) polypeptides as well as 40 and 46 kDa polypeptides. The polypeptides of 55 and 68 kDa were considered as complexes of elafin a and degraded elastin (Figure 3, lane 3).

UVA irradiation and IL-1β induce elafin expression in cultured dermal fibroblasts

To define the regulation of elafin expression in actinic elastosis, elafin expression in cultured fibroblasts was determined by RT-PCR assays. We tested the effects of various cytokines, including IL-1β, tumor necrosis factor-α, transforming growth factor-β, and IFN-γ, on the expression of elafin. Untreated (control) cells expressed no significant amount of elafin messenger RNA (mRNA) (Figure 4a, lane 1). In addition, no elafin mRNA was detected in cells treated with transforming growth factor-β (lane 2), tumor necrosis factor-α (lane 3), or IFN-γ (lane 6). IL-1β alone was found to enhance the elafin mRNA level (297 bp) in cultured fibroblasts at doses of 10 and 100 ng/ml (Figure 4a, lanes 4 and 5). Elafin mRNA was detectable in UVA-irradiated fibroblasts irrespective of the dose of radiation (0.5 and 1 J) (Figure 4a, lanes 7 and 8), whereas UVB failed to induce elafin mRNA at a dose of 50 mJ (Figure 4a, lane 9). Elafin mRNA was expressed in the cultured keratinocytes, as described previously (Alkemade *et al.*, 1994), but to a lesser extent than in the treated fibroblasts (Figure 4a, lane 10).

To confirm the results of RT-PCR, real-time PCR was performed (Figure 4b). Because untreated control fibroblasts did not express detectable amounts of elafin mRNA, relative values from cells treated with IL-1β (10 ng/ml) were calculated. IL-1β-treated (100 ng/ml) cells, UVA-irradiated cells, and keratinocytes were found to express 1.4-, 11-, and 14-fold elafin mRNA respectively, compared with IL-1β-treated (10 ng/ml) cells.

Because elafin polypeptide contains a signal sequence at its N-terminal domain and was considered to be secreted mostly into the culture medium, we measured the elafin level present in the medium fraction. Western blot assays demonstrated that treatment of cultured fibroblasts with IL-1β (50 ng/ml) upregulated elafin polypeptide (12 kDa) in culture media, whereas no detectable elafin was observed with the untreated cells (Figure 5, lanes 1 and 2). Irradiation of cultured fibroblasts by UVA induced 12 kDa elafin-immunoreactive polypeptide in the culture media at doses of 0.5 and 1 J (Figure 5, lanes 3 and 4), in contrast to UVB irradiation (50 and 100 mJ) (Figure 5, lanes 5 and 6). Elafin polypeptide was undetectable in the untreated cells (Figure 5, lane 7). Cultured keratinocytes treated with IL-1β (50 ng/ml) showed

