N^{ε}-(Carboxymethyl)lysine Modification of Elastin Alters Its Biological Properties: Implications for the Accumulation of Abnormal Elastic Fibers in Actinic Elastosis

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Accumulation of degenerated elastic fibers in the sun-exposed skin designated as actinic elastosis is a histological hallmark of photodamaged skin. Previous studies have indicated that the elastic fibers of actinic elastosis interact with lysozyme and are modified by N^{ε}-(carboxymethyl)lysine (CML), one of the major advanced glycation end products (AGEs). We studied here how CML modification of elastin is involved in the pathogenesis of actinic elastosis. The CML-modified insoluble elastin became resistant to neutrophil elastase digestion, which was reversed by treatment with aminoguanidine, a potent inhibitor of AGE formation. In a temperature-dependent aggregation assay, CML-modified elastin rapidly formed self-aggregates, the size of which was larger than unmodified elastin. The elastic fiber sheets prepared from CML-modified α-elastin showed 3D wider diameter, tortuous appearance, and decreased elasticity on tensile tests. The CML-modified α-elastin, but not unmodified α-elastin, was found to bind to lysozyme *in vitro*, supporting the immunohistochemical findings that the antibodies for lysozyme and CML reacted simultaneously with the elastic fibers of actinic elastosis and UV-irradiated skin. The glycated elastin is likely to cause the accumulation of abnormally aggregated elastic fibers and unusual interaction with lysozyme in actinic elastosis.

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INTRODUCTION

Elastic fibers provide resilience and elasticity to many tissues, including the skin, lungs, ligaments, and arterial walls. On electron microscopic examination, it was observed that they comprise a central elastin core and peripheral microfibrils, the latter of which are organized by microfibrillar proteins, including fibrillins, microfibril-associated glycoproteins, fibulins, and latent TGF-β-binding proteins that serve as a template to promote elastin polymerization. The mechanism by which monomeric elastin assembles into the polymeric extracellular elastic matrix is not yet well understood, but self-assembly of elastin has been suggested to be important for the first stage of proper elastogenesis (Wagenseil and Mecham, 2007). Non-elastic protein, lysozyme, has been shown to associate with elastic fibers, but the mechanism and significance of its binding is unclear at present.

Long-term incubation of proteins with glucose *in vitro* leads to the formation of advanced glycation end products (AGEs) through early-stage products such as Schiff base and Amadori rearrangement products. Several AGE structures have been identified, including N^{ε}-(carboxymethyl)lysine (CML), pyrraline, and pentosidine (Monnier *et al.*, 1996). The formation of several AGEs requires oxidation, and is dependent on the presence of transition metals for the oxidation of glucose or the Amadori products. AGEs are thought to be involved in aging and age-enhanced diseases, such as diabetes mellitus, atherosclerosis, dialysis-related amyloidosis, and Alzheimer's disease (Baynes *et al.*, 2005). In the skin, the accumulation of degenerated elastic fibers in the upper dermis of sun-exposed skin by the age of 40 years, which is called ''actinic elastosis'', has been reported. Immunohistochemical and immunoelectron microscopic studies using an mAb for CML demonstrated that CML accumulates predominantly in elastic fibers, especially in the

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Abbreviations: AGE, advanced glycation end product; CML, N^{ε}-(carboxymethyl)lysine; MED, minimal erythema dose; PBS, phosphate-buffered saline

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amorphous electron-dense materials (elastin) of actinic elastosis (Mizutari *et al.*, 1997). How CML-modified elastin is involved in the development of actinic elastosis is uncertain.

Lysozyme (EC 3.2.1.17) is a 1, 4-β-*N*-acetylmuramidase capable of cleaving bacterial cell wall peptidoglycan. Lysozyme is found in virtually all organisms, including bacteria, insects, and mammals, and is found at high concentrations in a wide variety of tissues and body fluids (Jollès and Jollès, 1984). In human tissues, lysozyme has been detected by immunohistochemical studies in the epithelial cells, leukocytes, and connective tissue cartilage (Mason and Taylor, 1975). The association of lysozyme with elastic fibers has been demonstrated in the elastotic area of breast carcinoma, internal elastic lamina of vascular walls, and actinically damaged dermal elastic fibers (Davies *et al.*, 1983; Mera and Davies, 1987; Mera *et al.*, 1987; Albrecht *et al.*, 1991; Park *et al.*, 1996). The mechanism, and significance, by which lysozyme associates with elastic fibers is unknown, although previous studies suggest that the association occurs most often with abnormal elastic fibers resulting from tissue injury. It is unlikely that lysozyme participates as an integral component of elastic fibers, because its association with elastin occurs in a pattern suggesting a nonspecific absorption resulting in a gradual accumulation over time.

In this study, we attempted to determine how AGE modification alters elastin assembly, proteolytic degradation, physical properties, and the interaction with other proteins such as lysozyme. We discuss whether AGE-induced alterations of elastin can explain the histological accumulation of abnormal elastic fibers in photodamaged skin.

RESULTS

Degradation of CML-modified insoluble elastin by neutrophil elastase

We examined whether ribose or UV irradiation causes CML formation of α-elastin *in vitro*. Ribose treatment for 2 weeks induced the CML formation of α-elastin in a dose-dependent manner. UV irradiation also significantly stimulated the CML formation of α-elastin in the presence of ribose, but without ribose no significant stimulation was seen after the 2-week irradiation. However, for a longer period of irradiation of 3 and 4 weeks, UV significantly induced CML formation of α-elastin even in the absence of ribose, although to a lesser extent than the CML content produced in the presence of ribose (Figure 1a).

