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Ultraviolet radiation reduces desmosine cross-links in elastin

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ABSTRACT

Elastic fibers, a major component of the extracellular matrix of the skin, are often exposed to ultraviolet (UV) radiation throughout mammalian life. We report on an *in vitro* study of the alterations in bovine nuchal ligament elastic fibers resulting from continuous UV-A exposure by the use of transmission electron microscopy (TEM), histology, mass spectrometry, and solid state ^{13}C NMR methodologies. TEM images reveal distinct cracks in elastic fibers as a result of UV-A irradiation and histological measurements show a disruption in the regular array of elastic fibers present in unirradiated samples; elastic fibers appear shorter, highly fragmented, and thinner after UV-A treatment. Magic angle spinning ^{13}C NMR was applied to investigate possible secondary structural changes or dynamics in the irradiated samples; our spectra reveal no differences between UV-A irradiated and non-irradiated samples. Lastly, MALDI mass spectrometry indicates that the concentration of desmosine, which forms cross-links in elastin, is observed to decrease by 11 % following 9 days of continuous UV-A irradiation, in comparison to unirradiated samples. These alterations presumably play a significant role in the loss of elasticity observed in UV exposed skin.

Introduction

Elastin, the principal protein component of the elastic fiber, is an extracellular matrix protein of human tissues that require elasticity such as the arteries, lungs, and skin. Elastin plays an important role in providing these tissues, and others, the ability to stretch while maintaining healthy cells [1–5]. In the skin, the majority of elastin is located in the reticular dermis. Mature elastin is a system of interconnected fibers that are encompassed by other proteins such as elaunin fibers and oxytalan fibers [6]. Tropoelastin, the monomer of elastin, is a large 72 kDa protein which is cross-linked by desmosine or isodesmosine to form elastin. The vast majority of elastin is produced during fetal development, and the first few years of life, after which the expression of tropoelastin sharply decreases [7]. Consequently, connective tissues rely on elastin that is formed early in life [8]. When skin is damaged through UV exposure and/or oxidation it may lose elasticity, and low levels of tropoelastin production may result in irreparable damage. Recent research into repairing damaged elastic fibers has focused on integrating tropoelastin into the skin; one challenge in this approach relates to transferring the protein across the epidermis. Another approach has been to increase tropoelastin expression through the use of small molecules such as all-*trans* retinoic acid [9,10].

The effects of photoaging are closely connected to elastin damage through UV exposure. It has been found that the major histopathological alteration to photoaged skin is an accumulation of poorly organized elastin, termed solarelastosis. UV irradiation has been shown to induce the expression of matrix metalloprotenases (MMPs), which cause degradation of extracellular matrix such as elastin and collagen. Irradiation of skin was found to increase the experession of human macrophase elastase (MMP-12) mRNA 11.9 fold within 16 h of UV exposure [11]. Other studies have found that elastin production is increased after exposure to UV radiation, which may result in large amounts of abnormal elastic material in the skin. In one study, irradiation of mice with ultraviolet B(wavelengths = 290–320 nm) yielded an 8.5 fold increase in promoter activity, while ultraviolet A(wavelengths = 320–400 nm) only resulted in an 1.8 fold increase in promoter activity. These results indicate that ultraviolet B has a significant impact on the accumulation of elastosis, while ultraviolet

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A contributes as well, but to a lesser extent [12]. In another study, by tagging elastic material with antibodies and conducting immunofluorescence microscopy, it was found that solar elastosis is primarily derived from elastic fibers and not from preexisting or newly synthesized collagen [13].

While the relationship between UV radiation and photoaging of skin has been established, the exact change that elastin undergoes when exposed to UV radiation is still unclear. One common belief is that elastic fibers are denatured or cleaved by UV radiation, and that most of the elastotic material is produced after exposure to UV radiation or its disorganized structure gives aged skin its characteristic wrinkled appearance. In previously reported studies, electron microscopy, histology, TEM (transmission electron microscopy), and SEM (scanning electron microscopy) have been applied to probe macroscopic changes to the elastic fiber in aged and photodamaged skin [14–17]. For all biological damage associated to sun exposure, UV-B contributes 80% whereas UV-A contributes only 20% even though approximately 95 % of terrestrial UV-radiation is UV-A [18]; more work is needed to better understand the microscopic changes that UV-A irradiation causes to the structure of elastin. We conducted this *in vitro* study to uncover changes on elastic fibers that undergo when exposed to high intensity UV-A irradiation. Elastic fibers were then examined histologically and by TEM to observe macroscopic changes that resulted from irradiation. ^{13}C solid state NMR spectroscopy was performed to measure possible structural alterations of elastin. Mass spectrometry was also implemented to quantify changes in the relative amount of desmosine cross-links. These combined methods provide new information relating to the detrimental effects of UV-A irradiation to the structure of the elastic fiber and of elastin.