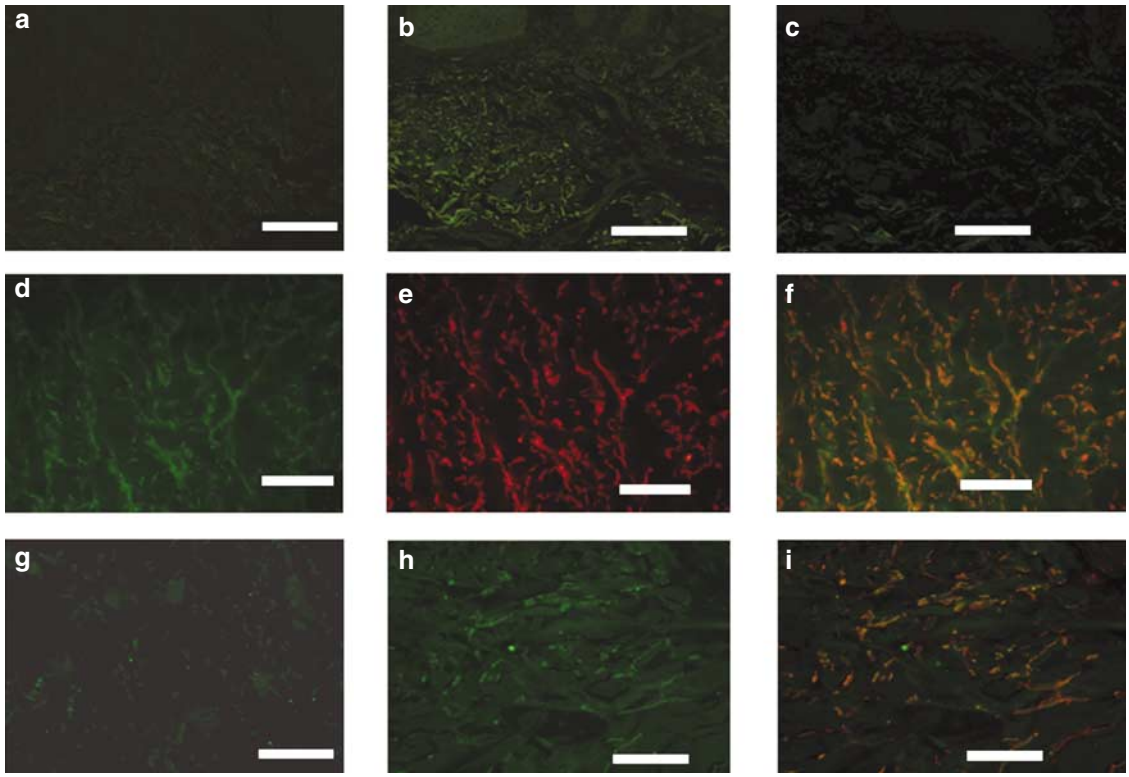

Figure 2. UVA induces elafin-immunoreactive fibers. (**a–c**) Normal skin from a sun-protected area (buttocks) of an 8-year-old patient were irradiated with (**a**) none or (**b**) UVA (10 J/cm^2), or (**c**) UVB (0.5 J/cm^2) immediately after surgical resection. After incubation at 37°C for 18 hours, skin specimens were embedded and incubated with monoclonal anti-elafin antibody (1:50). (**d–f**) A double immunofluorescence study was performed on an UVA-irradiated skin specimen taken from the buttock of an 8-year-old patient using (**d**) monoclonal anti-elafin antibody (1:50) or (**e**) polyclonal anti-elastin antibody (1:100), or (**f**) both. (**g–i**) The back skin of a 38-year-old patient with suspected photosensitivity was irradiated with UVA for 3 days at a total dose of 52.92 J/cm^2. Skin samples were taken by punch biopsy (**g**) before and (**h**) after irradiation, then processed for immunohistochemical study using anti-elafin antibody. (**i**) Double immunofluorescence was performed on UVA-irradiated skin using anti-elafin and anti-elastin antibodies. (**a–i**) Bars = 100 and 50 μm.

multiple 11–12 kDa polypeptides (Figure 5, lane 8). Heterogeneous molecular weights of elafin polypeptides have been reported, depending on the material studied: 6 kDa in psoriatic scales, ~9 kDa from skin extracts, and 8–10 kDa in cultured keratinocytes (Schalkwijk *et al.*, 1991; Alkemade *et al.*, 1994) and 4 kDa from tracheal mucous epithelium (Nara *et al.*, 1994). The molecular weight of elafin polypeptide in the cultured fibroblasts seems to be consistent with that in the cultured keratinocytes.

Degradation of elafin crosslinked insoluble elastin by leukocyte elastase

To prove the inhibitory activity of the crosslinked elafin against its target protease, radiolabeled insoluble elastin (specific activity 5.3×10^5 c.p.m./mg) was pretreated with recombinant full-length elafin and TGase, and then digested with neutrophil elastase. In the positive control experiments, radiolabeled elastin was found to be proportionately degraded within at least 4 hours by neutrophil elastase (Figure 6, ♦—♦), whereas, in the negative control experiments, no significant degradation was detected in the absence of elastase (Figure 6, ●●). When the elastin was incubated with elafin and TGase before elastase digestion, the degradation rate of elastin by elastase was significantly decreased (Figure 6, ■■). When the insoluble elastin was pretreated with elafin alone, it degraded at the same rate as the positive control (Figure 6, ▲▲).