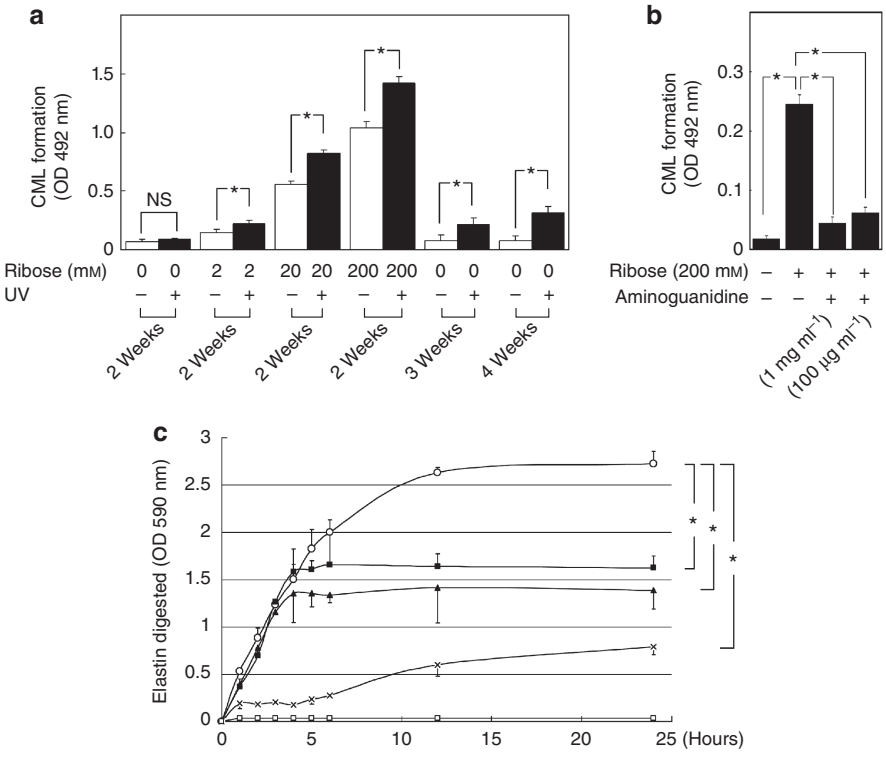


Figure 1. Elastase digestion of N^{ε}-(carboxymethyl)lysine (CML)-modified insoluble elastin. (**a**) α-Elastin-coated wells were treated for 2 weeks at 37 °C with indicated doses of ribose in the presence or absence of solar-simulated irradiation or treated with solar-simulated irradiation for longer periods of 3 and 4 weeks in the absence of ribose. The CML content of α-elastin was measured by non-competitive ELISA using the antibody against CML. (**b**) Orcein-labeled insoluble elastin (5 mg ml^{-1}) was incubated with 0.2 M ribose in the presence or absence of 100 μg ml^{-1} or 1 mg ml^{-1} aminoguanidine for 1 week at 37 °C. An aliquot was taken and placed on the wells overnight at 4 °C. The CML content of insoluble elastin coated on the wells was determined by ELISA. (**c**) The elastin was digested with leukocyte elastase (10 U per tube) at 37 °C for indicated periods. The optical density of the supernatant was measured at 590 nm. Indicated are unmodified elastin with (○–○) or without (□–□) elastase digestion, and CML-modified elastin in the absence (×–×) or presence of 1 mg ml^{-1} (■–■) or 100 μg ml^{-1} (▲–▲) aminoguanidine, followed by elastase digestion. The values are the means ± SD from triplicate assays. *Statistical significance at $P<0.01$. NS, not significant; OD, optical density.

Insoluble elastin was incubated with 0.2 M ribose at 37 °C for 1 week in the absence or presence of 100 μg ml^{-1} or 1 mg ml^{-1} aminoguanidine, an inhibitor of AGE formation. The CML content of insoluble elastin in the presence of 100 μg ml^{-1} or 1 mg ml^{-1} aminoguanidine decreased compared with the untreated samples (Figure 1b). CML-modified insoluble elastin was found to be very resistant to neutrophil elastase digestion, compared with unmodified insoluble elastin, during the digestion periods of 0–24 hours. When insoluble elastin was incubated with 0.2 M ribose in the presence of 100 μg ml^{-1} or 1 mg ml^{-1} aminoguanidine, the elastin was found to be moderately resistant to elastase, but less resistant than fully CML-modified elastin, thus suggesting that the resistance to elastase paralleled the degree of CML content (Figure 1c).

CML modification of α-elastin accelerates self-aggregation

Upon increasing the temperature of unmodified and CML-modified α-elastin, their absorption at 400 nm started to increase at 47 and 43 °C, respectively, reached a maximum level at 55 and 48 °C, respectively, and thereafter decreased gradually and promptly, respectively. The coacervation patterns of unmodified α-elastin in the presence or absence of 0.2 M ribose were very similar (open and closed circles in Figure 2a), which indicates that the presence of the high concentration of ribose (0.2 M) in the coacervation assays did not influence the coacervation rate of α-elastin. CML modification of α-elastin decreased the coacervation temperature (T_c; the temperature of approximately 50% of the maximum absorption; $T_c = 46$ °C for CML-modified elastin and $T_c = 50$ °C for unmodified elastin) and accelerated the precipitation rate at a higher temperature (Figure 2a). Staining of the aggregates with uranyl acetate showed that the appearance of unmodified elastin aggregates was relatively regular, with a rod-like structure (Figure 2b, left panel), whereas CML-modified elastin had a larger particle-like structure (Figure 2b, arrows in right panel). The sizes of aggregates of the CML-modified α-elastin on optical microscopy were 5- to 6-fold larger than the unmodified α-elastin after 2 and 8 minutes of incubation (Figure 2c).

