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ORIGINAL ARTICLE

Elastin changes during chronological and photo-ageing: the important role of lysozyme

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elastin, elastosis, human leucocyte elastase, lysozyme, solar simulated radiation, UVA

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Abstract

Background Cutaneous ageing, as a result of combined chronological and photo-ageing in sun-exposed areas, is accompanied by major modifications of the elastic fibres.

Aim We aimed to investigate qualitative and quantitative changes of dermal elastin fibres during cutaneous chronological and photo-ageing and the involvement of lysozyme in these processes.

Methods Morphological, age-related changes and variations in the relative elastin content in sun-protected (buttock) and sun-exposed (forearm and face) skin of healthy volunteers were studied (145 samples). The deposition of lysozyme in elastin fibres was studied using light and immuno-electron microscopy and taking into consideration the relative efficacy of different UV wavebands (UVA or SSR (solar simulated radiation)). Our studies also included the proteolytic degradation of elastin by human leucocyte elastase (HLE) *in situ*.

Results Our results indicate a reduction of elastin content with age in sun-protected and sun-exposed skin, associated for the latter with high elastin content, resulting in elastosis. Total UVA (320–400 nm), and in particular long wave UVA (UVA-1, 340–400 nm), induces lysozyme deposition in elastin fibres to a higher extent than solar simulated radiation (SSR, 280–400 nm). Immuno-electron microscopy revealed lysozyme association with the electron-dense granular amorphous elastin structures, corresponding to a basophilic degeneration induced by sun exposure. Lysozyme has no elastolytic activity *in situ*; however, its binding to elastin limits elastin degradation by human leucocyte elastase (HLE). In addition, a direct inhibitory effect of lysozyme on HLE was observed.

Conclusion Our data suggest that lysozyme prevents elastin degradation by HLE after binding to the damaged parts of the elastin network and by direct lysozyme–HLE interaction, which reduces HLE proteolytic activity. These observations contribute to a better understanding of the chronological and photo-induced changes of the dermal elastic network.

Introduction

Cutaneous ageing is a complex biological process that affects the different components of the skin. It involves two independent processes, chronological or innate ageing and sun-induced actinic damage.[1] Although clearly distinct in their biological, biochemical, and molecular mechanisms, both processes result in major changes of the elastic fibres.[2,3] Actinically damaged skin is characterized by an accumulation of elastotic material, while in sun-protected areas the number of elastic fibres is decreased.[4] The common age-associated feature in both processes is the loss of normal elastic fibre functions, which may well explain some of the manifestations of cutaneous ageing, including wrinkling and sagging of the skin, with a loss of resilience and elasticity.[1]

The histological hallmark of photo-ageing is dermal elastosis, which largely consists of thickened, tangled and ultimately granular amorphous elastin structures.[5–7] The magnitude of the progressive accumulation of elastic fibres depends on the degree of sun exposure. The mechanisms leading to the accumulation of elastotic material in actinically damaged skin are currently unclear.

Using immunohistochemical techniques, several studies have described (i), an increased lysozyme, alpha-1 antitrypsin and amyloid P deposition in elastic fibres in sun-exposed skin[8–10] and (ii), a correlation between the staining intensity and the extent of sun damage.[8,9,11] As lysozyme and alpha-1 antitrypsin at high concentrations inhibit the activity of collagenase and elastase[12] one might speculate that these proteins protect elastic fibres from proteolysis.[13]

The purpose of the present study was to evaluate qualitative and quantitative changes of dermal elastin fibres during cutaneous ageing. To better understand the involvement of lysozyme in this process, the deposition of lysozyme in elastin fibres was studied using light and electron microscopy, taking into consideration the relative efficacy of different UV wavebands. We also studied the proteolytic degradation of elastin by human leucocyte elastase (HLE) *in situ*.