DISCUSSION

We found elafin accumulation in the elastic materials of actinic elastosis in elderly subjects, but not in the sun-exposed skins of young people or in sun-protected skin of all ages. Because the deposited material in actinic elastosis has been reported to be derived from disintegrated elastic fibers, that is, caused by long-term UV irradiation, accumulation of elafin in sun-damaged skin may be related to UV irradiation. Negative immunoreactivity of anti-TIMP-1 and -2 antibodies with sun-damaged skin suggests that elafin rather than TIMP is involved in the accumulation of elastic fibers. Several investigators have shown that exposure of the skin to UV induces leukocyte infiltration (Woodbury *et al.*, 1994) and elastase secreted by leukocytes or dermal fibroblasts (Tsukahara *et al.*, 2001; Cavarra *et al.*, 2002; Teunissen *et al.*, 2002). Moreover, neutrophil elastase is considered to be an important mediator in the development of actinic elastosis, because elastic fibers in the leukocyte elastase-deficient mice were unaffected by exposure to UV irradiation, whereas in normal mice the elastin content was altered following UV

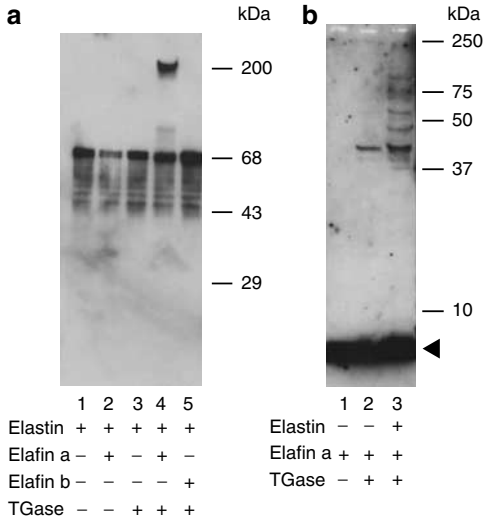

Figure 3. Transglutaminase-mediated crosslinking between elafin and elastin *in vitro*. Elafin peptides (elafin a, synthetic N-terminal crosslinking domain corresponding to amino acids 27–60; elafin b, synthetic C-terminal protease inhibitor domain corresponding to amino acids 61–117), and elastin were incubated for 1 hour at 37°C in the absence or presence of TGase. The products of the different assay sets were analyzed by SDS-PAGE followed by immunoblot using (**a**) polyclonal anti-elastin or (**b**) polyclonal anti-elafin antibody against the synthetic N-terminal domain. The arrowhead in (**b**) indicates the migration position of elafin a peptide.