Scanning election microscopy and measurement of physical parameters of CML-modified elastin sheets

Scanning electron microscopy (SEM) observation of elastin sheets prepared from the liquid-phase CML-modified α-elastin revealed 3D wider fibers than the unmodified sheets (0.82 ± 0.42 vs. 2.5 ± 0.7 μm in unmodified vs. CML-modified α-elastin; $P < 0.01$; Figure 3a). This is in agreement with the results of the liquid-phase coacervation assay in which the sizes of aggregates of CML-modified α-elastin were larger than unmodified α-elastin (see Figure 2b and c). When solid α-elastin fiber sheets were directly glycated with 0.2 M ribose for 2 weeks, the CML-modified elastin sheets showed a curling and tortuous appearance compared with the straight and linear elastic fibers of unmodified elastin sheets (Figure 3b). Measurement of the elastic modulus and rupture elongation of unmodified and CML-modified elastic fiber sheets revealed decreased elastic modulus and rupture elongation of the glycated sheets prepared from both liquid and solid phases of α-elastin (Figure 3c).

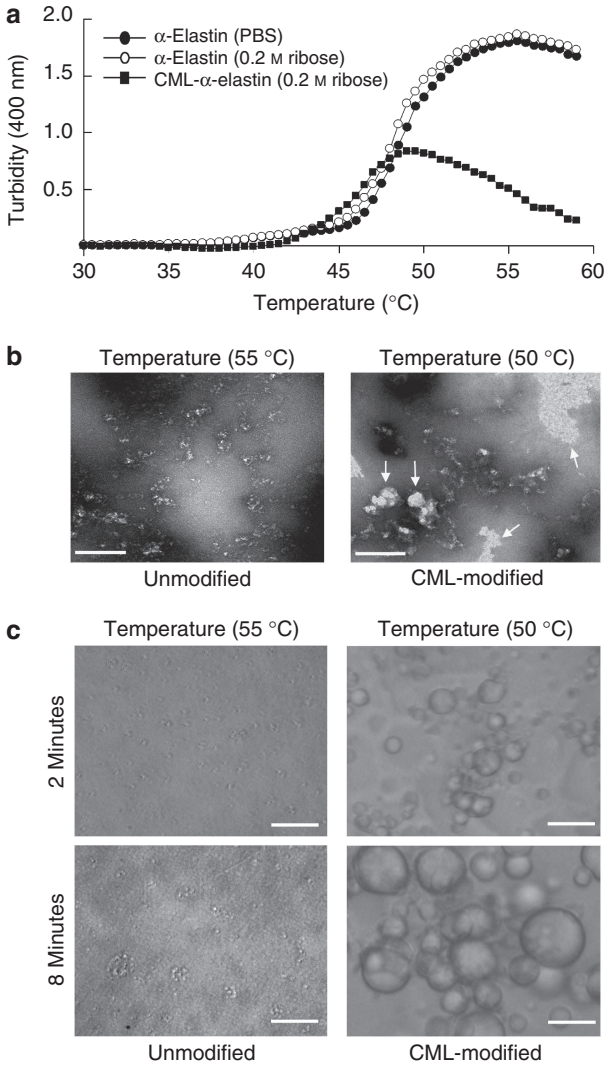
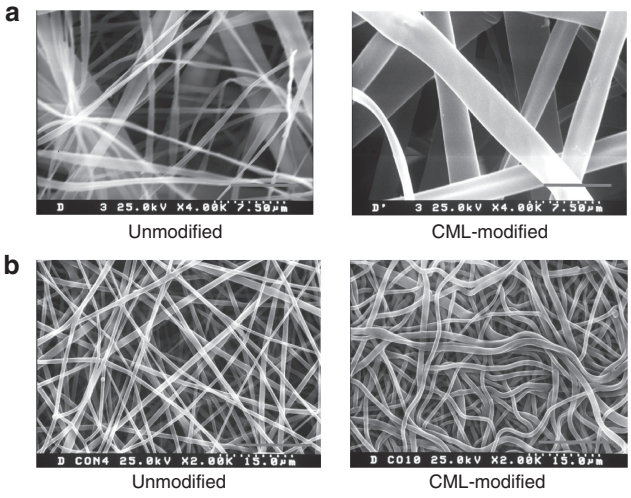


Figure 2. The coacervation and morphology of self-aggregates of N^{ε}-(carboxymethyl)lysine (CML)-modified α-elastin. (**a**) α-Elastin (10 mg ml^{-1} phosphate-buffered saline (PBS)) was treated at 37 °C for 2 weeks with PBS (designated as α-elastin (PBS)) or 0.2 M ribose (designated as CML-α-elastin). For a control experiment, 0.2 M ribose was added to α-elastin (PBS) solution just before the coacervation assay (designated as α-elastin (0.2 M ribose)). The coacervation assay was performed at different temperatures from 30 °C, increasing at a rate of 1 °C min^{-1}. (**b**) The self-aggregates of unmodified (left) or CML-modified α-elastin (right panel) were stained with uranyl acetate with warming at 55 or 50 °C, respectively, and then immediately observed by electron microscopy. Bar = 200 nm. (**c**) The self-aggregates of unmodified (left) or modified α-elastin (right panel) were incubated for 2 or 8 minutes at the indicated temperature (55 or 50 °C), and then observed by optical microscopy. Bar = 30 μm.