Materials and methods

Biopsies

Skin biopsies were obtained from healthy Caucasian volunteers (skin types II and III), enrolled after signing an informed consent that was approved by an ethics committee. A total of 145 skin biopsies were examined for the study of qualitative and quantitative changes in dermal elastin fibres during cutaneous ageing: 91 unexposed samples (buttock area) obtained from volunteers aged between 21 and 80 years (44 men and 47 women); 30 moderately sun-exposed samples (forearm area) obtained from volunteers between 22 and 64 years of age, and 24 severe sun-exposed samples (facial area) obtained from volunteers aged between 45 and 65 years. To study the effect of UV exposure on lysozyme deposition in elastin fibres, 122 skin biopsies obtained from the buttock area of young volunteers (20–40 years old) were also examined.

UV sources and dosimetry

In all the UV studies, a 1000-W, xenon arc, solar UV simulator (Oriel, Stratford, CT, USA), equipped with a dichroic mirror, was used. Short and long UV filtrations were adjusted using Schott filters (Schott, Clichy, France) as previously described.[14]

The spectral irradiance of UVR sources was measured at skin level with spectroradiometers (Macam Photometrics Ltd, Livingston, UK and Bentham Instruments Ltd, Reading, UK), which were calibrated using lamps traceable to the National Physical Laboratory. The lamp output was monitored using a Centra radiometer (Osram, Berlin, Germany) equipped with UVB and UVA sensors.

Immunohistochemistry and image analysis quantification

For indirect immunofluorescence analysis, cryostat sections were air dried, rinsed in phosphate buffered saline (PBS), pH 7.2 (Biomerieux Laboratories, Lyon, France) and immunolabelled for 60 min at room temperature with guinea pig antiserum against human elastin (IPL, Lyon, France) diluted in PBS at 1 : 50 and an undiluted rabbit antiserum against human lysozyme (Zymed, San Francisco, CA, USA). After washing with PBS, sections were incubated with a fluorescein isothiocyanate (FITC) conjugated goat anti-guinea pig IgG and a rhodamine (TRITC) conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark) (dilution 1 : 50 and 1 : 100, respectively) for 60 min, washed with PBS, and mounted. In negative controls, the primary antibodies were omitted and non-immune IgG was used instead.

For quantitative evaluation, computer-based software (Quantimet 570 system, version 2.02, Leica, Cambridge Ltd, Cambridge, UK) was used. The same operator analysed all samples. The results obtained were expressed in arbitrary units (a.u.) taking into account information about the number of pixels within the measurement frame and the level of illumination they represented (256 discrete grey levels).

Electron immunogold labelling

One biopsy, taken from the forearm of a 63-year-old volunteer presenting with moderate solar elastosis (relative elastin content = 35 a.u.), was immediately fixed in cold-buffered 1% glutaraldehyde (Polysciences, Eppelheim, Germany), pH 7.2, for 2 h at 4 °C. After dehydration, the sample was embedded in Lowicryl K4M (Polysciences) and polymerized at −20 °C with UV light. Sections were incubated in PBS-5% BSA (bovine serum albumin), and then incubated with rabbit antiserum against human lysozyme (undiluted, Zymed, San Francisco, CA, USA). After three rinses with PBS-1% BSA, sections were incubated with a 1 : 100 dilution of the secondary antibody, a goat anti-rabbit IgG coupled to 15-nm colloidal gold (AuroProbe GAR IgG-G15, Amersham, UK). Sections were rinsed in PBS and stained with aqueous uranyl acetate for 5 min and with lead citrate for 1 min. They were examined with a JEOL 100B electron microscope operating at 80 kV. Controls were performed by omitting or substituting the primary antiserum with non-immune serum.

Enzymatic digestion and morphometric analysis

Cryostat sections taken from sun-protected buttock skin (relative elastin content = 12 a.u.) and sun-exposed skin from the face (relative elastin content = 47 a.u.) were incubated with or without 500 ng of human leucocyte elastase (Elastin Products Company, Owensville, MO, USA) in 200 mM Tris-HCl buffer, pH 8, for 1h 30 min or 3 h at 37 °C before immunolabelling with elastin and lysozyme antibodies. Sections were also treated at room temperature for 2 or 5 min with 0.01 N HCl (pH 1.6) or 0.01 N NaOH (pH 11.5), or 70% ethanol, or 40% ethylene glycol, or 6 M urea-2 M thiourea-100 mM DTT before and after immunolabelling.