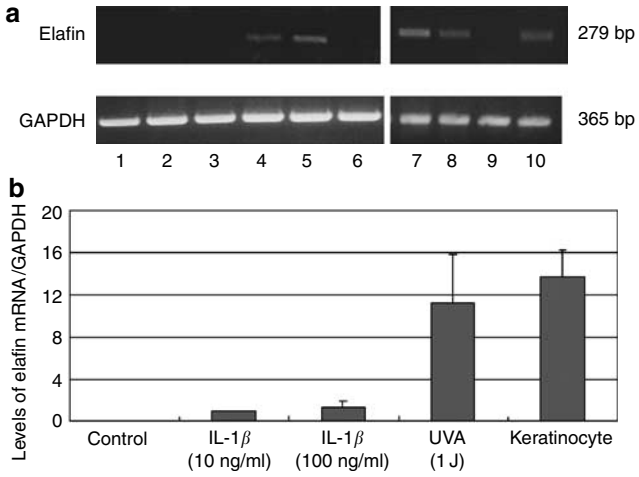

Figure 4. Induction of elafin mRNA in cultured skin fibroblasts. (**a**) Fibroblasts were treated for 24 hours without (lane 1) or with 10 ng/ml transforming growth factor-β1 (lane 2), 10 ng/ml tumor necrosis factor-α (lane 3), 10 ng/ml IL-1β (lane 4), 100 ng/ml IL-1β (lane 5), or 100 U/ml IFN-γ (lane 6), or irradiated with 0.5 J UVA (lane 7), 1 J UVA (lane 8), or 50 mJ UVB (lane 9). RNA was isolated from cultured fibroblasts (lanes 1–9) or cultured keratinocytes in KGM media containing growth factors (lane 10). The elafin and glyceraldehyde-3-phosphate dehydrogenase mRNA levels were determined by RT-PCR. (**b**) Real-time PCR amplification of elafin mRNA stimulated by UVA irradiation or IL-1β treatment. Fibroblasts were untreated, or treated with IL-1β (10 or 100 ng/ml) for 24 hours, or irradiated with UVA (1 J). The absolute values for elafin mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase. IL-1β-treated (100 ng/ml) cells, UVA-irradiated cells, and keratinocytes were found to express 1.4-, 11-, and 14-fold elafin mRNA, respectively, compared with IL-1β-treated (10 ng/ml) cells.

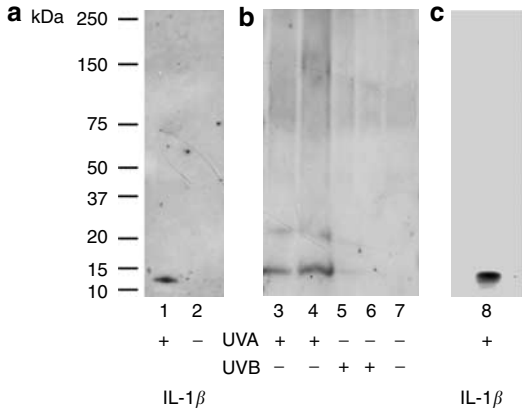

Figure 5. Detection of elafin polypeptide in cultured skin fibroblasts. (**a**) Cultured fibroblasts were treated for 24 hours without (lane 2) or with 50 ng/ml IL-1β (lane 1) in serum-free DMEM. (**b**) Cultured fibroblasts were irradiated with 0.5 J UVA (lane 3), 1 J UVA (lane 4), 50 mJ UVB (lane 5), 100 mJ UVB (lane 6), or none (lane 7). (**c**) Cultured keratinocytes were treated with 50 ng/ml IL-1β for 24 hours in KGM (lane 8). Cultured media were harvested and the proteins were precipitated with 10% trichloroacetic acid. The pellets were subjected to SDS-PAGE followed by immunoblot assay using polyclonal anti-elafin antibody.

irradiation (Starcher and Conrad, 1995). Therefore, the detection of elafin in the actinic elastosis of sun-damaged skin suggests that elafin, a potent inhibitor of leukocyte elastase, protects the elastic fibers from undesirable degradation by neutrophil elastase. An *ex vivo* experiment (single exposure to UVA of resected normal skin) and an *in vivo* experiment (repeated exposure to UVA of patient's skin) both induced expression of elafin, which readily binds to elastic fibers. Binding of elafin to elastic fibers represents the initial event of actinic elastosis. These immunohistochemical findings imply that the accumulation of elastic fibers in sun-damaged skin is caused by decreased degradation of elastic fibers rather than overproduction of elastic fibers.

The N-terminal domain of elafin (cementoin) contains the repeated consensus sequence unit GQDXVK, which is a good substrate for transglutaminase without requiring secondary or tertiary structures for recognition by transglutaminase. In this report, we found that elafin readily binds to elastin via transglutaminase-mediated crosslinking *in vitro*. It has been shown that elafin is secreted into the extracellular space and is associated with functionally essential proteins. The transglutaminase substrate domain of elafin is now known to bind to several extracellular matrix proteins such as laminin, fibronectin, β-crystallin, and to a lesser extent, collagen IV (Nara *et al.*, 1994; Guyot *et al.*, 2005). Elastic fibers are essential for maintaining the elasticity of the skin and consist of central amorphous material (elastin) and surrounding microfibrils (fibrillins, microfibril-associated glycoproteins, and fibulins). Tissue TGase plays a critical role in the organization and stabilization of elastic fibers by mediating the crosslinks between elastin and fibrillin-1, and elastin and microfibril-associated glycoproteins-1 (Brown-Augsburger *et al.*, 1994; Rock *et al.*, 2004). Accelerated degradation of elastic fibers by elastolytic enzymes causes the