Lysozyme binds to AGE-modified elastin, but not to unmodified elastin

When various amounts of radiolabeled lysozyme (1×10^4–5×10^5 c.p.m. per well) was incubated for 8 hours with CML-modified α-elastin, collagen type I, BSA, and



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	Elastic modulus (kPa)	Rupture elongation (%)
Liquid phase		
Unmodified sheet	90.8 ± 11.7 *	165.1 ± 39.6 *
CML-modified sheet	30.3 ± 9.6	74.5 ± 17.1
Solid phase		
Unmodified sheet	100.3 ± 5.0 *	199.6 ± 65.9 *
CML-modified sheet	89.4 ± 1.8	91.3 ± 18.9

Figure 3. Scanning electron microscopy (SEM) and physical parameters of N^{ε}-(carboxymethyl)lysine (CML)-modified elastic sheets. The α-elastin solution was incubated with or without 0.2 M ribose for 2 weeks at 37 °C. (**a**) The CML-modified or -unmodified α-elastin solutions were solidified to sheets, and then the 3D structure of the fibers was observed by SEM. Bar = 7.5 μm. The α-elastin solution was transformed to solid sheets and then glycated with 0.2 M ribose for 2 weeks at 37 °C. (**b**) The morphology of the glycated or non-glycated elastin sheets was observed by SEM. Bar = 15 μm. (**c**) The elasticity of the sheets (elastic modulus and rupture elongation percent) was measured. The values are the means ± SD of triplicate assays. *$P < 0.01$.

fibulin-5, the radioactivity of bound lysozyme was dose-dependently increased, whereas radiolabeled lysozyme did not bind to unmodified α-elastin, collagen type I, BSA, and fibulin-5 (Figure 4a). When radiolabeled lysozyme (2×10^5 c.p.m.) was incubated with CML-modified α-elastin, collagen type I, BSA, and fibulin-5 for 0, 2, 8, or 24 hours, the amount of bound lysozyme was increased with incubation periods, whereas the radioactivity of bound lysozyme to unmodified substrates was very low during the entire 0- to 24-hour time period (Figure 4b). The presence of 10 mM $CaCl_2$ in the binding assays did not alter the amount of bound lysozyme to CML-modified α-elastin (not shown).

Immunoreactivity of sun-exposed and sun-protected skin with anti-lysozyme and anti-CML antibodies

In the sun-exposed (face) skin, 39- and 80-year-old subjects showed positive reactions for both anti-lysozyme and anti-CML antibodies, but 20-year-old subjects showed negative reactions (Figure 5a, left and middle columns). The intensity of the immunoreactions increased with age. The localization of both immunoreactivities was found to be mostly identical by a double immunofluorescence study (Figure 5a, right columns). In the sun-protected area (buttocks), there was essentially no positive reaction with the antibodies for lysozyme and CML (figures not shown).

Immunohistochemical studies on the sun-exposed skin samples (15 cases) and sun-protected skin samples (7 cases) demonstrated that the immunoreactivity of anti-lysozyme and anti-CML antibodies were well correlated with the degree of actinic elastosis and both became positive in the subjects above the age of 40 years in the sun-exposed skins, whereas in the sun-protected skins the immunoreactivity with both antibodies was not observed (table not shown).

Immunoreactivity with anti-lysozyme and anti-CML antibodies in UV-irradiated human skin

Exposure of 2 minimal erythema dose (MED) of solar-simulated radiation on human back skin for 4 weeks resulted in weak but significant positive reactions with both anti-lysozyme and anti-CML antibodies (Figure 5b, lower panels), whereas no significant signals were found in the skins after 1 week of exposure (Figure 5b, upper panels). Both antibodies generally recognized identical fibrous materials (arrows in lower panels of Figure 5b).

DISCUSSION

Because elastin is a long-lived protein, mature elastic fibers undergo glycoxidation by chronic UV irradiation or reactive oxygen species exposure during human aging. It is very unlikely that newly synthesized elastin molecules (tropoelastin) undergo a glycoxidation reaction before the crosslinking reaction or elastic fiber formation. For this reason, we used insoluble elastin or α-elastin (insoluble, crosslinked elastin solubilized by oxalic acid), not tropoelastin, for the substrates of the elastase digestion, coacervation, and *in vitro* binding assays.

We demonstrated that UV irradiation of α-elastin in the absence of ribose enhanced CML formation. Because direct irradiation of UV to elastin or collagen causes the production of H_2O_2 at the level of 2–5 μM (Wondrak *et al.*, 2003), and because α-elastin prepared from bovine ligamentum nuchae may contain trace amounts of Amadori product, UV-induced CML formation will be mediated by oxidative cleavage by hydroxyl radical at the site of the carbon chain between C-2 and C-3 of carbohydrate chain, which is considered to be a major route of CML formation *in vivo* (Nagai *et al.*, 1997).

We also demonstrated that CML-modified elastin becomes resistant to elastase digestion, and that this is related to the degree of CML modification of elastin. In general, the accumulation of a protein in the skin can be attributed to either an increase in the production or a decrease of degradation of that protein. Although a 2- to 4-fold increase in elastin expression (mRNA level) has been previously reported in the photodamaged skin with actnic elastosis (Bernstein *et al.*, 1994), we believe that this 2- to 4-fold increase in elastin expression is not sufficient to explain the increase in the bulk of elastotic fibers from superficial to mid-dermis. Rather, a decrease in elastin degradation is more likely to be involved in the accumulation of elastin in photodamaged skin.