In another experiment, cryostat sections were overlaid with 10 µL of 200 mM Tris-HCl buffer, pH 8 (buffer control), or with the same buffer containing HLE (200–500 ng at 875 units/mg) as previously described[15] before or after pre-incubation of skin sections with lysozyme (0.01–1%; Boehringer Mannheim, Saint Didier, France). After incubation, the sections were stained with the polyphenolic catechin-fuchsin[16] and a quantitative estimation of the area fractions (A_A) occupied by elastic fibres, representing the surface of fibres as a function of the analysed tissue area, were performed on 10 fields, 0.7 × 0.7 mm in dimension, for each skin section.[17] Each experiment was repeated at least three times.

Statistical analysis

The effects of UV exposure on lysozyme deposition were assessed using an exact non-parametric Wilcoxon signed ranks test for intra-individual comparison. All the comparisons were made at a two-tailed significance level of 5%. These analyses were performed with SPSS release 9 statistical software.

Results

Immunofluorescent microscopy and image analysis

Elastin is one of the main constituents of elastic fibres in the papillary and reticular dermis. In unexposed young skin samples (buttock), elastin fibres were dispersed throughout the dermis. In the papillary dermis, thin elastin fibres ran vertically, then parallel to the skin surface to form the elaunin plexus from which fine oxytalan fibres branch perpendicularly towards the basement membrane of the epidermis (fig. 1a). In unexposed aged skin samples (buttock), most changes were seen in the reticular dermis, while the superficial network (oxytalan and elaunin fibres) seemed to be retained without signs of elastosis or 'Grenz zone' (fig. 1b). Moderate to severe elastosis was seen in sun-exposed skin samples (face), associated with a loss of the dermal superficial plexus (oxytalan and elaunin fibres). Elastin fibres increased in thickness and were detected as a broad band of clumped and densely stained material, separated from the epidermis by a thin, unstained zone ('Grenz zone') (fig. 1c).

Using a computer-assisted image analysis system, quantification of elastin after immunostaining on 91 unexposed skin samples from the buttock area of individuals of various ages and different sex revealed a decrease in the relative elastin content with age (fig. 2a). No difference was noticed between men and women. Quantification of 30 skin samples from moderate sun-exposed area (forearm) showed no age-related changes in the elastin content (16 ± 1 a.u. in the third decade and 17 ± 3 a.u. in the seventh decade). In comparison, severe sun-exposed skin (face, 24 samples) was characterized by an abnormally high elastin content (fig. 2b: comparison between 51–60- and 61–70-year-old group).

Double immunostaining, using an elastin antiserum combined with a lysozyme antiserum, showed a colocalization of lysozyme and elastin in the papillary and reticular dermis. Lysozyme immunoreactivity was mainly detected in sun-exposed, elastosic skin samples (fig. 3). Quantification of elastin and lysozyme immunostaining by computer-assisted image analysis of non-exposed and UV-exposed skin samples revealed that UVA (in particular UVA-1) induced a higher lysozyme deposition than solar simulated radiation (SSR) (at the same erythemogenic dose) (Table 1). Only UVA-1 induced a significant effect below the minimal erythemal dose (MED). It is important to mention that in all the above studies UV exposure never changed the initial elastin content (Table 1).