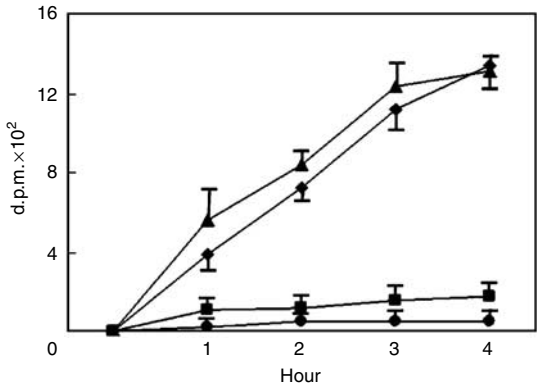

Figure 6. Elafin-crosslinked insoluble elastin is less susceptible to neutrophil elastase. [^{3}H]-labeled insoluble elastin (100 μg) was incubated with elafin (5 μg) in the presence or absence of tissue transglutaminase (20 mU) at 37°C for 1 hour, then digested with neutrophil elastase (5 μg) at 37°C for 0–4 hours. After centrifugation, the supernatant was counted. ■■; pretreated with elafin and tissue TGase before elastase digestion, ▲▲; pretreated with elafin alone before elastase digestion, ◆—◆; without pretreatment before elastase digestion, ●●; without pretreatment and elastase digestion. Values are mean ± range obtained from two separate assays.

acquired type of cutis laxa, in which loss of elasticity and multiple wrinkles are the major clinical signs (Fornieri *et al.*, 1994). In addition, we have also shown that elafin, which covalently crosslinks to insoluble elastin, nevertheless inhibits the activity of neutrophil elastase. This indicates that the transglutaminase substrate domain of elafin protects elastin from degradation by anchoring the inhibitor domain at its proper site of action. This kind of targeted immobilization of elafin at the elastic fibers of actinic elastosis appears to be very important for protecting against UV-induced aging processes. Accumulation of elafin in the elastic fibers of actinic elastosis may represent a protective response against undesirable elastolytic degradation.

Secretory leukocyte protease inhibitor is a proteinase inhibitor produced by epithelial cells (lungs, oral cavity, epidermis, and testis) and some non-epithelial cells (macrophages) (de Water *et al.*, 1986). This protein consists of a tandemly repeated four disulfide core motif, the second domain being structurally related to the C-terminal domain of elafin. However, this elafin-related proteinase inhibitor is only weakly expressed in the normal epidermis, but is strongly expressed in the tape-stripping or psoriatic epidermis. The dermal elastic fibers beneath the tape-stripping epidermis were found to be immunoreactive with anti-secretory leukocyte protease inhibitor antibody (Wingens *et al.*, 1998), indicating that secretory leukocyte protease inhibitorI and elafin are readily associated with elastic fibers in pathological conditions and prevent elastic fibers from degradation by the same mechanism.

Studies on elafin have been focused on the epithelial or epidermal cells but not on mesenchymal cells, because previous Northern blot assays and immunohistochemistry have shown that elafin is constitutively expressed in a number of normal human epithelia and is always negative in mesenchymal and neural tissues (Schalkwijk *et al.*, 1999). Restricted localization of elafin expression in epithelia may be related to its defensive role against external agents, including microorganisms (Pfundt *et al.*, 1996). We found in this report that skin fibroblasts are able to express elafin in restricted conditions of UVA and IL-1β exposure. It has been shown that UVB irradiation of normal human skin increases elafin expression by epidermal cells with a rapid induction of phosphorylation of c-jun and p38 mitogen-activated protein kinase (Pfundt *et al.*, 2001). The question of whether the elafin detected in actinic elastosis is of epidermal origin or dermal origin remains unanswered. It is possible that keratinocyte-derived elafin induced by UV irradiation is secreted from epidermal keratinocytes and accumulates in the superficial dermis. However, the elafin of the epidermis is known to crosslink to the major epidermal proteins keratin 1 and loricrin, being incorporated into the cornified cell envelope, and accounts for ~6% of all proteins in the cornified cell envelope (Steinert and Marekov, 1995; Nakane *et al.*, 2002). Further, UV-mediated induction of elafin in dermal fibroblasts rather than epidermal keratinocytes is involved in the accumulation of elafin in sun-damaged skin.