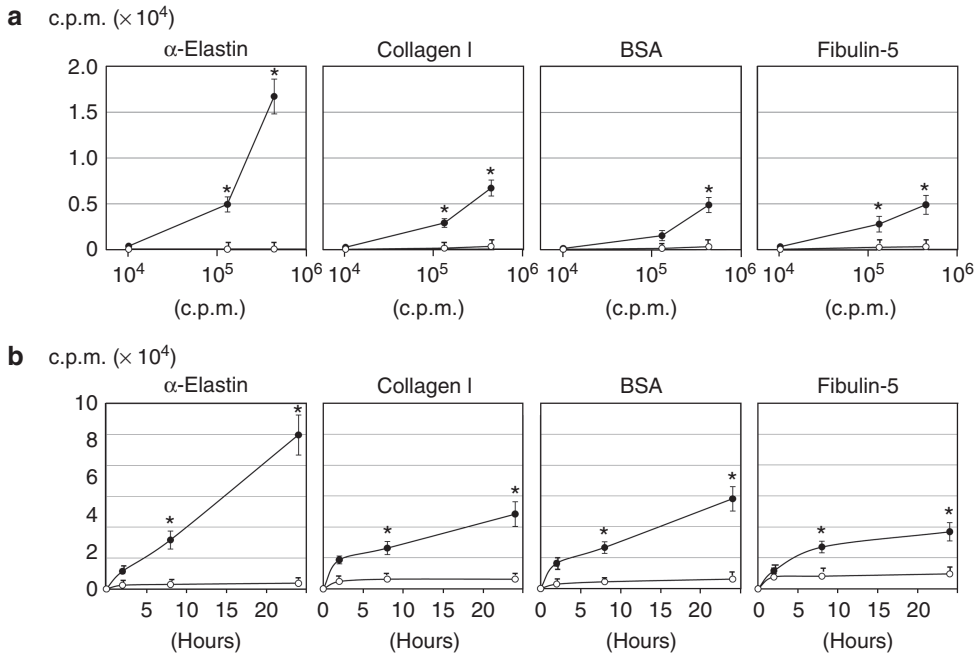

Figure 4. The interaction of N^{ε}-(carboxymethyl)lysine (CML)-modified elastin with lysozyme *in vitro*. Wells were coated with 10 μg ml^{-1} of α-elastin, collagen type I, BSA, and fibulin-5 at 4 °C for 24 hours. Half of the wells underwent CML modification by 0.2 M ribose treatment for 2 weeks at 37 °C. Lysozyme was radioiodinated with [^{125}I] using IODOGEN (specific activity, 1.2×10^4 c.p.m. ng^{-1}). CML-modified (●–●) and unmodified (○–○) substrates were incubated with various doses of [^{125}I] lysozyme (1×10^4, 2×10^5, and 5×10^5 c.p.m.) (**a**) for various periods (0, 2, 8, and 24 hours). (**b**) The radioactivities bound to the substrates were dissolved with Tris-Glycine buffer, pH 3.0, for 2 hours and counted. Indicated are the means ± SD from triplicate assays. *$P < 0.01$ versus unmodified substrates. c.p.m., counts per minute.

Elastin degradation is regulated by several elastin-related proteinases and their inhibitors. Elastin-degrading proteinases include neutrophil elastase, elastolytic matrix metalloproteinases (MMP-7 and -12), and skin fibroblast elastase (Tsukahara *et al.*, 2001; Cavarra *et al.*, 2002), but it is uncertain as to which is the major proteinase responsible for the degradation of dermal elastin *in vivo*. Elastase inhibitor would also be an important regulator of elastin degradation. We have previously demonstrated that elafin, an inhibitor of neutrophil elastase, is induced by UV irradiation, interacts with elastin via the transglutaminase substrate domain of elafin, and protects elastin from elastolytic degradation in the sun-damaged skin (Muto *et al.*, 2007). AGE formation in elastin and elafin binding to elastin will synergistically function as very strong inhibitors of elastolytic degradation, and will therefore cause elastic fiber hyperplasia during the initial stage (G1 of Kligman's grade) of actinic elastosis (Kligman, 1969).

Decreased degradation of elastin by itself, however, does not fully explain the accumulation of abnormally degenerated elastic fibers in actinic elastosis. The major pathogenesis of actinic elastosis might be due to the abnormal elastic fiber arrangement that occurs during elastogenesis. The property of self-assembly (elastin–elastin interaction) intrinsic to elastin itself is also important as a first step for its proper elastogenesis. Coacervation is dependent on the hydrophobic interactions between elastin polypeptides. Accelerated self-aggregation of CML-modified elastin may be due to the relative increase of hydrophobicity by the modification of lysine residue of elastin. Quicker self-assembly, larger sizes of self-aggregates, and 3D tortuosity of samples prepared from CML-modified α-elastin indicated an increased aggregation potential of CML-modified elastin polypeptides, which likely causes elastic fiber thickening and curling in the second stage (G2 of Kligman's grade) of actinic elastosis. CML modification of elastin also alters its physical properties to less elastic sheets, indicating that CML modification of elastic fibers is associated, at least a part, with a loss of dermal elasticity in photodamaged skin, which may be associated with an increase in wrinkle formation (Fujimura *et al.*, 2007; Ezure *et al.*, 2009).