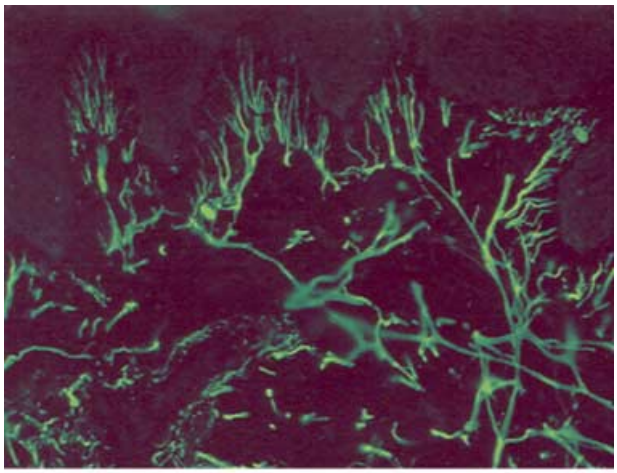
Comparison of unexposed (buttock) vs. sun-exposed (face) skin, under the same experimental conditions, revealed that only 25% (0.25 ± 0.03) of the elastin fibres in unexposed skin were covered with lysozyme compared to 66% (0.66 ± 0.05) in sun-exposed skin (Table 2), confirming a relationship between elastosis and lysozyme deposition.

Electron immunogold lysozyme labeling

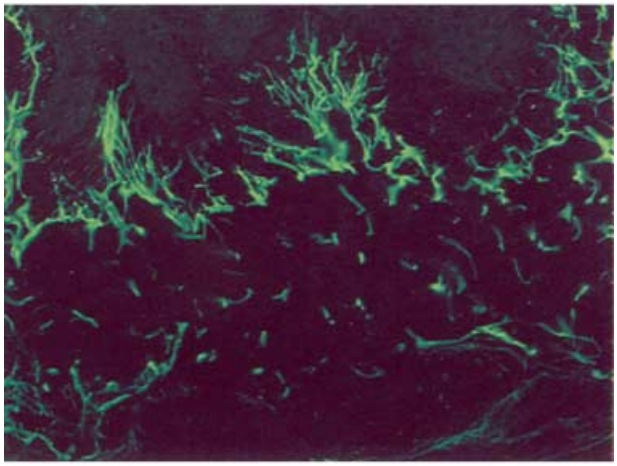
Immunogold labelling of a moderate elastosic skin sample (forearm of a 63-year-old woman) showed an amorphous electron-lucent elastin matrix with some electron-dense, irregular-shaped spots, exhibiting lysozyme immunoreactivity (fig. 4). The collagen network and papillary dermis were free of lysozyme deposition.

Enzymatic digestion and morphometric analysis

The fact that treatment of the immunolabelled sections with HCl, NaOH, ethanol, ethyleneglycol or urea did not



a



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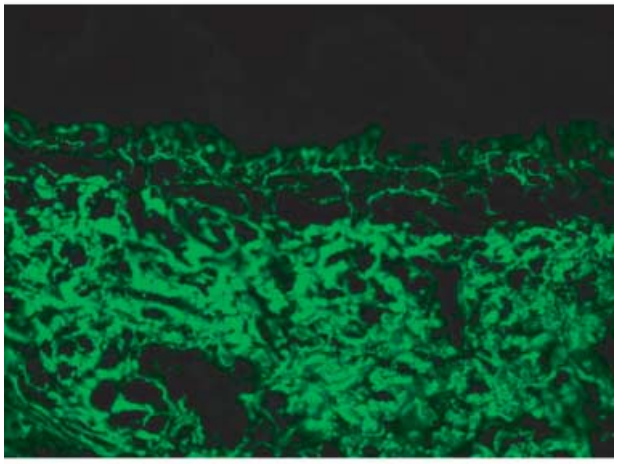
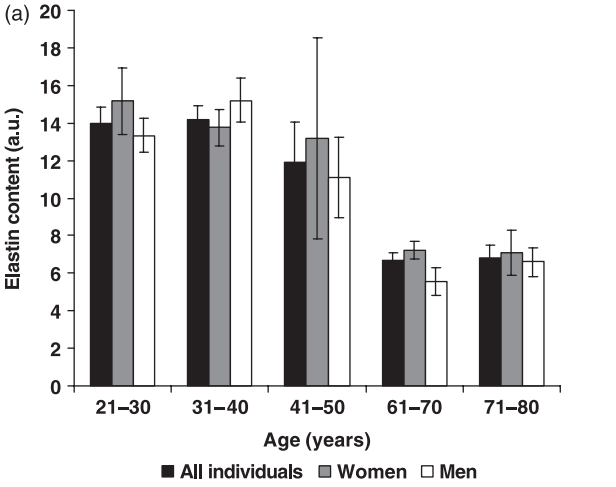