In this report, we demonstrated that IL-1β treatment and UVA irradiation both upregulated elafin expression in cultured fibroblasts. Upregulation of elafin expression by IL-1β or UV irradiation has been previously reported in the epidermis (Pfundt *et al.*, 2001), cultured keratinocytes (Tanaka *et al.*, 2000), and lung alveolar cells (Sallenave *et al.*, 1994). Because UV exposure of the skin or cultured keratinocytes or fibroblasts is known to upregulate various cytokines, such as IL-1β, tumor necrosis factor-α, and IL-6, -7, and -10 (Wlaschek et al., 1994; Kligman *et al.*, 1999; Brink *et al.*, 2000), it is possible that UV-induced elafin upregulation in cultured fibroblasts is mediated by UV-induced IL-1β stimulation. Similar autocrine stimulation has been reported in UVA-induced MMP-1 expression, in which the upregulation of MMP-1 by UVA is mediated by UVA-induced IL-1 and IL-6 (Wlaschek et al., 1994).

In conclusion, we present the possibility that the accumulation of abnormal elastic fibers in the sun-damaged skin is related to the decreased rate of elastin degradation. UV-induced elafin may play a major role in the decreased degradation of elastic fibers.

MATERIALS AND METHODS

Proteins and antibodies

Recombinant human elafin (amino-acid residues 1–117) containing two domains was purchased from R&D Systems Inc. (Minneapolis, MN). Synthetic human elafin peptide corresponding to the protease inhibitor domain (amino-acid residues 61–117) was purchased from HyCult Biotechnology BV (Uden, The Netherlands). mAb (clone No. TRAB 20, which recognizes the GQDPVK epitope) was purchased from HyCult Biotechnology BV. The N-terminal transglutaminase crosslinking domain, cementoin, consisting of 34 amino acids (amino acids 27–60, VPVKGQDTVKGRVPFNGQDPVKGQVSVKG QDKVKY) was chemically synthesized. Polyclonal antibody for the transglutaminase substrate domain (cementoin) was prepared as follows. A peptide segment corresponding to the amino-acid

sequence of cementoin was synthesized as a tetravalent multiple antigenic peptide (MMP-(cementoin)$_4$) and used for immunization of rabbits, because 34 amino acids is not large enough to produce a high titer antiserum (Nara *et al.*, 1994). The recombinant human tropoelastin used in this study was prepared and purified as previously described (Wachi *et al.*, 2004). Polyclonal antibody against human tropoelastin was purchased from the Elastin Products Company (Owensville, MI). Insoluble elastin extracted from bovine neck ligamentum was from Sigma-Aldrich Co. (St Louis, MI). mAbs against human TIMP-1 and -2 were purchased from Daiichi Fine Chemical Co. (Toyama, Japan).

Skin specimens and UV irradiation

Thirty-two paraffin-embedded skin specimens were obtained from sun-exposed and sun-protected areas in our department files between 2001 and 2005, under prior informed consent (Table 1). Normal-appearing perilesional areas of benign or premalignant tumors such as nevus, seborrheic keratosis, and actinic keratosis were subjected to immunohistochemical examination. The presence of actinic elastosis was examined by hematoxylin-eosin, and Elastica van Gieson stains. The degree of actinic elastosis was scored according to Kligman (1969) as follows: G0, no change; G1, increase in number without thickening; G2, greater hyperplasia with thickening and curling; G3, marked hyperplasia with thickening and curling, and frequent branching; G4, complete replacement of the dermis by a dense tangle of thickened, disorderly fibers accompanied by disorganization into murky amorphous masses.

