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Research paper

Molecular-level insights into aging processes of skin elastin

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Skin aging is characterized by different features including wrinkling, atrophy of the dermis and loss of elasticity associated with damage to the extracellular matrix protein elastin. The aim of this study was to investigate the aging process of skin elastin at the molecular level by evaluating the influence of intrinsic (chronological aging) and extrinsic factors (sun exposure) on the morphology and susceptibility of elastin towards enzymatic degradation. Elastin was isolated from biopsies derived from sun-protected or sun-exposed skin of differently aged individuals. The morphology of the elastin fibers was characterized by scanning electron microscopy. Mass spectrometric analysis and label-free quantification allowed identifying differences in the cleavage patterns of the elastin samples after enzymatic digestion. Principal component analysis and hierarchical cluster analysis were used to visualize differences between the samples and to determine the contribution of extrinsic and intrinsic aging to the proteolytic susceptibility of elastin. Moreover, the release of potentially bioactive peptides was studied. Skin aging is associated with the decomposition of elastin fibers, which is more pronounced in sun-exposed tissue. Marker peptides were identified, which showed an age-related increase or decrease in their abundances and provide insights into the progression of the aging process of elastin fibers. Strong age-related cleavage occurs in hydrophobic tropoelastin domains 18, 20, 24 and 26. Photoaging makes the N-terminal and central parts of the tropoelastin molecules more susceptible towards enzymatic cleavage and, hence, accelerates the age-related degradation of elastin.

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1. Introduction

Cutaneous aging is a complex biological process that occurs as intrinsic and extrinsic aging and affects different layers of the skin, in particular the dermis. Intrinsic or innate aging is a naturally occurring process, which is a result of slow tissue degeneration and is characterized by fine wrinkling, atrophy of the dermis and reduction of subcutaneous adipose tissue. Extrinsic aging, whose effects are superimposed on those of innate aging, is induced and accelerated by environmental influences, primarily ultraviolet (UV) radiation, but also smoking and air pollution [1–5]. UV-induced extrinsic aging is also called photoaging and leads to coarse wrinkling, furrowing and loss of elasticity along with an apparent thickening of the skin due to the accumulation of elastotic material in the upper and middle dermis. The latter process is referred to as solar elastosis or actinic damage [1–3]. Photoaging is associated with the skin pigmentation and is observed in particular in individuals who lead an outdoor lifestyle or live in sunny climates. While individuals with darker skin are less affected, photoaging is much more pronounced in individuals with light skin [6]. On the molecular level, both aging processes are not only connected with

Abbreviations: ANOVA, analysis of variance; CG, cathepsin G; EBP, elastin-binding protein; ECM, extracellular matrix; EDP, elastin-derived peptide; HLE, human leukocyte elastase; HCA, hierarchical cluster analysis; HPLC, high performance liquid chromatography; MMP, matrix metalloproteinase; NSP, neutrophil serine protease; LFQ, label-free quantification; MS, mass spectrometry; PC, principal component; PE, pancreatic elastase; PCA, principal component analysis; PR3, proteinase 3; SD, standard deviation; SEM, scanning electron microscopy; TE, tropoelastin; TFA, trifluoroacetic acid; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; UV, ultraviolet.

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phenotypic changes in cutaneous cells, but also with structural and functional changes of extracellular matrix (ECM) components such as collagens, elastin and proteoglycans that are required to provide tensile strength, elasticity and hydration to the skin, respectively. Due to their extreme longevity, damage to these ECM molecules results in significant changes in the mechanical properties of the skin [2].

As the core protein of elastic fibers, elastin provides elasticity and resilience to many vertebrate tissues such as aorta, lung and skin and is, thus, critical for their long-term function [7,8]. Due to its high hydrophobicity and cross-linked structure, elastin is insoluble, highly resistant to proteolytic degradation and shows virtually no turnover in healthy tissues [7]. Different tissues contain different amounts of elastin. The skin contains between 2% and 8% elastin [9], whereas the protein comprises even 30%–57% of the aorta (percentages based on dry weight of the tissue) [7]. Elastin is produced by various cell types including smooth muscle cells, fibroblasts and endothelial cells in the form of its monomeric precursor tropoelastin (TE), which occurs in different isoforms and is cross-linked after oxidative deamination at K residues by members of the lysyl oxidase family. In most mammalian tissues, the major part of elastin and elastic fiber formation takes place in a defined developmental window and reaches its maximum during early neonatal periods. In mature organs and tissues, elastin synthesis is repressed by post-transcriptional factors [8]. The low turnover and lack of continued elastin production upon maturity reflect the extreme durability and long half-life of elastic fibers that reaches the human lifespan of around 74 years [10]. Although TE expression may be reinitiated in response to wounding [11,12] or exposure to UV radiation [13,14], elastin production is aberrant in these cases and does not lead to the formation of normal elastic fibers. In the case of solar elastosis, an accumulation of large, clumped and potentially partially degraded fibers of aberrant composition characterized by increased amounts of elastin and fibrillin has been observed instead [14]. During formation of normal and in particular hypertrophic scars after wounding, the formation of large, thin elastin fibers that appear fragmented has been described [11].