c

fig. 1 Immunohistochemical visualization of elastin in (a) buttock skin of a 30-year-old woman (fourth decade) (×250), (b) buttock skin of a 75-year-old male volunteer (eighth decade) (×250), and (c) facial skin of a 65-year-old woman (seventh decade) (×220). Cryostat sections were stained using an antiserum against human elastin.



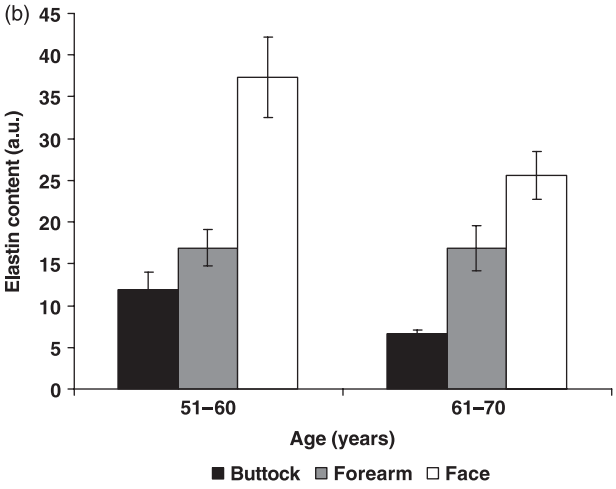


fig. 2 Relative elastin content in (a) buttock skin in individuals of different ages and sex, and (b) buttock vs. forearm and facial skin in individuals of sixth and seventh decade. The relative amount of elastin was determined using computerized image analysis in immunostained sections as illustrated in fig. 1. The values are mean ± SEM.

alter the fluorescent intensity of either elastin or lysozyme was taken as an indication of a particularly strong interaction between the two proteins (data not shown).

We observed a higher elastin digestion by human leucocyte elastase (HLE) in unexposed skin samples compared to sun-exposed samples (Table 2 and Table 3, study 1) indicating a protective effect of elastin-bound lysozyme against proteolysis of elastin by HLE. The fact that the relative lysozyme content was not markedly altered when the samples were treated with HLE (Table 3) was taken as a further indication that lysozyme-free elastin fibres for the most part were those accessible for proteolytic digestion.

Following incubation of skin sections with increasing concentrations of HLE (200–500 ng), we observed a