Five normal skin samples were obtained from 8-, 18-, 21-, 23-, and 24-year-old patients with an epidermal cyst or nevus on the buttocks or back. Immediately after surgical resection of the tumors, normal-appearing peripheral skin was irradiated with UVA or UVB at a single dose of 10 or 0.5 J/cm^2, respectively. The source of UV irradiation was a Dermaray M-DMR-1 (Toshiba Medical Supply, Tokyo, Japan) with lamps of FL20S·E30 (UVA) and FL20S·BLB/DMR (UVB). After irradiation, the skin samples were incubated in a humidified chamber for 18 hours at 37°C, and then embedded in optimum cutting temperature compound for immunohistochemical studies.

For *in vivo* experiments, the back skin of a 38-year-old male patient, who presented dermatitis on sun-exposed (face and arms) areas, was used. To examine the presence of photosensitivity, the back skin was irradiated with UVA for 3 days at a total dose of 52.92 J/cm^2. After 24 hours, skin samples were collected by punch biopsy before and after irradiation, embedded in paraffin, and then subjected to immunohistochemical examination.

Immunohistochemistry

Specimens embedded in paraffin or optimum cutting temperature were cut into 5 μm sections. The sections were washed with phosphate-buffered saline and then stained with polyclonal anti-elastin/monoclonal anti-elafin antibodies for 24 hours at 1:50 dilution with elafin antibody and at 1:100 dilution with elastin antibody. Bound antibodies were visualized with rhodamine-conjugated goat anti-rabbit IgG (1:20 dilution) (Dako, Glostrup, Denmark) and fluorescein-conjugated goat anti-mouse IgG (1:10 dilution) (Dako). Fluorescence was evaluated with a confocal laser scan microscope (LSM 410, Carl Zeiss, Jena, Germany).

Binding assay

For cross-binding experiments, 0.5 μg of synthesized human elafin peptide corresponding to the transglutaminase substrate domain (amino acids 27–60) (elafin a) or protease inhibitor domain (amino acids 61–117) (elafin b), 0.1 μg tropoelastin, and 1 mU of guinea-pig liver transglutaminase (Sigma-Aldrich Co., St Louis, MI) or various combinations of the three components were mixed in 20 μl of 50 mM Tris-HCl, pH 7.5. The reaction was initiated by the addition of 1 mM $CaCl_2$. After incubation for 1 hour at 37°C, the reaction mixtures were diluted with SDS-sample buffer containing 1 mM dithiothreitol, and then boiled for 2 minutes. Samples were subjected to SDS-PAGE and transferred to membranes. The blots were incubated with polyclonal anti-elafin or anti-elastin antibody.

Treatment of cultured skin fibroblasts

A fibroblast cell line established from a 4-year-old normal healthy child was cultured in DMEM supplemented with 10% calf serum. Cells grown at a confluent density were washed twice with phosphate-buffered saline and covered for 24 hours with fresh, serum-free DMEM containing IL-1β (10 or 100 ng/ml), tumor necrosis factor-α (100 ng/ml), transforming growth factor-β1 (100 ng/ml), or IFN-γ (100 U/ml). In some experiments, cells were irradiated with UVA at a total dose of 500 or 1,000 mJ/cm^2, or UVB at a total dose of 50 or 100 mJ/cm^2. During irradiation, cells were incubated in phosphate-buffered saline and maintained at 35–37°C in a thermostatic chamber. Following irradiation, phosphate-buffered saline was replaced with fresh 10% calf serum DMEM or serum-free DMEM, then the cells were further incubated for the last 24 hours.

For a positive control experiment, normal human keratinocytes purchased from Sanko Junyaku (Tokyo, Japan) were cultured in serum-free, low-calcium (0.1 mM) modified MCDB-153 medium supplemented with growth factors including epidermal growth factor, insulin, and bovine pituitary extract (designated as KGM).