Immunohistochemical examinations of normal skin derived from subjects of various ages and various sites demonstrated that elastic fibers are a major dermal component that reacted with the monoclonal anti-CML antibody, and that the appearance of CML-modified elastic fibers is limited to the sun-exposed areas of individuals over the age of 40 years, which was in agreement with a previous paper (Mizutari *et al.*, 1997) in which they demonstrated that elastin and CML, although both present within degenerated elastic fiber of actinic elastosis, do not strictly colocalize, as observed by the immunoelectron microscopic study; hence, the possibility still remains that other elastin-related proteins, such as microfibrillar proteins, proteoglycans (Bernstein *et al.*, 1995), and amyloid P protein (Dahlbäck *et al.*, 1990), may also undergo glycation. We further demonstrated that CML-modified elastic fibers and lysozyme-immunoreactive elastic fibers were generally colocalized, and simultaneously appeared in the

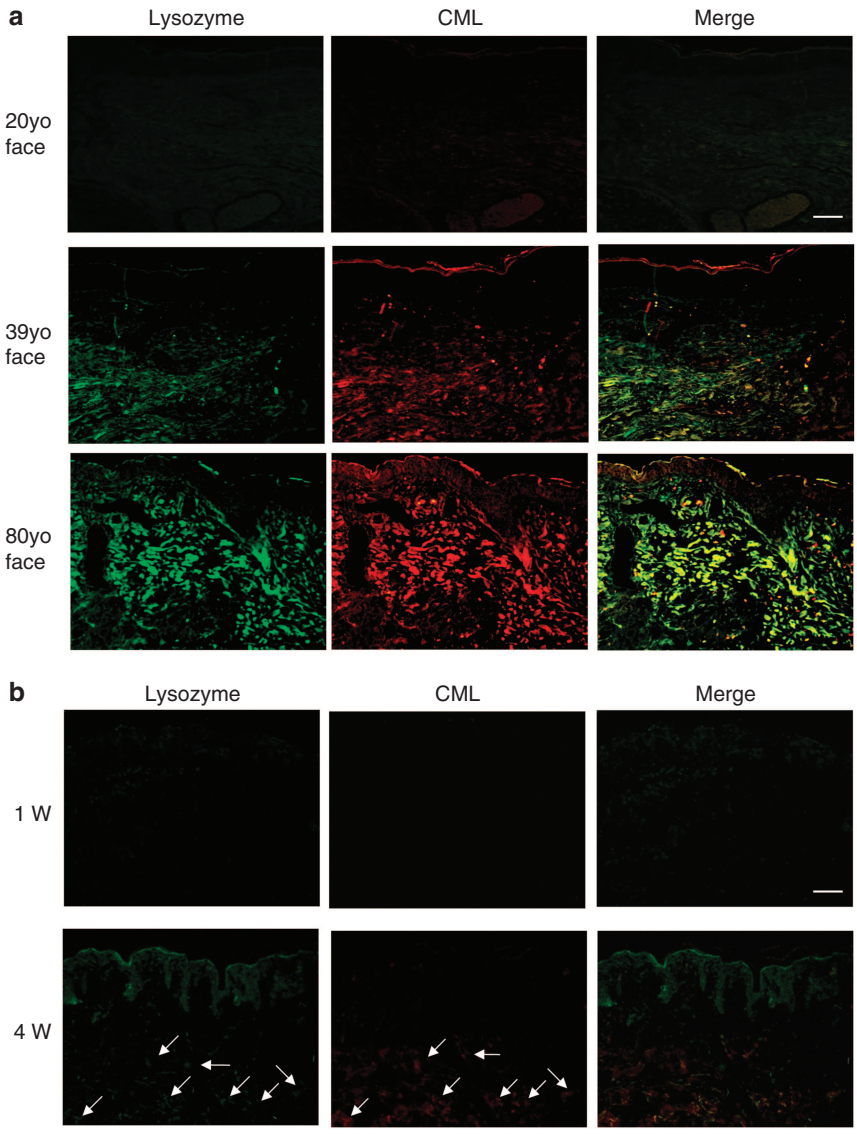

Figure 5. Simultaneous appearance of lysozyme- and N^{ε}-(carboxymethyl)lysine (CML)-immunoreactive elastic fibers in human skin. (**a**) Immunohistochemical detection of lysozyme- and CML-immunoreactive elastic fibers in the sun-exposed skin of subjects of various ages. Paraffin-embedded normal skin slices were incubated with polyclonal anti-lysozyme (left panels) and monoclonal anti-CML antibodies (middle panels) or merged (right panels). Bar = 100 μm. (**b**) Detection of lysozyme and CML after UV irradiation of human skin. The back skins of normal healthy men (23 or 24 years old (yo)) were irradiated with solar-simulated light at doses of 2 minimal erythema dose (MED) for 1 (upper panels) or 4 (lower panels) weeks (W), and then subjected to immunohistochemical examination using anti-lysozyme (left panels) and anti-CML antibodies (middle panels) or merged (right panels). Bar = 50 μm.

sun-exposed areas of the subjects older than 40 years, suggesting that the interaction between elastin and lysozyme is mediated by CML.