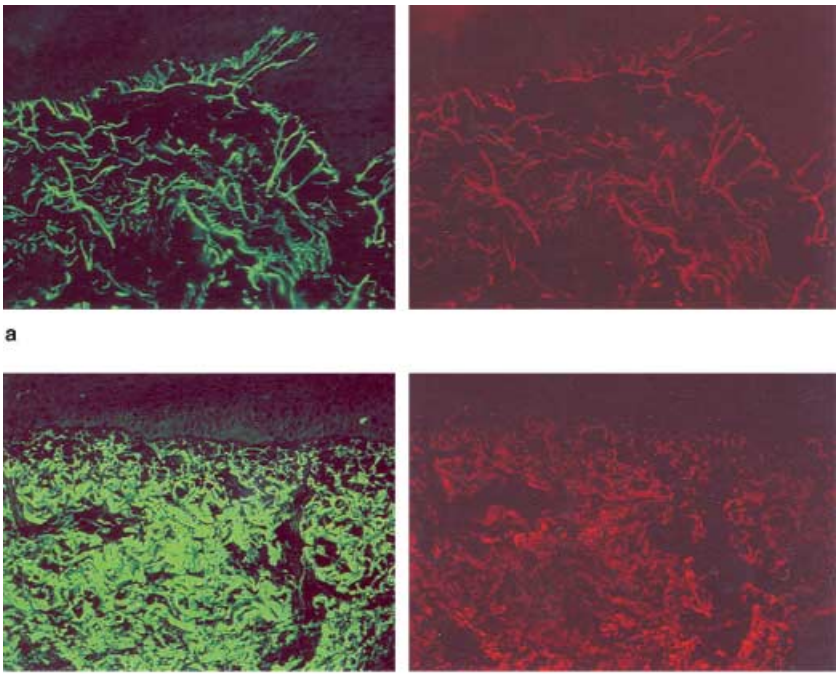


fig. 3 Immunohistochemical visualization of lysozyme deposition in elastin fibres in (a) forearm skin of a 24-year-old woman (×250), and (b) facial skin of a 69-year-old woman (×250). Cryostat sections were stained simultaneously with antiserum against human elastin (revealed by FITC – green pattern) and with antiserum against human lysozyme (revealed by TRITC – red pattern).

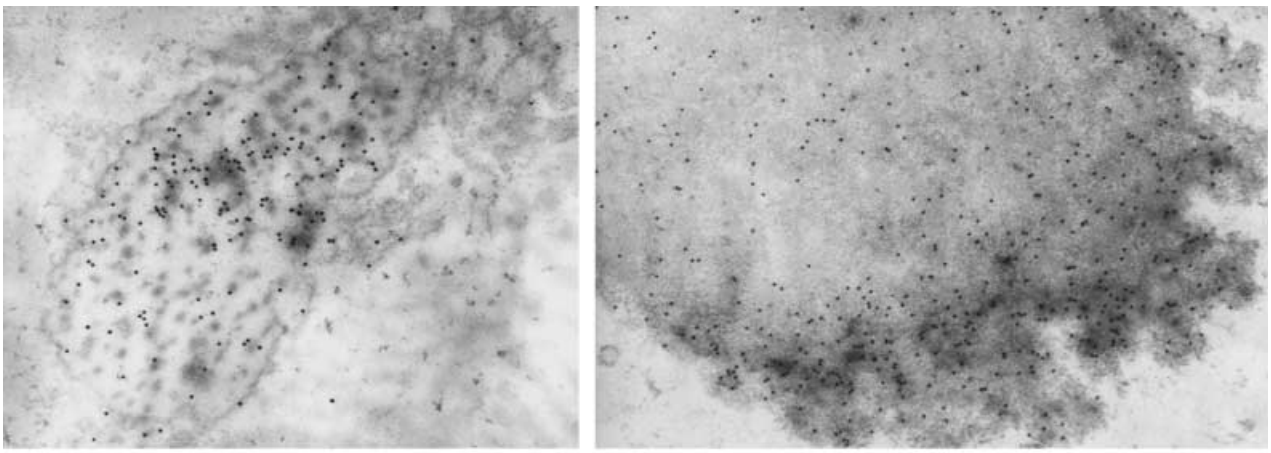


fig. 4 Slightly degenerated elastic fibres in the dermis of a 63-year-old woman, presenting with moderate solar elastosis (relative elastin content = 35 a.u.), after immunostaining with a specific lysozyme antibody. Gold particles are associated with the still-recognizable electron-dense amorphous component of the elastic fibres, indicating a strong association between elastin and lysozyme (fig. 4a, ×68000). Lysozyme is detected in the condensed spots, especially in the electron-dense core of the degenerative spots, and at the irregular periphery of the fibres (fig. 4b, ×54000).

dose-dependent digestion of elastin using morphometric analysis (Table 3, study 2). Incubation of skin sections with 1% of lysozyme (Table 3, study 2) had no effect on the network of elastic fibres. Furthermore, pre-incubation of skin sections with lysozyme did not provide additional protection against HLE digestion of elastin (Table 3, study 2).

An interesting observation was the fact that pre-incubating HLE with lysozyme before incubation with the skin sections resulted in up to 38% reduction of HLE elastolytic activity (25% vs. 40%, Table 3, study 3), indicating a lysozyme–HLE interaction.