Elastin does not only influence the architecture and biomechanical properties of the ECM, but also plays an active role in various physiological processes [15]. Elastin-derived peptides (EDPs) that occur upon proteolytic degradation of elastin *in vivo* have been shown to be involved in the regulation of various cell activities such as cell adhesion, chemotaxis, proliferation, protease activation, angiogenesis and apoptosis [16,17]. In particular EDPs containing the GXXPG motif display biological effects due to their ability to interact with the elastin-binding protein (EBP), which mediates biological activities [17]. Damage to elastin induced by enzymatic action in combination with the biological processes triggered by EDPs may contribute to the development and progression of various pathological conditions, which eventually leads to compromised function or even loss of function of tissues and organs. In fact, cancer progression and severe cardiovascular diseases such as lung emphysema, chronic-obstructive pulmonary disease, aortic stenosis and atherosclerosis have been shown to be associated with enzymatically induced elastin degradation [7,8,16,18]. Enzymatic fragmentation of elastic fibers is also a hallmark of intrinsic and extrinsic skin aging [18]. Elastolysis as well as degradation of other ECM components such as collagens occurs through cleavage by different members of the families of serine proteases, matrix metalloproteinases (MMP) and cysteine proteases [16]. Studies have indicated that increased expression of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12 and MMP-13 is associated with degradation of ECM components or accumulation of elastotic material during photoaging of the skin [19–23]. Furthermore, it was found that the expression of the neutrophil serine proteases (NSPs) cathepsin G (CG) and human leukocyte elastase (HLE) is increased during photoaging [24,25]. Of the mentioned proteases, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12 and the NSPs CG and HLE are known to degrade elastin [26,27].

In this study, human skin elastin samples from differently aged individuals derived from sun-exposed or sun-protected areas of the body were compared by scanning electron microscopy (SEM) and on the molecular level to gain insights into skin aging by evaluating the effects of intrinsic and extrinsic factors on the protein. All samples were subjected to enzymatic degradation by pancreatic elastase (PE) as elastin cannot be analyzed intact, and the resultant peptide mixtures were investigated by mass spectrometric techniques as well as label-free quantification (LFQ) to identify age-related changes in the peptide patterns of enzymatic digests of the elastin samples. Statistical methods were used to visualize differences between the samples and to identify marker peptides, whose abundances increase or decrease with increasing age of the subject.

2. Materials and methods

2.1. Materials

Twelve skin biopsies (5 mm in diameter) were obtained postoperatively from the tumor-free border of excised skin cancer tissue from different skin areas of individuals aged 19–90 years, and 5 foreskin samples (8 mm × 8 mm) were derived from children aged 6–13 years (Table 1). Subjects were Caucasians with light to mild pigmentation. The study was approved by the ethics committee of the Medical Faculty of the Martin Luther University Halle-Wittenberg (Germany) and carried out in compliance with the Helsinki Declaration. All experiments were undertaken with the understanding and written consent of each subject or each subject's legally authorized representative. Elastin was isolated from the tissue biopsies as previously described using a gentle method to remove other components of the ECM and prevent damage to elastin [28]. In brief, tissue samples were treated with different organic solvents and cleaved by cyanogen bromide and trypsin from porcine pancreas (all purchased from Sigma-Aldrich, Steinheim, Germany). Isolated elastin samples were dried under laminar air flow and stored at −26 °C until further analysis. Porcine PE was purchased from Elastin Products Company (Owensville, MO, USA), and analytical grade 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) and formic acid were purchased from Merck (Darmstadt, Germany). HPLC-grade acetonitrile (VWR Prolabo, Leuven, Belgium) was used. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich. Additional chemicals used were of analytical grade.

2.2. Scanning electron microscopy

Six skin elastin samples were analyzed by SEM using an environmental scanning electron microscope ESEM XL 30 FEG (Philips, Amsterdam, Netherlands) as described previously [29].

2.3. Proteolysis of human elastin

Elastin samples were weighed and dispersed at a concentration of 1 mg mL^{-1} in 50 mM Tris buffer, pH 7.8. Incubation with PE was carried out at 37 °C for 48 h at an enzyme-to-substrate ratio of 1:50 (w/w). The elastin samples were fully degraded and solubilized under these conditions. All digestions were stopped by addition of TFA to a final concentration of 0.5% (v/v). Digested samples were stored at −26 °C until mass spectrometric analysis.

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