Conventional RT-PCR and real-time RT-PCR

RNA was isolated from the cultured fibroblasts or keratinocytes with guanidine thiocyanate (Chomczynski and Sacchi, 1987), then adjusted to a concentration of 1 μg/ml. Exon-specific primer pairs were synthesized: 5′-GTTCCTGTTAAAGGTCA-3′ for the upstream primer and 5′-TCACTGGGGAACGAAAC-3′ for the downstream primer of human elafin (Saheki *et al.*, 1992), and 5′-CCAGCC GAGCCACATCGCTC-3′ for the upstream primer and 5′-ATGAG CCCCAGCCTTCTCCAT-3′ for the downstream primer of glyceraldehyde-3-phosphate dehydrogenase (Tso *et al.*, 1985) as an internal standard. Total RNA (5 ng) was used for the first-strand complementary DNA synthesis. RT-PCR was performed in the presence of 3 pmol of a 3′-oligonucleotide and 35 U of reverse transcriptase from avian myoblastosis virus (Takara Shuzo Co., Otsu, Shiga, Japan) in a total reaction volume of 20 μl (Kawasaki, 1990). The resulting complementary DNA was then subjected to the first PCR using 25 pmol 5′-oligonucleotide and additional 3′-oligonucleotide in a total volume of 100 μl. The complementary DNA was amplified for 35 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes. Analysis of all of the PCR products was performed by agarose (1.5%) gel electrophoresis followed by ethidium bromide staining.

Taq Man® Gene Expression Assays were purchased from Applied Biosystems (Foster City, CA). The primers and probes of the elafin gene are as follows: forward primers, 5′-GACTGCCCAGGAATCAA GAAGT-3′; reverse primers, 5′-CTGTGAAGGCTCTTGC-3′; and FAM probe, 5′-CTGTGAAGGCTCTTGC-3′. The glyceraldehyde-3-phosphate dehydrogenase primer/probe set was also purchased from Applied Biosystems. Elafin and glyceraldehyde-3-phosphate dehydrogenase mRNA levels were quantified in triplicate using Applied Biosystems 7900 HT Real-Time PCR system according to the supplier's recommendations. Because elafin mRNA levels in the untreated control cells were found to be undetectable, relative values for IL-1β-treated (10 ng/ml) cells were presented.

Western blot

A medium of cultured fibroblasts grown in two 10 cm diameter dishes was pooled and mixed with a protease inhibitor cocktail (1 mM EDTA, *N*-ethylmaleimide, and phenylmethylsulfonyl fluoride). The proteins were precipitated with 10% trichloroacetic acid, resolved on a 4–20% or 10–20% gradient SDS-PAGE gel under reducing conditions, and transferred to nitrocellulose membranes (Millipore Co., Bedford, MA) in a tank blotter in 25 mM Tris/0.192 M glycine, pH 8.3/20% methanol at 30 V overnight. The filters were blocked with 5% BSA in 10 mM Tris-HCl, pH 8.0/150 mM NaCl/0.05% Tween (TBST) for 2 hours and incubated with anti-human elafin polyclonal antibody against the N-terminal transglutaminase substrate domain or anti-human elastin polyclonal antibody at 1:10,000 dilutions for 24 hours. The blots were then washed with TBST for 30 minutes and incubated with a second antibody (anti-rabbit Igs) (Dako) at 1:5,000 dilution for 1 hour. Antigens were visualized using chemiluminescence (ECL, Amersham).

Elastase digestion assay

Radiolabeling of bovine insoluble elastin was performed by reductive alkylation using [^{3}H]-sodium borohydride as described previously (Banda *et al.*, 1987; Park *et al.*, 1996). The specific activity of tritiated elastin was 5.3×10^5 c.p.m./mg. One hundred micrograms of radiolabeled insoluble elastin was incubated with elafin (10 μg) and tissue TGase (20 mU) at 37°C for 1 hour, then exhaustively washed with phosphate-buffered saline by centrifugation at 10,000 g for 10 minutes at 4°C, to remove unbound elafin. The precipitated elastin was suspended in 100 μl buffer (50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$), then incubated with human leukocyte elastase (5 μg) (Sigma-Aldrich Co.) at 37°C for 0–4 hours. The reaction was terminated by centrifugation to remove any remaining insoluble elastin. The radioactivity in the supernatant was determined by scintillation counting (LSC-5100, Aloka, Tokyo, Japan).

Patients and samples

The patients, parents, and normal control individuals participated in this study following informed consent. They gave their written informed consent. This study was conducted according to the Declaration of Helsinki Principles and has been approved by the Medical Ethical Committee of National Defense Medical College.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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