Materials and methods

Sample preparation

Bovine nuchal ligament elastic fibers purchased from Elastin Products Company, LLC (Owensville, MO) were used for this study. These samples were purified by Elastin Products Company using a known protocol [19] and were free of fat, collagen, smooth muscle cells, and other connective tissue. In a prior study, we showed that the protocol used for isolating elastin did not alter the structure of the protein, or the concentration of cross-links [20]. Elastic fibers were completely immersed in distilled water while being irradiated with a 3U40W UV-A lamp (Cnlight Co, China) with a center wavelength of 365 nm which was placed 10 cm above the sample. The wavelength distribution of the UV-A lamp is shown in Figure S1 (supplementary). During irradiation, the system was covered with a shield to ensure that the sample was isolated from other light and were submerged in water during irradiation. The irradiation intensity was 12 mW/cm^2 and samples were continuously irradiated for 9 days. The intensity of the UV-A lamp is therefore approximately 3 times higher than that of the sun, when directly overhead (located at the zenith).

Histology and microscopy

Unirradiated elastic fibers and fibers following 9 days of UV-A irradiation were used for the histological study. For histology, a small amount of sample was placed overnight in 100 ml phosphate buffered saline. The samples were then placed in Lieca Cryo-Gel (SPI supplies Product Ref-02694-AB), sectioned on a Leica CM1850 cryostat at 10 μm, and stained using the Sigma-Aldrich elastic stain kit (REF HT25A-1KT) following a modified version of a previously reported protocol [21]. The slides were gently rinsed with 95% ethanol, and then placed in xylene for a few seconds. Cover slips were then mounted using an Eukitt quick-hardening mounting medium (Sigma-Aldrich REF 03989) and left to dry overnight. All sections were photographed using a National Optical DC4-156-S digital microscope at a magnification of 10×.

Transmission Electron Microscopy (TEM)

Bovine nuchal ligament elastic fibers were immersed in 0.1 M phosphate buffered solution (pH-7.4) for 1 h. All the samples were stained with osmium tetroxide and embedded in epoxy resin. Samples were sliced along the plane that was perpendicular to the fiber axis, with a thickness of 60 nm and examined in a JEM-2000EX transmission electron microscope. The accelerating voltage used was 120 kV.

^{13}C NMR experimental parameters

Prior to the NMR experiments, unirradiated and 9 days UV-A irradiated samples were immersed in distilled water and solid-state NMR experiments were carried out on hydrated samples. ^{13}C NMR experiments were performed using a Bruker Avance (Billerica, MA) spectrometer at a magnetic field strength of 21.10 T. All the experiments were carried out using a 4 mm center packing rotor with an insert to keep the samples hydrated, as well as to center the samples with respect to the RF coil. ^{13}C MAS (magic angle spinning) spectra were measured using a DEPTH sequence (to suppress background carbon signals arising from rotor inserts and the probe head) [22,23] with 80 kHz TPPM decoupling [24] at (300 ± 1) K and direct polarization. The spinning speed was set to 14.5 kHz for all the samples and spectra were acquired by accumulating 18,800 scans. The ^{13}C $\pi/2$ pulse was 54 μs and the recycle delay was 6 s. Analysis of data was performed using MATLAB and matNMR with a Gaussian multiplication broadening factor of 100 Hz. ^{13}C NMR spectra were referenced to adamantane (TMS = 0 ppm).

Sample hydrolysis and quantification with labeled desmosine

Elastic fiber samples for this study were lyophilized for 24 h prior to hydrolysis. Approximately, 2.1–2.2 mg of each sample was placed into a solution containing 300 μl of 6 M HCl and 1 μl of 0.5% w/w phenol solution. The sample and solution mixture was placed into a vacuum hydrolysis tube, flushed with nitrogen gas, and then evacuated. The samples were kept at 110 °C for 96 h, afterward the solvent was frozen in liquid nitrogen and lyophilized for 8–10 h. After lyophilization, each sample was suspended in a 50 μl solution of 94.5% 0.14 M sodium acetate, 0.5% triethylamine, and 5% acetonitrile (v/v/v) at a pH of 7.5. Resuspended samples were diluted to 100 fold. Labeled d_4-desmosine standard (Toronto Research Chemicals, Toronto, Canada) at final concentration of 10 pmol/μl was mixed with the diluted samples in three different ratios 1:1, 3:1, and 1:3. The relative amount of desmosine in each sample was quantified with respect to mass spectrometric peak intensity of standard desmosine in MS^2 mode. For statistical analysis of the data, a *t*-test was used assuming our data followed a normal distribution, and using the standard deviations in each samples studied. Null hypothesis probability was measured indicating the level of significance of our data.

MALDI-MS quantitative analysis

MALDI-MS^2 experiments were performed using a Thermo LTQ XL ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a vacuum MALDI source. α-Cyano-4-hydroxycinnamic acid (CHCA) purchased from Sigma-Aldrich (St. Louis, Missouri, USA) was recrystallized and used to prepare the matrix solution. Solid CHCA was added in a solution of 0.1% trifluoroacetic acid, 70% acetonitrile, and 29.9% HPLC grade water until saturation. The solution was centrifuged and supernatant liquid was used as the matrix solution. 1 μl of sample mixture containing different ratio of standard and sample were placed in 9 μl of CHCA matrix solution. The final solution was vortexed, and 1 μl of mixture was spotted on the MALDI plate. Spotted points were air dried prior to inserting the plate into the MALDI mass spectrometer for analysis.

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