Discussion

Variations in elastin content with age in both protected and sun-exposed skin of healthy volunteers have previously

Table 1 Effect of UV exposure on dermal elastin content and lysozyme deposition in elastin fibres

Treatment (20–40-year-old subjects)	Elastin (a.u.)	Lysozyme (a.u.)	Lysozyme/elastin
1. Unexposed ($n = 52$)	14.1 ± 0.6	1.2 ± 0.2	0.09 ± 0.01
2. UVA:			
Acute UVA exposure:			
60 J/cm^2 or 1.25 MED ($n = 10$)	12.9 ± 1.7	3.9 ± 1.3*	0.26 ± 0.05*
Semi-chronic UVA exposure:			
8 × 12.5 J/cm^2 or 8 × 0.5 MED ($n = 6$)	10.2 ± 1.4	1.3 ± 0.3	0.15 ± 0.04
8 × 25J/cm^2 or 8 × 1 MED ($n = 6$)	9.3 ± 1.2	2.1 ± 0.2*	0.23 ± 0.02*
Semi-chronic UVA-1 exposure:			
8 × 25J/cm^2 or 8 × 0.8 MED ($n = 6$)	10.3 ± 0.6	2.4 ± 0.3*	0.24 ± 0.04*
Chronic UVA exposure:			
39 × (10–50 J/cm^2) ($n = 6$)	19.9 ± 0.8	7.9 ± 1.1*	0.41 ± 0.06*
3. SSR:			
Semi-chronic SSR exposure:			
9 × 0.75 DEM ($n = 12$)	11.3 ± 0.7	2.9 ± 1.5	0.25 ± 0.03
20 × 0.5 DEM ($n = 12$)	11.5 ± 0.8	2.9 ± 0.7	0.23 ± 0.04
Chronic SSR exposure:			
30 × 1MED ($n = 12$)	21.6 ± 0.9	4.3 ± 1.3*	0.20 ± 0.06*

Data are means ± SEM; *$P \leq 0.05$ vs. unexposed area (same volunteer). UVA (320–400 nm); UVA-1 (340–400 nm); SSR (280–400 nm).

Table 2 Protective effect of lysozyme on human leucocyte elastase (HLE) degradation of elastin fibres

Treatment	Elastin (a.u.)	Lysozyme (a.u.)	Lysozyme/Elastin	Hydrolysis
Unexposed skin (buttock – $n = 19$)	12 ± 1	3 ± 1.0	0.25 ± 0.03	
Unexposed skin incubated with HLE 500 ng (1h 30 min: 37 °C)	8 ± 1	3 ± 0.4	0.39 ± 0.04	33%
Sun-exposed skin (face – $n = 19$)	47 ± 2	32 ± 3.0	0.66 ± 0.05	
Sun-exposed skin incubated with HLE 500 ng (1h 30 min: 37 °C)	37 ± 3	28 ± 3.0	0.76 ± 0.06	21%
Sun-exposed skin incubated with HLE 500 ng (3h: 37 °C)	19 ± 1	24 ± 1.0	1.31 ± 0.11	60%

Data are means ± SEM. Each experiment was repeated at least three times.

Table 3 Protective effect of lysozyme on degradation of elastic fibres by HLE and direct inhibitory effect of lysozyme on HLE