Immunohistochemical detection of lysozyme on the elastic fibers in UV-treated human skin has been reported previously, and the disappearance of lysozyme immunoreactivity has been the focus of evaluations of the effectiveness of sunscreen products (Fourtanier *et al.*, 1992; Seité *et al.*, 2000; Seité and Fourtanier, 2008). In this study, using the same UV-irradiation protocol, we found CML- and lysozyme-immunoreactive fibers after 4 weeks of irradiation. The strong immunoreactivity of the epidermis with the antibody for lysozyme after 4 weeks of irradiation (see Figure 5b) will be related to the activation of lysozyme expression by UV irradiation. The simultaneous appearance and colocalization of CML- and lysozyme-immunoreactive elastic fibers in sun-damaged skin samples in subjects over the age of 40 years *in vivo* and UV-irradiated sun-protected (back) skin specimens of young volunteers *in vivo* will be in agreement with an *in vitro* direct interaction of CML-modified elastin with lysozyme. However, in *in vitro* binding assays, lysozyme seems to be able to bind to AGE-modified BSA, fibulin-5, or collagen type I as well, although to a lesser extent than CML-modified α-elastin (see Figure 4). Past studies demonstrated that lysozyme is capable of binding to a number of AGE-modified proteins. In fact, lysozyme-linked affinity matrix has

been used for removing AGE-modified proteins such as AGE-modified IgG molecules from the sera of diabetic patients with end-stage renal disease (Mitsuhashi *et al.*, 1997). The biological significance of the lysozyme–CML interaction in photodamaged skin is unknown. Because lysozyme has no elastolytic activity (Park *et al.*, 1996) and its enzymatic activity is inhibited by glycated proteins (Li *et al.*, 1995a, b), lysozyme–CML interaction appears to be related to the protection from tissue injury caused by overexpressed lysozyme derived from UV-induced inflammation.

MATERIALS AND METHODS

Proteins and antibodies

The mAb against CML (clone no. 6D12) and polyclonal antibody against human lysozyme prepared from neutrophils were purchased from Sigma-Aldrich (Tokyo, Japan). α-Elastin from bovine neck ligaments was purchased from Elastin Product Company (Owensville, MO), which is fractionated from hot oxalic acid-soluble elastin followed by repeated coacervation (Partridge *et al.*, 1955). Human collagen type I was purchased from Nihon Millipore KK (Tokyo, Japan). Recombinant human fibulin-5 was produced according to the previously described procedures (Wachi *et al.*, 2008). Orcein-labeled insoluble elastin prepared from bovine ligamentum nuchae was purchased from Elastin Product.

Preparation of glycated protein-coated dishes

The protein solutions, α-elastin (10 μg ml^{-1} phosphate-buffered saline (PBS)), collagen type I (10 μg ml^{-1} 0.3% acetic acid), BSA (10 μg ml^{-1} PBS), and fibulin-5 (10 μg ml^{-1} PBS), were aliquoted onto 6- or 96-well plates for 24 hours at 4 °C, and then washed with PBS four times. The adherence of proteins on the dishes was confirmed by ELISA using the respective antibodies. In some experiments, commercially available elastin-coated 6- and 96-well plates were purchased (BD BioCoat Cellware, Bedford, MA). To prepare plates coated with glycated proteins, the plates were incubated under sterile conditions with 0.2 M D-ribose (SERVA, Heidelberg, Germany) and were maintained at 37 °C for 1–3 weeks. In some experiments, elastin-coated plates were exposed to solar-simulated radiation (UVA 5 J per day + UVB 200 mJ per day) for 1–3 weeks. In some assays, elastin-coated plates were incubated with 0.2 M ribose in the presence of aminoguanidine hydrochloride (100 μg ml^{-1} or 1 mg ml^{-1}; Sigma-Aldrich), a potent inhibitor of AGE formation. Excess ribose was removed by washing several times with sterile PBS. A non-competitive ELISA was performed to determine the CML content of the protein. Briefly, the wells were blocked with 100 μl of 1% BSA in washing buffer (0.05% Tween-20 in PBS) at room temperature for 1 hour and incubated with 100 μl of anti-CML antibody (6D12) (1 μg ml^{-1}) at room temperature for 2 hours. After washing with washing buffer three times, the wells were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody at room temperature for 1 hour. The antigen–antibody complex was visualized by 1, 2-phenylenediamine dihydrochloride, and the absorbance at 492 nm was read with a plate reader.

Elastase digestion assay

The digestion of insoluble elastin was performed essentially as described in a previous paper (Banda *et al.*, 1987). Orcein-labeled insoluble elastin was suspended in PBS at 5 mg ml^{-1}, and then incubated with 0.2 M ribose at 37 °C for 1 week in the presence or absence of 100 μg ml^{-1} or 1 mg ml^{-1} aminoguanidine (Matsumoto *et al.*, 1997; Hou *et al.*, 1998). After washing with PBS exhaustively, the glycated or unglycated insoluble elastin (5 mg per tube) was digested with leukocyte elastase (10 U per tube) (Sigma-Aldrich) in 0.05 M Tris-HCl, pH 7.4, containing 20 mM $CaCl_2$, for 0–24 hours at 37 °C. The optical density of the supernatant fraction of the reaction mixture was measured at 590 nm.

Coacervation test

The coacervation assays were performed as described previously (Bellingham *et al.*, 2001). α-Elastin (10 mg ml^{-1} PBS) was treated with or without 0.2 M ribose for 2 weeks. The CML-modified and -unmodified α-elastin solutions were transferred into a 50-μl quartz cuvette for turbidity measurements at 400 nm in a temperature-controlled spectrophotometer (V-550; JASCO, Tokyo, Japan) and assayed at specific temperatures between 30 and 60 °C. The temperature was increased from 30 °C at a rate of 1 °C min^{-1}. The coacervation temperature (T_c) was determined as the temperature at 50% maximal absorbance. To examine the effects of 0.2 M ribose on the coacervation pattern of α-elastin, coacervation was performed with or without 0.2 M ribose.

Optical and electron microscopy

Optical microscopic observation of elastin aggregates was performed as described previously (Clarke *et al.*, 2006; Cirulis *et al.*, 2008; Tu and Weiss, 2008). Self-aggregates being formed in unmodified and CML-modified α-elastin were placed on the glass slides that were pre-warmed to 55 or 50 °C, respectively, by a hot plate, and then incubated for 2 or 8 minutes at respective temperatures, because coacervate droplet size increases with maturation time. The size of droplets in 20 random, non-overlapping fields was measured under an optical microscope. For electron microscopic observation, elastin aggregates were placed on the copper grids (mesh × 400) with warming by a hot dryer at respective temperature, and then negatively stained with 4% uranyl acetate for 3–4 seconds during warming (Gheduzzi *et al.*, 2005). Immediately after removal of excess staining solution with a filter paper, the aggregates were examined with a Hitachi model HU-11A electron microscope.

Measurement of the elasticity of α-elastin sheets

Two different procedures were used to prepare the CML-modified α-elastin sheets: α-elastin solution was first glycated with 0.2 M ribose for 2 weeks as described above, and then the solution was transformed to solid sheets (liquid-phase CML-modified α-elastin sheets) by the electrospinning method (Buttafoco *et al.*, 2006; Miyamoto *et al.*, 2009), or the α-elastin solution was directly solidified to sheets, which were then glycated with 0.2 M ribose for 2 weeks (solid-phase CML-modified α-elastin sheets). Unmodified or modified elastin fiber sheets were observed using an SEM at 5 kV (JSM-6340F; JEOL, Tokyo, Japan). The physical parameters of elasticity (elastic modulus and rupture elongation) of the sheets were determined by a tensile tester (FG-1; ECM Laboratories, Mie, Japan).

Solid-phase binding assay

Lysozyme (Sigma-Aldrich) from human neutrophils was radio-iodinated with Na [^{125}I] using IODOGEN (Pierce Biotechnology,

Rockford, IL) as described by the supplier. Briefly, 14 μg of lysozyme in 100 μl of Tris-buffered saline (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) was incubated with 300 μCi of Na [^{125}I] in an IODOGEN-coated microcentrifuge tube for 10 minutes at room temperature. Unincorporated [^{125}I] was separated from labeled lysozyme by PD-10 column chromatography. The specific activity of the radiolabeled lysozyme was approximately 1.2×10^4 c.p.m. ng^{-1}.

The wells coated with α-elastin, collagen type I, BSA, and fibulin-5 were incubated with 0.2 M ribose at 37 °C for 2 weeks. The CML content of each substrate was found to have comparable values (3.5, 3.0, 2.8, and 2.5 in α-elastin-, collagen type I-, BSA-, and fibulin-5-coated wells, respectively). The binding of lysozyme to substrates was assessed by incubating radiolabeled lysozyme with coated CML-modified or -unmodified substrates in 200 μl of assay buffer (Tris-buffered saline with or without 10 mM $CaCl_2$). The duration of assays (0–24 hours) and amount of input radiolabeled lysozyme (1×10^4–5×10^5 c.p.m.) are described in the respective figure legends. The binding assays were terminated by washing exhaustively with Tris-buffered saline. The associated lysozyme was eluted with Tris-Glycine buffer, pH 3.0, for 2 hours at room temperature. The radioactivity of the Tris-Glycine buffer was measured with a gamma counter (ARC-2000; Aloka, Tokyo, Japan).

Skin specimens and immunohistochemistry

In all, 22 paraffin-embedded skin specimens were obtained from sun-exposed (15 cases) and sun-protected (7 cases) areas of subjects of various ages from our department files. Normal-appearing perilesional areas of benign tumors such as a nevocellular nevus and seborrheic keratosis were selected and subjected to immunohistochemical examination. The degree of actinic elastosis was scored according to the Kligman's criteria (Kligman, 1969). The paraffin-embedded skin specimens were cut into 10 μm sections, washed with phosphate-buffered saline, and then stained with a monoclonal anti-CML antibody or polyclonal anti-lysozyme antibody for 24 hours at a 1:100 dilution. Bound antibodies were visualized with rhodamine-conjugated goat anti-rabbit IgG (1:20 dilution; Dako, Glostrup, Denmark) or 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).

Sequential UV irradiation of human skin

The procedures used for UV irradiation of human skin were followed as described previously (Lavker *et al.*, 1995; Phillips *et al.*, 2000; Seité *et al.*, 2000, 2004). A solar simulator (Model XES-1002S; San-Ei Electric Corporation, Tokyo, Japan) equipped with a 1,000 W Xenon lamp power supply (Model UXL-10SCB; Ushio, Tokyo, Japan) and a VIS–infrared bandpass-blocking filter was used for the study. The areas on the lower part of the back of two healthy volunteers (23- and 24-year-old Japanese men, skin type II) were exposed to 2 MED of solar-simulated radiation (UVA 1.0 J + UVB 404 mJ per exposure) for 5 days per week for 1 and 4 weeks. The initial irradiation dose was 0.5 MED, which was determined individually before treatment. After 3 hours from the last irradiation, skin samples were collected by punch biopsy, and then stored at −80 °C until use. The samples were subjected to immunohistochemical examination using the antibodies for lysozyme and CML as described above.

Patients and samples

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

CONFLICT OF INTEREST

The authors state no conflict of interest.

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