Treatment	Area fraction (A_A %)	Hydrolysis
4. Study 1:		
Unexposed skin incubated with control buffer (3 h: 37 °C)	7 ± 1	
Unexposed skin incubated with HLE 350 ng (3 h: 37 °C)	1 ± 0.5	86%
Sun-exposed incubated with control buffer (3 h: 37 °C)	20 ± 3	
Sun-exposed skin incubated with HLE 350 ng (3 h: 37 °C)	6 ± 1	70%
5. Study 2 (sun-exposed skin):		
Control buffer	19 ± 3	
HLE digestion (3 h: 37 °C):		
200 ng	15 ± 3	21%
350 ng	11 ± 3	42%
500 ng	5 ± 2	74%
Lysozyme 1% (1h 30 min: 37 °C)	18 ± 3	5%
Lysozyme 1% (1h 30 min 37 °C) before HLE digestion 350 ng (3 h: 37 °C)	9 ± 3	53%
6. Study 3 (unexposed skin):		
Control buffer	10 ± 1.5	
HLE digestion 350 ng (1h 30 min: 37 °C)	6 ± 1	40%
Incubation of lysozyme 1% with HLE 350 ng before digestion (1h 30 min: 37 °C)	7.5 ± 1	25%

Data are means ± SEM. Each experiment was repeated at least three times.

been reported. El-Domyati *et al.*[18] showed an age-related elastin reduction of up to 38% in abdominal skin samples. Our data, based on 91 buttock skin samples, showed an even higher reduction of elastin (−51% between 20 and 80 years of age). The difference in results may be because of the difference in the anatomic sites of the skin samples examined (abdomen vs. buttock) and the phototype of the donors (Egyptian vs. Caucasian skin types II and III).

In general, our study clearly showed that in unexposed buttock skin, elastin content decreased with age (−44% between 50 and 70 years of age). A similar decrease was seen in severe sun-exposed skin (face) (−31% between 50 and 70 years of age), which presents a high elastin content as a result of sun exposure. Interestingly, the elastin content in moderately sun-exposed areas (forearm) did not change during ageing. This phenomenon might be explained by a combination of age-induced reduction and sun-dependent increase in elastin.

From the above results, one is tempted to conclude that UV (sun)-exposure has a direct effect on elastin content. However, even after a 13-week exposure to increasing UVA doses (total dose 1237 J/cm^2), we were unable to detect any changes in the relative elastin content in skin samples obtained from young volunteers. Other authors even report a significant elastin reduction after repeated UVA exposure.[19]

An important observation, made by us and by others, was the UV-induced deposition of lysozyme in elastin fibres.[11,20,21] It is important to note that lysozyme preferentially binds to those parts of the elastin network that show UV-induced damage.[10] These observations led us to investigate *in situ* whether elastin-bound lysozyme prevents elastin from enzymatic degradation. A protective role of lysozyme has been speculated by others and demonstrated *in tubo* by Park *et al.*[13]

Our *in situ* experiments clearly demonstrated, for the first time, a direct reverse relationship between lysozyme deposition and elastin degradation by human leucocyte elastase (HLE). We further detected that free lysozyme was able to reduce the catalytic activity of HLE. Taken together, our results contribute to a better understanding of the age-related changes of elastin content in protected and sun-exposed body sites.

In sun-protected skin areas elastin is not modified and, in consequence, does not bind lysozyme, which protects it from proteolysis. Under these conditions, the presence of low amounts of degrading enzymes leads to the observed slow reduction in elastin content with increasing age. In severe sun-exposed areas, several events contribute to the detected accumulation of elastin or elastosis. UV-exposure damages elastin fibres and stimulates at the same time an increased synthesis of elastin.[22] The UV-damaged parts of the elastin network trigger the binding of released lysozyme, which prevents proteolytic degradation of damaged fibres. Furthermore, lysozyme, the level of which is particularly high after an inflammatory response of the skin, interacts with HLE, resulting in a partial inhibition of proteolytic activity of the latter.

The fact that the relative elastin content in moderately sun-exposed skin areas remains unchanged with age, might result from a combination of the above described events in protected and severe sun-exposed areas. The slow degradation of elastin with age is compensated for by a slight increase during periods of sun exposure.

In summary, the observed increase in elastosis with age in sun-exposed skin is a combined effect of daily exposure to UVA (during 'indoor' activities) – which, even at low doses, induces lysozyme deposition in damaged elastin fibres – and severe sun exposure during summer time (sunbathing), contributing to the release of additional lysozyme. Lysozyme, in turn, interacts with and reduces HLE elastolysis.

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